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ANALYSIS OF CHROMOSOMAL REARRANGEMENTS AFTER REPLICATION RESTART

SAED MOHEBI

SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
UNIVERSITY OF SUSSEX
AUGUST 2014
I hereby declare that this thesis has not been and will not be, submitted in whole or in part to another University for the award of any other degree.

Signature:………………………………………………
I would like to express my deepest gratitude to my supervisor Jo Murray for the opportunity to undertake my PhD in her laboratory, and for her continuous and generous support, guidance, and patience throughout the process, without which the completion of this work would not be possible.

I would like to extend my appreciation to Tony Carr for his advice and support during my research.

I would also like to thank all the people who helped me in completion of this PhD, Ken’Ichi Mizuno, and Adam Watson for their contributions towards my research, and all of the members Murray and Carr laboratories.

Special thanks to my parents, my brother, Sara Mohebi, Lena Erchen, and Eric Cartman.
Impediments to DNA replication are known to induce gross chromosomal rearrangements (GCRs) and copy-number variations (CNVs). GCRs and CNVs underlie human genomic disorders and are a feature of cancer. During cancer development, environmental factors and oncogene-driven proliferation promote replication stress. Resulting GCRs and CNVs are proposed to contribute to cancer development and therapy resistance.

Using an inducible system that arrests replication forks at a specific locus in fission yeast, chromosomal rearrangement was investigated. In this system, replication restart requires homologous recombination. However, it occurs at the expense of gross chromosomal rearrangements that occur by either faulty template usage at restart or after the correctly restarted fork U-turns at inverted repeats. Both these mechanisms of chromosomal rearrangement generate acentric and reciprocal dicentric chromosomes. The work in this thesis analyses the timing of replication restart and appearance of chromosomal rearrangements in a single cell cycle after induction of fork stalling. This research also identifies the recombination-dependent intermediates corresponding to the two pathways of rearrangements. Moreover, the DNA integrity checkpoint responses after replication fork arrest, homologous recombination dependent replication restart, and the accumulation of GCRs are investigated.
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<tr>
<td>2DGE</td>
<td>two-dimensional gel electrophoresis</td>
</tr>
<tr>
<td>AAA+</td>
<td>ATPase associated with various activities</td>
</tr>
<tr>
<td>AAD</td>
<td>ATR activating domain</td>
</tr>
<tr>
<td>APC/C</td>
<td>anaphase promoting complex/cyclosome</td>
</tr>
<tr>
<td>ARS</td>
<td>autonomous replicating sequence</td>
</tr>
<tr>
<td>AT</td>
<td>ataxia telangiectasia</td>
</tr>
<tr>
<td>BER</td>
<td>excision repair</td>
</tr>
<tr>
<td>BIR</td>
<td>break induced replication</td>
</tr>
<tr>
<td>bp</td>
<td>base-pair</td>
</tr>
<tr>
<td>CAK</td>
<td>CDK activating kinase</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>CFS</td>
<td>common fragile sites</td>
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<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<tr>
<td>CMG</td>
<td>Cdc45-MCM-GINS</td>
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<tr>
<td>CNV</td>
<td>copy-number variation</td>
</tr>
<tr>
<td>DDK</td>
<td>Dbf4-dependent kinase</td>
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<tr>
<td>DDR</td>
<td>DNA damage response</td>
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<tr>
<td>dHJ</td>
<td>double Holliday junction</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSB</td>
<td>double strand break</td>
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<tr>
<td>DSBR</td>
<td>double strand break repair</td>
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<tr>
<td>dsDNA</td>
<td>double-stranded DNA</td>
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<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>FXS</td>
<td>Fragile X syndrome</td>
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<tr>
<td>GCR</td>
<td>gross chromosomal rearrangements</td>
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<tr>
<td>HR</td>
<td>Homologous recombination</td>
</tr>
<tr>
<td>HU</td>
<td>Hydroxyurea</td>
</tr>
<tr>
<td>IR</td>
<td>ionizing radiation</td>
</tr>
<tr>
<td>LOH</td>
<td>loss of heterozygosity</td>
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<tr>
<td>M</td>
<td>mitosis</td>
</tr>
<tr>
<td>mb</td>
<td>mega-base</td>
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<tr>
<td>MCM</td>
<td>minichromosome maintenance proteins</td>
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<td>MMR</td>
<td>mismatch repair</td>
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<tr>
<td>NAHR</td>
<td>non-allelic homologous recombination</td>
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<tr>
<td>NER</td>
<td>excision repair</td>
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<tr>
<td>NHEJ</td>
<td>non-homologous end joining</td>
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<tr>
<td>ORC</td>
<td>origin recognition complex</td>
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<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulsed field gel electrophoresis</td>
</tr>
<tr>
<td>PIKK</td>
<td>phosphoinositol 3’-kinase-like-kinase</td>
</tr>
<tr>
<td>PIP</td>
<td>PCNA interacting peptide</td>
</tr>
<tr>
<td>pre-RC</td>
<td>pre-replicative complexe</td>
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<tr>
<td>PRR</td>
<td>post-replication repair</td>
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<tr>
<td>RFB</td>
<td>replication fork barriers</td>
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<td>RFC</td>
<td>replication factor C</td>
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<tr>
<td>RF</td>
<td>replication fork</td>
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<tr>
<td>RI</td>
<td>replication intermediate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>RMCE</td>
<td>recombination-mediated cassette exchange</td>
</tr>
<tr>
<td>RNR</td>
<td>ribonucleotide reductase</td>
</tr>
<tr>
<td>RPA</td>
<td>replication protein A</td>
</tr>
<tr>
<td>RTS1</td>
<td>replication termination site one</td>
</tr>
<tr>
<td>S</td>
<td>DNA synthesis</td>
</tr>
<tr>
<td>SAC</td>
<td>spindle assembly checkpoint</td>
</tr>
<tr>
<td>SDSA</td>
<td>synthesis dependent strand annealing</td>
</tr>
<tr>
<td>SMC</td>
<td>structural maintenance of chromosome</td>
</tr>
<tr>
<td>SSA</td>
<td>single strand annealing</td>
</tr>
<tr>
<td>SSB</td>
<td>single stranded binding protein</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single-stranded DNA</td>
</tr>
<tr>
<td>TBZ</td>
<td>thiabendazole</td>
</tr>
<tr>
<td>TLS</td>
<td>translesion synthesis</td>
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CHAPTER 1 – INTRODUCTION

The genetic material, deoxyribonucleic acid (DNA), encodes all the proteins essential to life. Therefore to pass on this genetic material to the next generation, each cell has to duplicate its DNA in a process known as DNA replication before segregating it into daughter cells. DNA replication is highly regulated and monitored during the cell cycle to ensure that the genetic material is passed on free of mistakes and genome integrity is kept intact. DNA replication can be compromised by a great variety of endogenous or exogenous factors such as oxidative products of cell metabolism or ionizing radiation (IR). To maintain genome integrity cells have developed signaling networks of DNA damage responses (DDR), which includes several aspects of DNA metabolism e.g. checkpoints, DNA repair, and transcriptional control (Bartek & Lukas, 2007; Branzei & Foiani, 2008).

In this introduction I will first give a brief over view of fission yeast as the model organism used in this study. Then I will go on to introduce cell cycle regulation, DNA replication, replication fork barriers that can cause replication fork arrest, the checkpoints that ensure the integrity of genomic material by preventing progression of cell cycle, and replication restart. I also will introduce site-specific protein-DNA barriers and systems to study replication restart. In this work, I will distinguish between proteins from different organisms by referring to proteins from Schizosaccharomyces pombe with sp, Saccharomyces cerevisiae with sc, and human with an h.
1.1 — *Schizosaccharomyces pombe* as a model organism

Fission yeast *S. pombe* of *Schizosaccharomyctaceae* family is a rod shaped unicellular eukaryotic organism and the first of its genus, *Schizosaccharomyces* to be discovered. The last common ancestor between *S. pombe*, the budding yeast, *S. cerevisiae*, and metazoans diverged 420 to 330 million years ago (Sipiczki, 2000). This ancestral divergence between the two main yeast model organisms and metazoans has been of great importance to discovery of biological pathways conserved throughout the evolution. The 13.8Mb *S. pombe* genome was sequenced in 2002 and consists of 4940 genes on three chromosomes (Wood et al., 2002). *S. pombe* is an haploid organism that spends the majority of its cell cycle, which takes between two to four hours in laboratory conditions, in G2. A very short G1 follows M phase and S phase coincides with the formation of the septum and cell division (Figure 1.1). Since the 1950’s, *S. pombe* has been extensively studied and due to the ease of genetic manipulation and the relative small size of genome has served as one of the best model organisms to study cell cycle control, DNA replication, repair, and recombination (Wood et al., 2002). Most *S. pombe* laboratory strains used are derived from the isolate that Urs Leupold used for his studies in the 1940’s and 1950’s (Leupold, 1993).
Figure 1.1 | *S. pombe* cell cycle. Diagram showing the stages of the *S. pombe* cell cycle. *S. pombe* cells where the DNA is stained with DAPI are superimposed on the diagram at the appropriate place in the cell cycle. G1 is very short and S phase is coincident with the formation of septum. Newly separated short early G2 cells grow lengthwise until they reach the critical size for cell division.
1.2 – Cell cycle and its regulation

In order to proliferate, cells must undergo cell division once every cell cycle. For ease of study, the cell cycle is divided into four main phases: DNA synthesis (S), mitosis (M), and gaps phases separating the two known as G1 and G2. G1 phase is between M and S phase and during this time cells prepare for DNA replication. In S phase cells replicate their genetic material to sister chromatids. In G2, cells continue to grow and ensure that the replication of genetic material is complete and error free. In M cells equally divide the duplicated genetic material to the two opposite poles of the cell, after which cytokinesis takes place (Nurse, 1991).

In eukaryotes, progression through the cell cycle is regulated by serine/threonine cyclin dependent kinases (CDKs). This regulation ensures the precise replication and appropriate segregation of the genetic material. In the event of damage to the genetic material, cell cycle progression is halted to allow cells time to overcome the damage. In yeasts, the activity of a single CDK (spCdc2, scCdc28) drives cell cycle progression, whereas in metazoans different CDKs regulate the passage through different stages of the cell cycle. Depending on their level of activity, CDKs phosphorylate specific targets at specific times during the cell cycle and this confers the transition between the stages of the cell cycle. CDK activity oscillates through the cell cycle: in G1 there is little CDK activity but this gradually increases until the level at which S phase starts. CDK activity continues to increase through G2 to allow initiation of M. This activity drops in M to the levels seen in G1 restarting the cell cycle. Many aspects of DNA metabolism such as replication (discussed in section 1.3.2) and repair are directly affected by the CDK activity.
CDKs kinase activity is dependent on the binding to the regulatory subunits known as cyclins. There are two main categories of cyclins: G1 cyclins are required for G1-S transition and G2 cyclins facilitate G2-M progression. In *S. pombe* G1 cyclins include *spCig1* and *spPuc1*, and G2 cyclins *spCig2* and *spCdc13*. G2 Cyclins show cell cycle stage specific patterns of expression, and this determines the activity of their partner CDKs at different stages of the cell cycle. G2 cyclins are also subject to anaphase promoting complex/cyclosome (APC/C)-dependent ubiquitination, which targets them to the protein degradation machinery during mitosis. Although essential, cyclin binding is not the only regulatory pathway of CDK activity. CDK activity is also determined by phosphorylation of various residues. For example, phosphorylation by CDK activating kinase (CAK) stimulates CDK activity. In contrast, CDK is also negatively regulated by tyrosine phosphorylation. An example is the inhibition of mitosis in fission yeast, which is mechanistically conserved in higher eukaryotes. *spWee1/spMik1* phosphorylates Cdc2 on Tyr15, which inhibits the Cdc2-Cdc13 activity. The G2-M transition is brought about by dephosphorylation of Tyr15 by Cdc25, which allows full activation of Cdc2-Cdc13 (Branzei & Foiani, 2008; Diffley, 2004; Lees, 1995; Nurse, 1991; Nurse, 1997; Nurse, 2002; Rhind *et al*., 1997). Inhibition of Cdc2-Cdc13 after DNA damage occurs through a double block mechanism whereby Wee1 is kept active and Cdc25 is kept inactive (Calonge & O’Connell, 2008).

### 1.3 – DNA Replication

The fundamentals of the process of DNA replication are conserved through all three domains of life archaea, prokaryotes, and eukaryotes. Replication initiates at specific
sites, known as replication origins, and double-stranded DNA (dsDNA) is unwound bidirectionally by DNA helicases to give rise to single-stranded DNA (ssDNA). DNA polymerases, which move behind the helicases, catalyze base insertion and pairing hence the formation of the new DNA chain. DNA replication is semi-conservative and continuous on the leading strand but discontinuous on the lagging strand owing to the fact that DNA polymerases only incorporate nucleotides in the 5’→ 3’ direction (Meselson & Stahl, 1958; Bessman et al. 1958; Okazaki et al., 1968; D. Leipe et al., 1998). In this section I will introduce a brief overview of prokaryotic and eukaryotic DNA replication.

1.3.1 – Prokaryotic DNA Replication

In all bacterial chromosomes and plasmids examined to date, DNA replication initiates at a single origin of replication. This initiation event is followed by bidirectional replication by diverging replication machineries (Reyes-Lamothe et al. 2008). In E. coli, replication of the 4.7 mega-base (Mb) chromosome initiates at oriC, a 260 base-pair (bp) sequence distinguished by two sequence motifs: DnaA binding repeats, and an AT-rich DNA unwinding element which marks where the replication bubble forms (Bramhill & Kornberg, 1988; Kowalski & Eddy, 1989; Gille & Messer, 1991).

DnaA, a structurally conserved protein and related to eukaryotic Orc proteins is an AAA+ (ATPase associated with various activities) protein. DnaA is required for recognition of oriC, initiation of replication and initial melting of the DNA duplex at oriC, allowing the assembly of the replisome (Felczak & Kaguni, 2004; Duderstadt et al., 2011). Prior to the onset of replication, ATP or ADP bound DnaA binds to the
DnaA binding motifs of oriC. As the replication initiates, additional copies of ATP-DnaA are recruited to oriC forming a helical complex that wraps duplex DNA around itself (Erzberger et al., 2006; Grimwade et al., 2007; Samitt et al., 1989; Scholefield et al., 2012). Binding of ATP-DnaA is essential to melt the DNA duplex at the DNA unwinding element of the oriC. ATP-DnaA can also directly bind the DNA unwinding element of oriC and actively unwind the duplex in an ATP-dependent manner (Bramhill & Kornberg, 1988; Ozaki et al., 2008; Speck & Messer, 2001). ADP-DnaA acts as a negative regulator of initiation. Despite the dependence of replication initiation on ATP-DnaA binding to oriC, DnaA remains tightly bound to ADP after initiation and this disfavours its assembly to oriC, thereby preventing reinitiation at oriC (Katayama et al., 2010).

Once DnaA recognises and processes oriC, DnaB helicase is loaded onto the single stranded region of the DNA unwinding element of oriC by the cooperative action of the DnaA and DnaB loader, DnaC (Marszalek & Kaguni, 1994; Wang et al., 2008; Wickner & Hurwitz, 1975). Primase DnaG is then recruited to DnaB, expelling DnaC and synthesizing an RNA primer, which serves as the loading site for the clamp loader, γ complex. The γ complex is comprised of five different subunits (γδιδ′ιχιφι) and loads the β sliding clamp (the processivity factor for PolIII) onto the primed DNA in an ATP dependent manner. Once the β sliding clamp is loaded onto the primed DNA, the clamp loader dissociates from the complex, leaving the closed clamp on DNA (Hingorani & O'Donnell, 1998; Jeruzalmi et al., 2001; Marians, 1992; Turner et al., 1999).

Polymerase PolIII and the γ complex have been shown to bind to the same interface of the sliding clamp and compete for the clamp, however, the ATP hydrolysis-dependent dissociation of the clamp loader complex allows PolIII to bind to the β clamp
(López de Saro et al., 2003; Naktinis et al., 1996). Speed and processivity of PolIII increases by coupling to the β clamp compared to when un-coupled from the sliding clamp (Maki & Kornberg, 1985; Stukenberg et al., 1991). In E. coli, PolIII drives both leading and lagging strand synthesis and is a trimeric complex of the α polymerase, ε proofreading exonuclease, and θ subunits (Studwell-Vaughan & O'Donnell, 1991). In case of circular chromosomes, appropriate replication termination is important to prevention of over-replication and maintenance of genome integrity. In E. coli, Ter sites direct the replication termination (discussed in section ‘1.4.5’). Tus protein binds to Ter sequences and blocks replication forks in a polar manner (Neylon et al., 2005).

1.3.2 – Eukaryotic DNA Replication

Unlike the situation in prokaryotes, in eukaryotes replication initiates at multiple origins of replication on each linear chromosome. Eukaryotic origins appear to be defined by the chromatin structure and local DNA topology rather than specific DNA sequences (Mechali, 2010). However budding yeast, S. cerevisiae, is known to have defined origins, also known as autonomous replicating sequences (ARSs), which are recognized and bound by the origin recognition complex (ORC) (Bell & Stillman, 1992; Stinchcomb et al., 1979). In fission yeast, S. pombe, origins often have an AT-rich sequence to which Orc4 binds, but deletion of these stretches has minimal effects on origin firing (Chuang & Kelly, 1999; Heichinger et al., 2006). In higher eukaryotes, origins of replication show even less sequence dependency but some reports indicate the presence of AT/CG rich sequences at some of the sites of ORC enrichment on chromosomes (Kong et al., 2003; MacAlpine et al., 2010).
Orc complex was first purified from budding yeast and the genes encoding the proteins identified (Bell & Stillman, 1992; Diffley & Cocker 1992; Bell et al., 1993). During G1, six subunits of ORC (Orc1-6) in association with scCdc6/spCdc18 and Cdt1 recognize and bind the replication origins and facilitate the ATP-dependent loading of the minichromosome maintenance proteins (Mcm2-7), which act as the main replicative helicase (Cocker et al., 1996; Liang et al., 1995; Maiorano et al., 2000; Nishitani et al., 2000; Speck et al., 2005). The MCM complex is assembled on DNA in an inactive double hexamer form that encircles the DNA duplex (Ervin et al., 2009; Gambus et al., 2011; Remus et al., 2009). The assembly of inactive MCM onto the ORC bound origins to form pre-replicative complexes (pre-RCs) is referred to as origin licensing (Diffley, 2004). This and initiation of replication is summarized in Figure 1.2.

In S phase, two kinases, cyclin dependent kinase (CDK) and Cdc7-Dbf4 (Dbf4-dependent kinase (DDK)), govern the activation of inactive MCM and hence origin firing. Several subunits of MCM are phosphorylated by DDK. The inhibitory domain of Mcm4 is inactivated by DDK phosphorylation of the N-terminal domain of the protein (Sheu & Stillman, 2010). Moreover, DDK phosphorylation sites on Mcm4 and Mcm6, which require prior phosphorylation by other kinases such as Mec1 (hATR), have been identified. Mutation of these DDK phosphorylation sites gives rise to a severe growth defect in the mcm4/mcm6 double mutant (Randell et al., 2010).

MCM activation involves recruitment of Cdc45 and GINS complexes to the MCM to form the CMG complex. Cdc45 and GINS proteins travel with replication forks and are essential for progression of the replication complex, also known as the replisome (Gambus et al., 2006; Moyer et al., 2006; Pacek et al., 2006; Tercero et al., 2000). It has been shown that the CMG complex exhibits significantly stronger helicase activity compared to that of MCM alone, suggesting that CMG is the functionally active
form of the helicase. Side by side comparison of activity of purified *Drosophila* MCM and CMG showed that CMG is several hundred fold more active as a helicase than MCM (Ilves *et al.*, 2010). While the inactive MCM complex encircles the duplex DNA, MCM in the active form encircles the leading strand and displaces the lagging strand (Fu *et al.*, 2011).

Several additional factors including Sld2/hRecQ4L, Polɛ, Sld3/hTreslin, MCM10, and scDpb11/spRad4/hTopBP1, transiently associate with MCM/Cdc45/GINS to form the active CMG complex. In both fission and budding yeasts CDK phosphorylates Sld2 and Sld3 creating conformational changes, which provide a binding site for scDbp11/spRad4 (Fukuura *et al.*, 2011; Masumoto *et al.*, 2002; Tanaka *et al.*, 2007; Yabuuchi *et al.*, 2006; Zegerman & Diffley, 2007). Sld2 and a non-catalytic unit of Polɛ direct GINS recruitment and Sld3 and Sld7 promote Cdc45 assembly to MCM (Handa *et al.*, 2012; Muramatsu *et al.*, 2010; Nakajima & Masukata, 2002; Tanaka *et al.*, 2011). Human Treslin has been shown to be subject of phosphorylation by CDK and the residues are conserved in yeast Sld3. This phosphorylation is essential for replication (Boos *et al.*, 2001; Kumagai *et al.*, 2011). Once the CMG complex is assembled, it drives unwinding of the DNA at the origin exposing ssDNA, which is coated by ssDNA binding protein, replication protein A (RPA). The MCM complex and Cdc45 then facilitate loading of Polα/primase onto ssDNA at the origin and initiation of a short (~10 nucleotide) RNA primer synthesis by Pri1 subunit of Polα. The 3’ end of the nascent strand then translocates from the primase active site (Pri1) to the polymerase active site (PolI). PolI subunit of Polα subsequently extends the RNA primer by approximately 20 nucleotides (Collins & Kelly, 1991; Frick & Richardson, 2001; Melendy & Stillman, 1993). This initiation event leads to loading of proliferating cell nuclear antigen (PCNA), the sliding clamp,
onto dsDNA by the clamp loader complex, replication factor C (RFC). PCNA was initially characterized as the processivity factor for Polδ (Prelich et al., 1987; Tan et al., 1986). PCNA, a homotrimeric ring shaped molecule, acts as a coordinator and binding platform for replicative and translesion polymerases as well as many other proteins involved in replication, repair, and cell cycle regulation (Moldovan et al., 2007). The clamp loader consists of five subunits (RFC1-5) which are homologous to each other and to the γ and δ′ subunits of E. coli γ complex (Cullman et al., 1995; O’Donnell et al., 1993). In short, the RFC complex loads PCNA onto the 3’ primer-template junction in an ATP dependent manner (Podust et al., 1998; Tsurimoto & Stillman, 1991). In S. cerevisiae, in a stepwise fashion, the RFC complex first binds two ATP molecules and then a third one when PCNA binds the complex. The complex then binds a fourth ATP when it binds the primer-template junction. DNA binding commits RFC to ATP hydrolysis, which then causes the RFC to eject leaving the closed PCNA ring encircling the dsDNA (Chen et al., 2009; Gomes et al., 2001). One of the replicative polymerases, Polε or Polδ is then recruited to PCNA, and binding to PCNA stimulates their activity (Chilkova et al., 2007). Polε synthesizes the leading strand, and Polδ catalyzes lagging strand formation (Miyabe et al., 2011; Miyabe et al., in press; Nick McElhinny et al., 2008; Pursell et al., 2007).

In eukaryotic cells, since replication commonly initiates from multiple origins on multiple chromosomes (Sclafani & Holzen, 2007), an inappropriate re-initiation event from a single origin can lead to gene amplification and genetic rearrangements or cell death (Green et al., 2010). Temporal separation of origin licensing and firing is the key to limiting DNA replication to exactly one round per cell cycle. CDKs and anaphase promoting complex/cyclin (APC/C) play direct roles in achieving this regulation. Licensing occurs only during the window between late M and G1 phase.
where CDK activity is low and the APC/C is still active and Mcm2-7 assemble on ORC-bound origins. Origin licensing is inhibited outside G1 via three different mechanisms. Firstly, CDK phosphorylates multiple pre-RC subunits, which inhibits licensing via different mechanisms. CDK dependent phosphorylation of MCM components during S, G2, and M, promotes the nuclear export of the free Mcm2-7, therefore inhibiting re-licensing (Hennessy et al., 1990; Labib et al., 1999; Liku et al., 2005; Nguyen et al., 2000; Tanaka & Diffley 2002). Phosphorylation of Cdc6 by CDK targets the protein for degradation, thereby inhibiting licensing outside G1 (Drury et al., 1997; Elsasser et al., 1999; Jang et al., 2001; Perkins et al. 2001). CDK phosphorylation inhibits the interaction between Cdt1 and Orc6 to inhibit helicase loading (Chen & Bell, 2011). Secondly, in higher eukaryotes, Cdt1 is stoichiometrically inhibited by geminin outside G1, however, ubiquitination by the APC/C during G1 targets geminin for proteolysis, allowing licensing to occur only in G1 (De Marco et al., 2009; Lee et al., 2004; Wohlschlegel et al., 2000). During S phase, Cdt1 is targeted to the protein degradation machinery by ubiquitination by the Cul4 ubiquitin ligase. PCNA is essential for Cul4 dependent degradation of Cdt1 (Arias & Walter, 2005; Arias & Walter, 2006). Lastly, as previously explained, origin firing and MCM activation requires CDK activity, which is cell cycle regulated.

Despite the fact that all potential origins are licensed before the start of the S phase, not all of the origins fire at the initiation of S phase, but rather fire in a coordinated and regulated manner. Some origins fire early in S phase and some are late firing (Blow & Dutta, 2005). Several factors contribute to enforcement of this regulation such as checkpoint signaling (which will be discussed in detail in the ‘checkpoint’ section), chromatin structure, nuclear positioning of chromatin in
mammals, and expression of certain proteins like those involved in origin firing e.g. Rif1 (Yamazaki et al., 2013).

In budding yeast, Sir3, which interacts with Sir2 histone deacetylase, suppresses firing at subtelomeric origins (Stevenson & Gottschling, 1999). Hyper-acetylation of H3 caused by the lack of H3 deacetylase Rpd3 enhances genome-wide origin firing in a manner independent of checkpoint signaling (Aparicio et al., 2004; Knott et al., 2009; Vogelauer et al., 2002). In fission yeast, telomeric repeats recruit Taz1, which inhibits Hsk1 dependent loading of Sld3 onto nearby origins. Paradoxically, heterochromatic centromere regions are replicated early in fission yeast due to Swi6/HP1 dependent recruitment of Hsk1 (Hayashi et al., 2009; Tazumi et al., 2012). These results emphasize the regulatory role of chromatin structure on origin firing.
Figure 1.2 | Initiation of DNA replication. A- Origin licensing: ORC binds origin of replication and in concert with Cdc6 and Cdt1 loads MCM complex to form Pre-RC. B & C- DDK & CDK-dependent association of GINS and Cdc45 with Pre-RC. This reaction requires catalytic activity of Sld2, Sld3, Dpb11, and Polɛ. D- Origin melting by activated CMG complex. E- recruitment of Polα to unwound origin results in primer synthesis and this in turn recruits PCNA and replicative polymerases to initiate DNA synthesis.
Little is known about the mechanism of replication termination in eukaryotes but it is thought to occur either at random when two converging replication forks meet between origins or when replication fork reaches the telomeric sequences at the end of the chromosomes (Edenberg & Huberman, 1975). Replication termination also occurs at programmed replication fork barriers (discussed in section ‘1.4.5’).

### 1.4 – Replication Fork Barriers

Once replication is initiated, replication forks (RFs) can encounter obstacles that result in RF slow down or arrest. In the literature, two terms are used to define the conformation of the arrested RFs: “collapsed” fork is used for the situation in which the replication holoenzyme (replisome) is dis-assembled from the nascent DNA strand and “stalled” fork defines the situation where the RFs are stabilised and the replisome remains at the site of last incorporated base. It must be noted that a collapsed fork does not contain a break in the nascent DNA strand which is the case for a “broken” fork. A number of factors commonly referred to as replication fork barriers (RFB) can compromise DNA replication. These obstacles can be divided into template lesions, secondary DNA structures, alternative DNA metabolism, replication inhibitors, and non-histone protein-DNA complexes (Lambert & Carr, 2005). They could either affect the replicative helicase activity or DNA polymerase progression. Uncoupling of the replicative helicase and polymerase, and processing of arrested RFs results in formation of ssDNA ahead of or behind the RF.
The replication machinery can be blocked by a variety of template lesions that are introduced due to endogenous or exogenous damaging agents like oxidative metabolic products, UV light, ionising radiation, MMS, and CPT. These agents compromise replication by creating DNA damage such as abasic sites, pyrimidine dimers, interstrand crosslinks. Depending on the type of the lesion, the replication machinery action is compromised either through blocking the helicase activity or the polymerase itself (Barbour & Xiao, 2003; Lambert & Carr, 2005; Setlow et al., 1963).

DNA sequences such as trinucleotide repeats (e.g. GGG or GAC), inverted and direct tandem repeats can undergo structural transitions and form secondary structures such as cruciforms, G quartets, hairpins, triplex DNA, left-handed Z-DNA. Again these can inhibit replication by compromising the action of either the replicative helicase or lagging or leading DNA polymerases (Pearson & Sinden, 1996; Trinh & Sinden, 1991; Sinden, 1994).

Since replication and transcription machineries share the same template, occasional collisions between the two are inevitable and these can interfere with fork progression (Brewer, 1988). Experimental evidence shows head-on collisions between DNA and RNA machineries to occur both in prokaryotes, and at the tRNA genes and rDNA loci of eukaryotes (Deshpande & Newlon, 1996; Takeuchi et al., 2003). Other observed transcriptional related phenomenon that can cause fork stalling are stable R loops and transcription-dependent replication inhibition at poly G/C repeats. Stable R-loops form when mRNA displaces the non-template strand, giving rise to a loop structure (Santamaria et al., 1998). In *E. coli* RNA mediated replication inhibition was reported at the site of G/C repeats, especially when the nascent RNA harbored G repeats (Krasilnikova et al., 1998).
Hydroxyurea (HU) is the most widely used replication inhibitor and acts through inhibiting the ribonucleotide reductase (RNR), hence disturbing the nucleotide pool in the cell. HU treatment in checkpoint proficient cells results in stably stalled forks but in checkpoint deficient cells stalled forks are unstable and collapse forming chicken foot structures (Lopes et al., 2001).

Non-histone protein-DNA complexes provide natural pause sites that are active in each cell cycle. In *E. coli* (as mentioned in section 1.2.1) replication terminates at Ter sequences. Similarly, in *Bacillus subtilis* a homodimer of RTP binds Ter sequences and arrests replication (Lewis et al., 1989; Smith et al., 1992). The *E. coli* genome harbours ten 23 bp Ter sites located in a region diametrically opposite to *oriC*. Ter sites act as polar fork barriers and are orientated in such way so as to allow the replication fork to pass through one cluster of Ter sites orientated in a non-active direction before meeting the Ter sites orientated in the non-permissive direction. This creates a ‘trap’ for replication forks enhancing the termination of the replication. Termination is achieved when the converging RF meets the arrested fork (Hill, 1992; Mulcair et al., 2006). Tus has been shown to interact with DnaB helicase in a yeast two hybrid assay and a Tus mutant was shown to have lower affinity for DnaB binding and lower barrier activity despite its normal binding affinity to Ter (Mulugu et al., 2001). Moreover, Tus has been shown to possess contra helicase activity specific to DnaB (Bedrosian & Bastia, 1991).

On the other hand, it has been shown that Tus interacts with a conserved cytosine residue in the Ter sequences which flips out and binds a pocket within Ter when RFs are passing in the non-permissive direction (Mulcair et al., 2006). These data suggest that the activity of the barrier is achieved via both physical hindrance of the helicase movement, and the inhibition of the helicase activity of DnaB.
In eukaryotes, the rDNA polar RFBs prevent collisions between the replication and transcription machineries and are conserved throughout evolution (Tsang & Carr, 2008). The *S. cerevisiae* rDNA barriers were the first eukaryotic site-specific replication barriers to be discovered. Budding yeast rDNA consists of approximately 150 tandem repeats of 9.1kb, in one array on chromosome XII. Each repeat contains 5S and 35S ribosomal genes separated by two non-transcribed spacers, one of which contains RFBs 1-3 that overlap with HOT1 recombination hotspot and the other contains the autonomously replicating sequence (rARS). Replication initiates at rARS but the leftward moving fork is arrested at Fob1 dependent programmed polar barriers RFB1 and RFB2 (Brewer & Fangman, 1988; Kobayashi & Horiuchi, 1996; Tsang & Carr, 2008). The activity of Fob1 has been shown to depend on Tof1 (*spSwi1*) (Hodgson *et al.*, 2007). In *S. pombe*, rDNA arrays reside at the two ends of the chromosome III.

Similar to budding yeast rDNA, each repeat contains the 35S transcriptional unit and an ARS within the non-transcribed region. Four RFBs are found in each of the *S. pombe* rDNA repeats at 5’ end of 35S transcriptional unit, the first is dependent on Swi1 and Swi3 members of replication fork protection complex (*ScTof1/*hTimeless & *ScCsm3/*hTipin respectively), and the second and third dependent on the *scFob1* homologue *spReb1*, and Swi1 and Swi3, and the final pause site is dependent on transcription of ribosomal RNA (Krings & Bastia, 2004; Planta *et al.*, 1995). The fission yeast mating type switching locus also contains a polar fork barrier known as replication termination site one (*RTS1*). Replication stalling at *RTS1* requires Swi1, Swi3, Rtf1, and Rtf2 proteins and is discussed in detail in section 1.11 (Codlin & Dalgaard, 2003; Dalgaard & Klar, 2000).
1.5 – Stabilisation of Arrested Fork

In eukaryotes, the replication checkpoint plays an important role in stabilizing arrested RFs and that in the absence of a proficient replication checkpoint replication forks undergo collapse at the site of the damage (Desany et al., 1998; Tercero et al., 2003). In both *S. cerevisiae* and *S. pombe* aberrant pathogenic structures accumulate and replication fails to resume in checkpoint deficient backgrounds in response to HU treatment (Lopes et al., 2001; Meister et al., 2005). Checkpoint defective budding yeast cells showed a considerable increase in the formation of X-shaped molecules corresponding to gapped regressed replication forks, when compared to a checkpoint proficient population (Cotta-Ramusino et al., 2005; Sogo et al., 2002). Moreover, the observation that checkpoint proficient replication mutants (such as polα) accumulate similar replication intermediates to that of HU treated checkpoint defective cells, suggests the replisome stabilizing function of the checkpoint proteins (Lopes et al., 2001; Sogo et al., 2002). Taken together, these studies exemplify the importance of the checkpoint response for survival of replication stress in both yeasts. Furthermore, studies have shown the high conservation of DDR in higher eukaryotes. Although the pathways are more elaborate in mammalian cells, the principles remain similar (Harper & Elledge, 2007). The intra-S (replication) checkpoint maintains the genome integrity by stabilization of replication forks, inhibition of late origin firing, and reducing the fork progression speed (Tercero et al., 2001). In response to replication perturbation, checkpoint prevents entry into mitosis by inhibiting activation of CDK, ensuring the completion of replication before cell division (Enoch et al., 1992, Lindsey et al., 1998; O’Connell et al., 2000). In addition, damage created due to problems in S phase, signals
through the G2/M DNA damage checkpoint, also known as the DNA integrity checkpoint to prevent entry into mitosis (Carr, 2002).

1.5.1 – DNA DAMAGE AND REPLICATION CHECKPOINTS

The DNA damage response is the signal transduction pathway that regulates different aspects of DNA metabolism with cell cycle progression. Checkpoints, elaborate network of proteins that sense and signal DNA perturbations, sit at the heart of this regulatory mechanism. While the observation that cells from ataxia telangiectasia (AT) patients did not delay the mitotic onset in response to radiation (and presence of DNA damage) predicted the existence of a DNA damage checkpoint (Painter & Young, 1980), the concept was not formalized until Weinert and Hartwell showed in S. cerevisiae that the rad9 mutant unlike wild type cells did not delay mitosis in response to DNA damage (Weinert & Hartwell, 1988). In a key experiment they showed that the radiation-induced loss of viability in rad9 mutant could be suppressed by allowing cells time to repair the DNA damage when mitosis was artificially blocked. Weinert and Hartwell then proposed the term ‘checkpoint’ to define regulatory pathways that delayed mitosis in response to DNA damage or replication stress (Weinert & Hartwell 1988; Weinert & Hartwell, 1989). Much of what is known about the checkpoint response was first discovered in yeasts but is highly conserved, here I summarise the mechanism of checkpoint activation.
Table 1.1

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<tr>
<th>Human</th>
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<th>S. cerevisiae</th>
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<td>MUS81</td>
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Table 1.1 | Table of nomenclature. Table showing the names of human, S. pombe, and S. cerevisiae functionally analogous proteins. Note Cds1 is the orthologue of CHK2 and Rad53 but functionally the equivalent of hChk1.
Checkpoint proteins form complexes in the vicinity of the DNA lesion. Primary to both DNA damage and replication checkpoints are “sensors”: the proteins that sense the DNA damage and replication stress. Mediator proteins facilitate recruitment of effector kinases to the sensor proteins. Sensors then activate the effector kinases, which in turn phosphorylate downstream targets resulting in inhibition of origin firing, replication fork stabilization, and cell cycle delay. Recruitment of particular mediators defines which effector kinase is used, thereby activating the damage or replication checkpoint.

Simplistically, the DNA damage checkpoint detects DNA lesions whereas the DNA replication checkpoint is activated when fidelity of replication is compromised (Branzei & Foiani, 2009; Harper & Elledge, 2007; Kai & Wang, 2003; Lambert et al., 2007). Central to checkpoint pathways are two members of phosphoinositol 3’-kinase-like-kinase (PIKK) family of proteins. ATM and ATR and their yeast orthologues Tel1 and spRad3/scMec1 respectively (see Table 1.1), along with their binding partners act as checkpoint sensors of DNA damage and initiate the checkpoint cascade. The main substrate for ATM is DSBs, therefore ATM is referred to as the sensor of the DNA damage checkpoint. The substrate for ATR is RPA-coated ssDNA exposed at arrested replication forks or processed DSBs, therefore ATR is referred to as the sensor of DNA replication checkpoint. In yeasts however, the role of Tel1 (ATM) in checkpoint activation is minimized and it is involved in maintenance of telomere stability. In contrast, spRad3 and scMec1, which were shown to be related to ATR, are critical for both the replication and DNA damage response. (Bentley et al., 1996; Greenwell et al., 1995; Morrow et al., 1995).

In higher eukaryotes, ATM is found in cells in the form of inactive dimers. Upon DNA damage, ATM is recruited to the DSBs via interaction with Nbs1 of MRN complex which results in its autophosphorylation (on S367, S1981, and S1893),
monomerisation, and hence activation (Bakkenist & Kastan, 2003; Kozlov et al., 2006; Uziel et al., 2003). Activated ATM then phosphorylates H2AX on S139 to give rise to γH2AX, which in turn recruits MDC1 to the site of the damage (Burma et al., 2001; Stewart et al., 2003). ATM then phosphorylates MDC1, which results in recruitment of RNF8. RNF8 in turn ubiquitinates H2A, H2AX, and γH2AX resulting in recruitment of RNF168 and mediator proteins BRCA1 and 53BP1 (Doil et al., 2009; Huen et al., 2007; Mailand et al., 2007). ATM then phosphorylates both BRCA1 and 53BP1, which results in recruitment of Chk2. ATM dependent phosphorylation of Chk2 results in its activation. Chk2 then phosphorylates CDC25A, which leads to its degradation and thereby prevention of cell cycle progression into mitosis (Cortez et al., 1999; Matsuoka et al., 2000; Xia et al., 2001). As previously mentioned (section 1.2), CDC25 phosphatase activity is required for the G2-M transition.

ATR and its yeast homologues (spRad3/scMec1) are found in cells in a complex with their binding partners hATRIP/spRad26/scDdc2 (Cortez et al., 2001; Edwards et al., 1999; Paciotti et al., 2000). In the presence of stalled or broken RFs or DNA damage, ATRIP facilitates the binding of the complex to the exposed RPA-coated ssDNA at the site of the damage. Similarly in budding yeast Ddc2 is required for recruitment of Mec1-Ddc2 to RPA-ssDNA and \( ddc2Δ smlΔ \) shows similar phenotype to that of \( mec1Δ smlΔ \) (Cortez et al., 2001; Paciotti et al., 2000; Zou & Elledge, 2003). Mec1-Ddc2 recruitment to RPA is dependent on a conserved domain of Ddc2. However, mutations in the conserved FAT domain of Mec1 resulted in decreased interaction of Mec1-Ddc2 with RPA, suggesting enhancement of Mec1-Ddc2-RPA interaction by Mec1 (Ball et al., 2005; Zou & Elledge, 2003). ATR-ATRIP recognition of ssDNA at the site of the damage is necessary for activation of the checkpoint but not sufficient. Once recruited to the site of the damage, ATR and its yeast homologues only
posses a basal kinase activity, which is not sufficient for full activation of a checkpoint cascade, and therefore ATR needs to be fully activated. Recruitment of a second sensing complex, the 9-1-1 complex, is essential for ATR checkpoint activation (Caspari et al., 2000; Kai et al., 2007; MacDougall et al., 2007; Melo et al., 2001). Independently of ATR, Rad17 (spRad17/scRad24) and four small subunits of replication factor C (RFC2-5) form the checkpoint clamp loader and recognize and bind to the 5’ junction between ssDNA and dsDNA dependent on RPA at the site of DNA damage. The Rad17-RFC complex then deposits the 9-1-1 complex (sp/hRad9-Hus1-Rad1, scDdc1-Mec3-Rad17), which shares structural similarities with PCNA, onto DNA (Doré et al., 2009; Majka et al., 2006A; Zou et al., 2003). Mechanistically similar to PCNA loading by RFC(1-5), 9-1-1 loading onto ssDNA-dsDNA junctions requires ATP hydrolysis by Rad17 which results in opening of the 9-1-1 ring and its loading on DNA (Bermudez et al., 2003; Bloom, 2009; Ellison & Stillman, 2003). In fission yeast Rad3 phosphorylates C-terminal of Rad9 on T412 and S423 and these phosphorylation are important for the DNA damage but not the replication checkpoint (Furuya et al., 2004). In S. cerevisiae, the C-terminus of Ddc1 was shown to stimulate the kinase activity of Mec1 and activate the checkpoint, and artificial Ddc1-dependent recruitment of Mec1-Ddc2 to chromatin resulted in checkpoint activation (Bonilla et al., 2008; Majka et al., 2006B). In higher eukaryotes, Rad9 is also phosphorylated but independently of ATR. Rad9 phosphorylation results in recruitment of TopBP1/spRad4/scDbp11, which is important for ATR kinase activity (Kumagai et al., 2006; Pfander & Diffley, 2011; Puddu et al., 2008; Takeishi et al., 2010). ATR also phosphorylates ATRIP on S68 and S72 (Edwards et al., 1999; Itakura et al., 2004), and itself on various residues including T1989. This autophosphorylation site was shown to be important for activation of ATR kinase activity by TopBP1. Rad9 recruited TopBP1 binds phosphorylated T1989 of
ATR. The interaction between the ATR activating domain (AAD) of TopBP1 and ATR-ATRIP stimulates ATR kinase activity. ATR then phosphorylates TopBP1, which in turn further stimulates the kinase activity of ATR (Delacroix et al., 2007; Kumagai et al., 2006; Liu et al., 2011). Moreover, in response to replication stress in budding yeast Dna2 nuclease-helicase has been shown to be able to activate Mec1 independent of its nuclease or helicase activities (Kumar & Burgers, 2013).

In response to replication stress, once ATR and its yeast homologs are recruited to the site of fork arrest and activated, they phosphorylate the mediator protein hClaspin and sp/scMrc1 (Errico et al., 2007; Kumagai & Dunphy, 2000; Xu et al., 2006; Zhao et al., 2003). Claspin and Mrc1 then recruit hChk1, spCds1, and scRad53, the effector kinase of the replication (intra-S) checkpoint (Alcasabas et al., 2001; Kumagai & Dunphy, 2000; Kumagai et al., 2004; Tanaka & Russell, 2001; Xu et al., 2006; Zhao et al., 2003). spCds1 and scRad53 are then phosphorylated by spRad3 and scMec1 respectively. This results in spCds1 dimerization and autophosphorylation, hence its full activation (Xu et al., 2006; Yue et al., 2011; Zhao et al., 2003). Similarly, in higher eukaryotes Claspin is required for Chk1 activation indicating the conservation of this pathway (Kumagai et al., 2004). However, the situation in metazoans is slightly different to yeasts, as during the course of evolution Chk2 (orthologue of spCds1/scRad53) has swapped roles with Chk1 and acts downstream of ATM (Labib & De Piccoli, 2010).

In response to DNA damage, ATR and its homologues spRad3 and scMec1 are also recruited to ssDNA at resected DSBs. In yeasts, this forms the major DNA damage response. As previously explained, once ATR and the 9-1-1 complex are recruited to the site of the lesion and activated, hTopBP1, spRad4 and scDbp11 are recruited by h/spRad9/scDdc1 and phosphorylated by ATR and its homologues, and this facilitates
the recruitment of the mediator proteins h53BP1, spCrb2, and scRad9 (not a part of 9-1-1 checkpoint clamp) respectively (Du et al., 2006; Mochida et al., 2004; Pfander & Diffley, 2011). In both yeasts, the functional form of spCrb2/scRad9 is a homodimer (Du et al., 2004; Soulier & Lowndes; 1999) and in S. pombe Crb2 recruitment to Rad4 is dependent on a CDK-dependent phosphorylation and hence is cell cycle regulated (Du et al., 2003). spCrb2 and scRad9 are then phosphorylated by spRad3 and scMec1 respectively, and this results in recruitment of spChk1 and scRad53. spRad3 and scMec1 then phosphorylate spChk1 and scRad53 respectively (Emili, 1998; Lopez-Girona et al., 2001; Qu et al., 2012; Schwartz et al., 2002; Vialard et al., 1998). Similarly, in metazoans ATR phosphorylates Chk1 (Guo et al., 2000). Chk1 phosphorylation results in its dimerization and full activation (Lee et al., 2003; Schwartz et al., 2003). Chk1 then targets Cdc25 and Wee1 to inhibit CDK and prevent entry to mitosis. It is worth noting that in budding yeast, Rad53 carries out most of the functions of Chk1 and Chk2, and acts as the main effector kinase of both the replication and damage checkpoints (Rhind & Russell, 2000). The S. pombe DNA integrity checkpoint pathways are summarized in Figure 1.3.
Figure 1.3 | DNA damage and replication checkpoints in *S. pombe*. A- model for the DNA damage and DNA replication checkpoints. In response to DNA damage, Rad3–Rad26 and Rag9–Rad1–Hus1 complexes bind to DNA independently of each other. Crb2 mediates the signalling to Chk1. Following phosphorylation, Chk1 subsequently regulates the kinases and phosphatases that determine the activity of Cdc2 resulting in cell cycle regulation. B- During DNA replication, the same core checkpoint proteins may associate with the replication complex. When replication stalls, Rad3 phosphorylates Cds1, a process mediated by Mrc1. Cds1 activity results in mitotic delay and also in the regulation of replication proteins.
1.5.2–CHECKPOINT RESPONSES TO DNA REPLICATION PERTURBATIONS

The replication checkpoint prevents firing of late origins and formation of new RFs in response to replication stress. In AT cells, unlike wild type, DNA synthesis is not inhibited in response ironizing radiation. This checkpoint defect was shown to be partly due to an inability to inhibit late origin firing (Larner et al., 1999; Lee et al., 1997; Zachos et al., 2003). Similarly, in budding yeast in response to DNA damaging agents late origins fire only in checkpoint mutants (Shirahige et al., 1998; Tercero & Diffley, 2001). Inhibition of late origin firing is brought about by inhibitory scRad53-dependent phosphorylation of Dbf4 and Sld3 in response to replication stress (Lopez-Mosqueda et al., 2010; Zegerman & Diffley, 2010).

Several lines of evidence suggest that the DNA replication checkpoint protects arrested forks probably by affecting the phosphorylation state of replication fork proteins, and keeping the stably stalled replisome at the site of nucleotide incorporation. This prevents the fork collapse and the need for reloading the replisome (Cobb et al., 2005; Enoch et al., 1992; Luca et al., 2004). When fission yeast cells are treated with HU, the DNA damage checkpoint is only activated in the absence of the replication checkpoint effector kinase, Cds1, suggesting that replication checkpoint protects arrested RFs in ‘stalled’ state and prevents formation of toxic DNA intermediates which activate the damage checkpoint (Lindsay et al., 1998).

In contrast, in budding yeast where the Cds1 homologue, Rad53, is required for both the intra-S and DNA damage checkpoints, loss of Rad 53 leads to both replication fork collapse and loss of cell cycle delay. In rad53 mutants, electron microscopy and two-dimensional (2D) electrophoresis showed the accumulation of aberrant DNA intermediates in response to HU treatment (Lopes et al., 2001; Sogo et al., 2002).
Moreover, chromatin immunoprecipitation (ChIP) showed that both leading and lagging strand DNA polymerases (polε and polδ) dissociate from the site of nucleotide incorporation in HU treated mec1 mutants (Cobb et al., 2003; Cobb et al., 2005; Luca et al., 2004). Similarly, it was recently shown in fission yeast that chicken foot structures accumulated in cds1 null cells in response to HU (Hu et al., 2012). Surprisingly, a more recent study in budding yeast showed the stable association of replisome with replication fork during replication stress in the absence of Rad53 or Mec1, and authors suggested the checkpoint regulation of replisome function rather than stability in response to replication stress (De Piccoli et al., 2012).

Studies on a separation of function allele of Mec1, mec1-100 which is unable to prevent late origin firing, showed that despite the defect in origin inhibition, forks are stably stalled in this mutant in response to replication stress, showing that the intra-S checkpoint influences fork stability independently of its role in the inhibition of late origin firing (Paciotti et al., 2001; Tercero et al., 2003). Replication fork-associated proteins that are not essential for replication but are necessary for maintaining the genome integrity include spSwi1/scTof1/hTimless, spSwi3/scCsm3/hTipin and sp/scMrc1/hClaspin. In both yeasts, Mrc1 and spSwi1/scTof1 proteins have been shown to travel with the replication fork and prevent the uncoupling of the replicative apparatus from the site of nucleotide incorporation, which indicates a role for checkpoint proteins in DNA replication (Gambus et al., 2006; Katou et al., 2003; Noguchi et al., 2003; Noguchi et al., 2004). In budding yeast, Mrc1 loading to replication forks requires Tof1 and Csm3 (Calzada et al., 2005). Stalling of arrested replication forks at programmed protein-DNA RFBs in both yeasts requires spSwi1/Swi3 and scTof1/Csm3 (Calzada et al., 2005; Shimmoto et al., 2009). The observations that the HU sensitivity of fission yeast mrc1Δ mutants is suppressed by
mutations in $cdc45$ and $mcm$, and that in budding yeast Mrcl forms a complex with Cdc45 and MCM, could suggest that Mrcl inhibits the Cdc45-MCM-GINS complex when the action of polymerase is compromised (Katou et al., 2003; Nitani et al., 2006). In fission yeast, regulation of cellular responses to replication arrest also requires the $spHsk1-Dfp1$ ($scCdc7-Dbf4$) kinase complex. Fission yeast Hsk1-Dfp1 has been shown to physically interact with the Swi1-Swi3 complex. In response to HU treatment, fission yeast Hsk1 and Dfp1 are phosphorylated by Cds1 and in budding yeast Rad53 phosphorylates Dbf4 (Lei et al., 1997; Shimmoto et al., 2009; Snaith et al., 2000; Takeda et al., 1999; Weinreich & Stillman, 1999).

Evidence suggests the checkpoint dependent down regulation of potentially dangerous recombinogenic events at stalled forks to prevent inappropriate fork processing. In fission yeast, Rad60, which is involved in the regulation of HR repair along with the Smc5/6 complex, is phosphorylated by Cds1 in response to HU and this results in its nuclear delocalization. RF arrest confers lethality in a $rad60$ mutant that inhibits regulation by Cds1 (Boddy et al., 2003). However, Miyabe et al. showed that a non-phosphorylatable $rad60$ mutant, which was located in the nucleus in HU, had no affect on viability (Miyabe et al., 2009). In checkpoint proficient fission yeast cells, Cds1 also phosphorylates the TXXF motif of Mus81 in response to HU induced replication stress and this results in Mus81 delocalization from chromatin. Mutations in the TXXF motif result in a hyper recombinogenic phenotype (Kai et al., 2005). Loss of Mus81 in $S. pombe$ results in a reduction in rates of spontaneous deletions between direct repeats, consistent with RNAi depletion of Mus81 in human cells that resulted in a reduction in rates of recombination between direct repeats (Blais et al., 2004; Doe et al., 2004). In addition, Dna2 activity is regulated via Cds1 phosphorylation, recruiting it to chromatin to prevent fork regression (Hu et al., 2012). In response to HU treatment in
both budding yeast and human cells Exo1 is phosphorylated in a Mec1/ATR dependent manner and this results in its degradation and negative regulation (El-Shemerly et al., 2008; Morin et al., 2008). Similarly, in *S. pombe* Rad3 is important to restrain Exo1-dependent resection at collapsed replication forks (Tsang et al., 2014). Together, these data suggest that the fork collapse seen in checkpoint defective cells in HU is due to the misregulation of multiple proteins.

It has also been shown that checkpoints up-regulate the dNTP pool in response to replication defects by modulating the activity of the RNR complex. In budding yeast, the RNR complex is inhibited by a small protein, Sml1, which is degraded upon entry to S phase and after DNA damage. Mec1 phosphorylates Dun1 kinase, which in turn phosphorylates Sml1. Dun1 dependent phosphorylation of Sml1 leads to Sml1 degradation and activation of RNR (Anderson et al., 2010; Zhao et al., 1998; Zhao et al., 2001). Moreover, checkpoint activation results in phosphorylation of Dif1, which facilitates the nuclear import of the small subunit of RNR. Phosphorylation of Dif1 results in its degradation, which allows the cytoplasmic interaction of the small RNR subunit with the large, hence activation of RNR (Lee et al., 2008; Wu & Huang, 2008). The evidence above demonstrates that replication checkpoint employs multiple pathways to ensure maintenance of perturbed RFs in a ‘stalled’ state that is competent to resume replication.

### 1.6 – RESTART OF ARRESTED REPLICATION FORKS

Replication fork barriers impose a great danger to replication progression and genome stability. Processing of arrested forks can result in genomic rearrangements. This
replication stress induced genome instability underlies a great proportion of the abnormalities in cancer cells (Branzei & Foiani, 2010). Cells have evolved multiple pathways to restart the arrested forks. Prokaryotic and eukaryotic replication restart pathways function in slightly different manners since firstly unlike the situation in eukaryotic cells, prokaryotic replication initiates from a single origin of replication and terminates at defined sites. Hence there is no converging fork to rescue the arrested forks. Secondly, prokaryotic cells lack the checkpoint functions that stabilize the arrested forks but instead readily reassemble collapsed RFs. In eukaryotes, the majority of arrested fork are held competent to resume replication by the action of the intra-S (replication) checkpoint and restart replication once the RFB is relieved (Lambert et al., 2007).

Stalled forks formed due to covalent modifications of DNA can activate specific repair mechanisms known as post-replication repair (PRR) which facilitates the damage bypass to avoid prolonged stalling of RFs which could lead to aberrant processing and DSBs (Yeeles et al., 2013). However, replication forks do collapse and in this case replication can be rescued by dormant origin firing and converging RFs. In the absence of a converging fork such as in subtelomeric regions, or in regions of poor origin density such as common fragile sites (CFSs) in humans, homologous recombination is required to set up a new replication fork (Carr & Lambert, 2013). In the following sections, I briefly summarize homologous recombination, prokaryotic replication restart, and eukaryotic processes involved in the restart of arrested forks including post-replication repair, and the role of recombination at collapsed replication forks in eukaryotic fork rescue is discussed.
1.7 – HOMOLOGOUS RECOMBINATION

Homologous recombination (HR) plays a central role in double strand break (DSB) repair, and the restart of stalled replication forks in all life forms. In eukaryotes, it is also important for the segregation of homologous chromosomes during meiosis, hence maintaining genome integrity. HR is induced in response to DSBs introduced endogenously, for example in the process of mating type switching in *S. cerevisiae* or during meiosis to facilitate chromosome segregation. It is also induced after exogenously-generated DSBs, for example as a result of exposure to mutagenic reagents or RF collapse. While HR is the main pathway for DSB repair in yeasts, in higher eukaryotes non-homologous end joining (NHEJ) is the preferred repair pathway in G1 and G2 but HR is essential in S phase (Kakarougkas & Jeggo, 2014).

HR relies on the presence of the homologous sister chromatid and is proposed to occur in several steps (Figure 1.4). In the first step, DNA ends at the site of DSB are processed in a polar manner (5’→3’) to produce 3’ overhangs. This step is known as ‘end processing’ or ‘resection’. In the strand invasion stage, one of the 3’ overhangs searches for the homologous sequence in the donor molecule and replaces the complementary strand. This results in the formation of an intermediate known as a ‘D-loop’. In the synthesis dependent strand annealing (SDSA) model of repair, once the missing information has been copied by the invading strand by repair synthesis, the strand is displaced and reanneals with the original template (Figure 1.4). The complementary strand is then replicated resulting in a non-crossover repaired DNA molecule. In the Szostak model of double strand break repair (DSBR) after formation of D-loop the second end is captured and forms a structure known as a double Holliday junction (dHJ). Resolution of the dHJ results in formation of crossover and non-
crossover products. In contrast, dissolution of dHJs through the action of RecQ helicase and TopoisomeraseIII gives rise only to non-crossover products (Hartlerode & Scully, 2009; Paques & Haber, 1999; van den Bosch et al., 2002; Wu & Hickson, 2003). The heart of the HR machinery is composed of the RAD52 epistasis group proteins (Rad50, Xrs2/Nbs1, Rad51, Rad52, Rad54, Rad55, Rad57). The genes encoding these proteins were first identified in budding yeast as IR or X-ray sensitive mutants that were HR defective. Homologues of these proteins have been identified in archaea, prokaryotes, and eukaryotes demonstrating the conservation of HR pathways throughout evolution (reviewed in Symington, 2002).
Figure 1.4 | Models of homologous recombination repair. DSB repair is initiated by 5'→3' resection of the broken ends creating 3' overhangs, which are initially coated with Rpa and subsequently Rad51. Rad51-dependent homology search and strand invasion creates the D-loop structure. In SDSA model of repair the invasive strand is displaced and repair synthesis occurs. In DSBR model of repair second end capture leads to formation of dHJ. Resolution of dHJ gives rise to crossover and non-crossovers as oppose to non-crossover production when dHJ is dissolved by helicase and topoisomerase.
1.7.1 – Proteins Involved in Resection

The MRN (Mre11, Rad50, Nbs1) complex (in *S. cerevisiae* MRX (Mre11, Rad50, Xrs1)) has distinct roles in telomere maintenance and DNA damage repair. In higher eukaryotes it is also required for checkpoint activation by recruiting ATM and consequently downstream signaling factors. MRN acts as the primary sensor of DSBs (Borde, 2007; Lavin, 2007; Petri & Stracker, 2003). The Mre11 subunit has been shown to associate tightly with coiled coil region of Rad50 (Lammens *et al.*, 2011; Lim *et al.*, 2011; Williams *et al.*, 2011), to have a distinct affinity for DNA ends (de Jager *et al.*, 2001A), and possess 3’→5’ exonuclease activity (Paull & Gellert, 1998). RAD50 consists of walker A and B ATP-binding cassettes that come together by antiparallel intervening coiled coil, and forms a homodimer that has structural homology to SMC (structural maintenance of chromosome) proteins (Hopfner *et al.*, 2000; Murray & Carr, 2008). It has been suggested that RAD50 in collaboration with Mre11 tethers the DNA ends together at the site of the DSB (de Jager *et al.*, 2001B). Nbs1, which harbors FHA, BRCT, and Mre11 and ATM interacting domains, has been shown to play a role in the translocation of the MRN complex to the nucleus and recruitment of DNA repair and damage checkpoint proteins to DSBs (Desai-Metha *et al.*, 2001; Lloyd *et al.*, 2009; Tsukamoto *et al.*, 2005).

It is believed that the MRN complex functions in HR by collaborating with *Sp*Ctp1/ScSae2/hCtIP to initiate end resection by endonucleatic cleavage of the 5’ ends internal to DSB ends. Ctp1 is phosphorylated both in a cell cycle and DNA damage dependent manner (Akamatsu *et al.*, 2008; Chen *et al.*, 2008; Clerici *et al.*, 2005; Huertas *et al.*, 2008; Limbo *et al.*, 2007; Sartori *et al.*, 2007; Symington, 2014). The MRX complex was reported to stimulate Sae2 activity in processing DNA hairpin
RecQ family helicases and Exo1 are implicated in the next stage of DNA processing in HR. Exo1 exhibits 5’→3’ exonuclease activity and is also involved in mismatch repair, degradation of collapsed RFs, telomere maintenance, and control of crossovers during meiosis (Cotta-Ramusino et al., 2005; Maringele & Lydall, 2002; Tran et al., 2004). Studies show the role of Exo1 in extensive resection of DSBs (Gravel et al., 2008; Nimonkar et al., 2008; Mimitou & Symington, 2008; Zhu et al., 2008). The observation of residual recombinational activities in exo1 mutants suggests that further parallel pathways were involved in end processing in HR. Human BLM and budding yeast Sgs1 (RecQ helicases) have been suggested to function in resection in concert with Dna2, which harbors 5’→3’ exonuclease activity. The helicase unwinds the DNA duplex and Dna2 degrades the 5’ strand (Nimonkar et al., 2008; Mimitou & Symington, 2008; Zhu et al., 2008). However, yeast cells lacking both Sgs1 and Exo1 show DNA break ends that are shorter than the initial cut fragment (Mimitou & Symington, 2008; Zhu et al., 2008). These data suggest a model of resection where MRN in collaboration with Ctp1 carries out initial processing of DSB, and Exo1 and Sgs1-Dna2 carry out long-range resection.

It is worth noting that end resection is a crucial step that determines the balance of repair choice between HR and NHEJ pathways. In yeast, HR functions mainly in S and G2, when the sister chromatid is available as template strand, whereas NHEJ is the main repair mechanism through G1 (Aylon et al., 2004; Ira et al., 2004). In mammalian cells NHEJ is also the main repair pathway in G2 but HR is required for repair of DSBs in heterochromatin (Goodarzi & Jeggo, 2013) and is essential in S phase. Cyclin dependent kinases are suggested as regulatory factors affecting this choice of pathways.
by periodically phosphorylating and dephosphorylating Ctp1 (Ctp1 is activated when phosphorylated). Several studies have made identical observations in budding yeast and human cells (Baroni et al., 2004; You & Bailis, 2010). Conversely, excessive resection in HR can lead to gross chromosomal rearrangements (GCRs), therefore, the extent to which resection is carried out needs to be regulated. This regulation is achieved by activation of checkpoint proteins. Excess exposure of ssDNA as a result of resection recruits RPA (replication protein A), which initiates checkpoint signaling. This leads to activation of the downstream checkpoint kinase cascade. Exo1 activity is modulated by checkpoint proteins (El-Shemerly et al., 2008; Morin et al., 2008; Tsang et al., 2014).

1.7.2 – Proteins involved in strand invasion

Recombinase Rad51, the homologue of bacterial RecA, is central to strand invasion and the homology search in mitotic HR. Rad51 exhibits ATPase activity and forms a right-handed nucleofilament on RPA-bound ssDNA, known as the presynaptic filament (Bianco et al., 1998; Bleuit et al., 2001; Shinohara et al., 1992; Yu et al., 2001). Despite the fact that RPA, the eukaryotic single stranded binding protein (SSB), that binds ssDNA, has been shown to stimulate RAD51 loading onto ssDNA by preventing formation of secondary structures, it has a higher affinity for ssDNA and is in abundance in vivo. This leads to a requirement for Rad52 and Rad55-Rad57 yeast mediator proteins to overcome the inhibitory effect of RPA and enhance Rad51 loading onto RPA-coated ssDNA (Gasior et al., 1998; Krejci et al., 2003; Bleuit et al., 2001; Symington, 2002).
Rad52 binds RPA-coated ssDNA and also interacts with Rad51, and this interaction is required for Rad51 loading onto RPA-bound ssDNA (Krejci et al., 2002; Seong et al., 2008; Shinohara & Ogawa, 1998; Shinohara et al., 1992). Substoichiometric amounts of Rad52 catalyze Rad51 nucleofilament formation by polymerization of nucleated Rad51 molecules (Song & Sung, 2000; Sugiyama et al., 1998; Sung et al., 2003). Rad52 also exhibits Rad51-independent strand annealing activity which mediates single strand annealing (SSA), a sub-pathway of HR, and promotes second end capture in post Rad51-dependent invasion steps (Ivanov et al., 1996; McIlwraith & West, 2008; Sugiyama et al., 2006). Similar to Rad52, the Rad55-Rad57 heterodimer directly interacts with Rad51 and can load Rad51 onto RPA-coated ssDNA. Although Rad55-Rad57 exhibits ATPase activity, it cannot catalyze the strand exchange (Hays et al., 1995; Johnson & Symington, 1995; Sung, 1997). Co-filamentation of Rad55-Rad57 with Rad51 stabilizes the presynaptic filament against Srs2 anti recombinase activity (Fung et al., 2009).

Strand invasion and homology search starts once Rad51 is successfully loaded on ssDNA. Rad51 with the assistance of two members of Snf2/Swi2 family of DNA-dependent ATPases, Rad54 and Rdh54, then catalyzes the formation of the D-loop structure (Petukhova et al., 1998; Van Komen et al., 2000). Rad54 and Rdh54 were also found to stabilize Rad51-coated ssDNA and promote branch migration in early stages of HR (Bugreev et al., 2007; Mazin et al., 2003; Mazin et al., 2010; Solinger & Heyer, 2001). However, Rad54 acts as a negative regulator of Rad51 in late stages of HR by preventing inappropriate Rad51 binding and removing Rad51 from dsDNA to promote DNA synthesis (Chi et al., 2006; Heyer et al., 2006; Li & Heyer, 2009; Solinger et al., 2002). Similarly, the Fbh1 and Srs2 helicases were found to have Rad51-antagonistic activity, which prevents Rad51 filament formation. Srs2 binds Rad51 and promotes
ATP hydrolysis within the presynaptic filament causing its weakening and disassembly of the Rad51 filament. This activity is of particular importance as inappropriate non-allelic or ectopic recombination increases loss of heterozygosity (LOH) and GCRs. These helicases channel break repair through the SDSA pathway, which reduces the production of crossovers (Antony \textit{et al.}, 2009; Gangloff \textit{et al.}, 2000; Lorenz \textit{et al.}, 2009; Osman \textit{et al.}, 2005).

An additional complex, Swi5-Sfr1, is required for HR in fission yeast and mammalian cells (Akamatsu & Jasin, 2010; Kurokawa \textit{et al.}, 2008). The Swi5-Sfr1 complex interacts directly with Rad51 and stimulates strand exchange. The equivalent \textit{S. cerevisiae} complex, Sae3-Mei5, is meiosis-specific, interacts with Dmc1 and promotes assembly of Dmc1 on meiotic chromosomes (Hayase \textit{et al.}, 2004; Tsubouchi & Roeder, 2004).

### 1.7.3 – PROTEINS INVOLVED IN RESOLUTION

Once strand invasion has occurred HR can proceed via two alternative pathways, in SDSA invading strand in the D-loop structure is displaced. Alternatively second end capture sets the preference of DSBR. Little is known about factors affecting this preference. It has been proposed that filament displacement in SDSA is mediated by \textit{scSrs2}, \textit{spFml1} or \textit{hBLM} & \textit{RTEL1}. It is notable that these helicases favour the formation of non-crossover products (Barber \textit{et al.}, 2008; Sun \textit{et al.}, 2008; van Barbant \textit{et al.}, 2000). In DSBR, the second end is captured to form a dHJ. Lao \textit{et al.} (2008) showed that formation of dHJs is promoted by Rad52 in \textit{S. cerevisiae}. Resolution or dissolution of dHJ in DSBR is the key step that predicts the production of non-
crossovers and crossovers based on symmetry of cleavage or helicase and topoisomerasesIII activity respectively. In bacteria, the RuvC resolvase carries out the symmetrical resolution of HJs. Although no structural homologue of RuvC has been reported in eukaryotes, in human and S. cerevisiae symmetric resolution of dHJs is carried out by structure specific endonucleases hGEN1 and scYen1 (Ip et al., 2008), and hMUS81-EME1 and scMus81-Mms4. GEN1 dimerises on HJs and resolves them by introducing dual nicks in the pair of non-crossing strands (Rass et al., 2010). S. pombe, on the other hand, has no known homologue of GEN1/Yen1. However, the Mus81-Eme1 complex has been proposed to carry out the resolution of dHJs. The role of the heterodimeric Mus81-ScEme1/ScMms4 endonuclease complex in cellular growth and processing of perturbed replication forks has been the subject of various studies (Boddy et al., 2000; Boddy et al., 2001; Kai et al., 2005; Kaliraman et al., 2001; Interthal et al., 2000). In vitro and in vivo studies have shown that the Mus81 complex has a substrate specificity for HJs, nicked HJs, D-loops, RFs with 5’ ends at the junction point, and 3’ flaps (Bastin-Shanower et al., 2003; Fricke et al., 2005; Osman et al., 2003; Whitby et al., 2003). Recently, Mus81-Eme1 has been shown to have resolvase activity in concert with Slx1-Sxl4 in mammalian cells (Castor et al., 2013; Garner et al., 2013; Wyatt et al., 2013).

RecQ helicases (SpRqh1, ScSgs1, hBLM) in conjunction with Topoisomerase III are proposed to catalyse dHJ dissolution, which also lead to non-crossover products. In this process, two individual junctions of dHJ are migrated towards each other by the helicase to form a hemicatenane, which is then disentangled by TopIII to produce non-crossovers (Hope et al., 2007; Oh et al., 2007; Wu & Hickson, 2003). However, single HJs that arise during SDSA by branch migration of the initial D-loop cannot be dissolved and require resolution by structure specific endonucleases (Schwartz & Heyer
In vivo analysis of meiotic and mitotic recombination intermediates has shown the presence of single and double HJs in both prokaryotes and eukaryotes (Bzymek et al., 2010; Cromie et al., 2006; Kobayashi & Ikeda, 1983; Schwacha & Kleckner, 1995). It must be noted that while the same steps are involved in meiotic and mitotic HR, in some cases additional or different factors are required for meiotic recombination or vice versa and these are not discussed here (see Ehmsen & Heyer, 2010 for more details on meiotic recombination).

1.8 – Prokaryotic replication restart

oriC is the initiation point of replication in the 4.7Mb circular E. coli chromosome. Since the replication starts in a bidirectional manner, each fork needs to replicate a distance of 2.3Mb within which many obstacles can compromise the progression of the fork and completion of DNA replication. Multiple pathways have been shown to take part in prokaryotic fork rescue.

It has been well established that in prokaryotes recombinational events play a major role in reassembling a unidirectional RF when forks collapse (Sandler & Marians, 2000; Xu & Marians, 2003). PriA, which was originally discovered as an essential factor in the primosome required for conversion of circular single stranded DNA of φX174 phage to double stranded DNA, plays a central role in replication fork restart (Arai & Kornberg, 1981). The primosome is composed of PriA, PriB, PriC, DnaB helicase, DnaC, DnaT, and DnaG primase (McMacken et al., 1977; McMacken & Kornberg, 1978). Please note that a different primosome is required for initiation of replication at oriC in E. coli (Bramhill & Kornberg, 1988). Studies of priA, priB, and
priC mutants suggest existence of at least two pathways for restarting the arrested replication forks in *E. coli*. *priA* dependent and independent pathways. *priA* mutants are viable but have a very sick phenotype and are induced for the SOS response. *priB* and *priC* mutants are also viable and exhibit a more normal phenotype. *priB* and *priC* double mutants are still viable but show a sick phenotype. *priA* and *priC* or rep double knockouts are lethal. Collectively, these results suggest that the PriA dependent fork restart pathway requires PriB/PriC, whereas PriA independent pathway requires PriC and Rep, and that the PriA dependent pathway is the major fork rescue pathway considering the severe phenotype of *priA* mutants (Gregg *et al.*, 2002; Heller & Marians, 2005A; Nurse *et al.*, 1991; Sandler, 2000; Sandler *et al.*, 1999).

Experimental evidence did not uncover a direct role for PriA in cellular replication, however it was revealed that PriA has a high affinity for D-loops and arrested fork-like structures and binding to these stimulates its ATP hydrolysis activity in presence of single stranded binding protein (SSB). These data suggest that PriA can sense the stalled replication forks and promote reloading of the replisome through either recombination or non recombination-dependent events. SSB is proposed as the recruitment factor of PriA to the site of damage as PriA can bind SSB through its SSB interacting zinc motif (Cadman & McGlynn, 2004; McGlynn *et al.*, 1997; Mizukoshi *et al.*, 2003; Tanaka *et al.*, 2003). The role of PriA in replication restart has been suggested to function in two ways, first by creating a single stranded nick on the lagging strand, creating a platform for DnaB loading, and second by enhancing the origin-independent loading of the replisome at the site of arrested forks (Heller & Marians, 2006). In the PriA-dependent replication resumption pathway, PriA, PriB, and DnaT catalyze reloading of the replisome onto D-loop structures or recombination joint molecules.
(Heller & Marians, 2005B). In the PriA independent pathway, PriC interaction with Rep coordinates the function of Rep and DnaB for fork restart (Heller & Marians, 2005A). In replication mutants (polIII or rep mutants), the fork reversal model of fork rescue has been proposed to restart the arrested forks. In this model, RecBCD prevents the RuvABC mediated chromosome linearization and an unidentified helicase enhances the fork reversal (RecA has been shown to mediate this reaction in dnaB mutants). The resulted ‘chicken foot’ structure is then recognized by PriA which promotes the reassembly of the replisome (Flores et al., 2002; Grompone et al., 2002; Gruss & Michel, 2001). In the UV induced fork arrest response, either RecFOR and RecA strand invasion activity mediates the bypass of the damage by nucleotide excision repair (NER), or the RecG catalyzed fork-reversal-mediated template strand switching assists the bypass of the damage, both of which requires PriA (Grompone et al., 2004; Singleton et al., 2001). However, in a gyrase mutant that accumulate positive supercoiling, no evidence of fork reversal has been observed, as RecBCD is not required for cell viability. The PriA dependent viability in this mutant suggests the direct PriA-dependent fork restart (Grompone et al., 2003).

1.9 – EUKARYOTIC REPLICATION RESTART

While cells employ multiple pathways to account for errors in replication fidelity (mismatch repair/MMR) or template lesions, the continuous endogenous and exogenous insults lead to occasional damage on DNA. This damage is usually repaired by base excision repair (BER) or nucleotide excision repair (NER). However, if left unrepaired, the damage can compromise replication progression. When RFs meet template lesions,
BER and NER can no longer be utilized since the duplex is unwound and the complementary strand would not available to serve as a template for resynthesizing the excised region. While template lesions on the lagging strand are not thought to impact on replication progression due to the discontinuous nature of lagging strand replication, post replication repair pathways rescue replication stalls at leading strand template lesions. This is achieved through covalent modifications of PCNA (see Figure 1.4). In the translesion synthesis (TLS) pathway of PRR, monoubiquitination of PCNA recruits specialized translesion polymerases to bypass the damage. Polyubiquitination of PCNA leads to activation of error-free damage avoidance pathways to bypass the damage (Lehmann & Fuchs, 2006; Branzei & Foiani, 2010; Ulrich, 2011).

Hoege et al. first reported in *S. cerevisiae* ubiquitination of PCNA on K164 in response to DNA damage (Hoege et al., 2002) and high conservation of K164 ubiquitination was shown in all eukaryotic species analyzed (Anderson et al., 2008; Arakawa et al., 2006; Frampton et al., 2006; Leach and Michael 2005). Mono-ubiquitination of PCNA is mediated by E2 ubiquitin conjugating enzyme Rad6 and its E3 ubiquitin ligase partner Rad18 (Rad6/Rad18 complex) (Frampton et al., 2006; Hoege et al., 2002; Kannouche et al., 2004; Watanabe et al., 2004). This monoubiquitination of PCNA promotes activation of Y-family (Polη, Polι, Polκ, and REV1) DNA TLS polymerases (Stelter & Ulrich, 2003). These polymerases lack 3′→5′ endonuclease proofreading ability, and have more open active site than replicative polymerases which enables them to accommodate and replicate irregular templates (Lehmann et al., 2007; Prakash et al., 2005). These characteristics enable TLS polymerases to replicate damaged templates but with low fidelity and increased mutagenesis. Therefore, TLS is also known as the error-prone pathway of damage tolerance (Ulrich & Takahashi, 2013). Y-family TLS polymerases harbor ubiquitin
binding motifs which can bind to ubiquitinated PCNA. Also, various TLS polymerases (Polη, Polι, Polκ) interact with PCNA via their PCNA interacting peptide (PIP) motifs. It has been shown that these motifs are important for PCNA binding and play a role in the regulation of function of TLS polymerases in response to DNA damage (Bienko et al., 2005; Guo et al., 2006; Parker et al., 2007; Plosky et al., 2006; Prakash et al., 2005; Wood et al., 2007). Zhuang et al. (2008) using a reconstituted yeast system showed that the switch between Polδ and Polη is dependent on K164-monoubiquitination of PCNA and fork stalling. Similarly, the ubiquitin dependent exchange of the replicative polymerase for a TLS polymerase was demonstrated in human cell extracts (Masuda et al., 2010).

In response to fork stalling, poly-ubiquitination of PCNA is promoted by the heterodimeric E2 complex Ubc13/Mms2 and E3 ligase Rad5 (Figure 1.5). Ubiquitin chain formation is catalyzed on mono-ubiquitinated K164 via a non-canonical K63 linkage, which is distinct from K48-linked ubiquitin chain involved in proteasome-dependent protein degradation (Frampton et al., 2006; Hoege et al., 2002; Hofmann & Pickart, 1999; Ulrich & Jentsch, 2000). In metazoans, while RAD18 and UBC13 are essential for PCNA polyubiquitination, MMS2 is not required and embryonic stem cells from mms2 knockout mice show PCNA polyubiquitination (Brun et al., 2008). However, human homologues of Mms2, HLTF and SHPRH, have been shown to take part in polyubiquitination of PCNA (Motegi et al., 2008; Unk et al., 2008). In budding yeast, rad5Δ, ubc13Δ, or mms2Δ mutants show increased spontaneous or damaged induced mutagenesis, and rev3 mutation confers a synergistic UV sensitivity in rad5Δ or ubc13Δ mutants (Broomfield et al., 1998; Brusky et al., 2000; Johnson et al., 1992). In higher eukaryotes, reduction of expression of HLTF/SHPRH or inhibition of PCNA polyubiquitination results in UV induced mutagenesis (Chiu et al., 2006; Motegi et al.,
The evidence above indicates that PCNA polyubiquitination promotes an error-free pathway of PRR. Polyubiquitination is proposed to control error-free PRR via either a recombination-mediated template switching mechanism where the blocked strand invades the sister chromatid to form a D-loop or dHJ intermediates which facilitates the lesion bypass, or a fork reversal model where a regressed chicken foot intermediate forms allowing the fork to restart (Atkinson & McGlynn, 2009; Branzei & Foiani, 2010; Zhang & Lawrence, 2005). Although the exact molecular machinery governing the error-free pathway of PRR remains illusive, evidence suggests the involvement of members of FANCM family of helicases in this pathway. In budding yeast, mph1 mutants show sensitivity to MMS, and increased spontaneous mutation rates that depend on TLS polymerases, suggesting the suppression of error-free PRR in the absence of Mph1. Interestingly while mph1 mutants are HR proficient, a rad51 mph1 double mutant shows the same rate of spontaneous mutations (Scheller et al., 2000; Schürer et al., 2004). Moreover, deletion of mph1 suppresses the Rad51-dependent sister chromatid exchanges formed when cells are exposed to DNA damaging agents (Ede et al., 2011). Together, this evidence suggests a role for Mph1 in HR dependent error-free pathway of PRR. Similarly in S. pombe, fml1 (the FANCM homologue) mutants show sensitivity to crosslinking agents and Fml1 promotes recombination at stalled forks suggesting its role in error-free PRR (Sun et al., 2008).

PCNA is sumoylated on K164 and to a lesser extent on K127 by the Sumo E2 ligase Ubc9 and Sumo E3 conjugator Siz1 in S. cerevisiae (Hoege et al., 2002) and this modification has also been reported in higher eukaryotes (Arakawa et al., 2006; Gali et al., 2012; Gohler et al., 2008; Leach & Michael 2005; Moldovan et al., 2012). In budding yeast, PCNA sumoylation recruits Srs2 to the stalled fork. As previously described Srs2 is a helicase with anti-recombinogenic properties and acts via direct
disruption and prevention of formation of Rad51 filaments (Krejci et al., 2003; Veaute et al., 2003). Srs2 was found to physically interact with sumoylated PCNA via its C-terminal SIM motif, and elevated levels of recombination and crossover events were seen upon loss of PCNA sumoylation, suggesting an inability in recruitment of Srs2 to the site of damage and an anti-recombinase role of Srs2 in repair choice (Le Breton et al., 2008; Papouli et al., 2005; Pfander et al., 2005; Robert et al., 2006). The anti-recombinase activity of Srs2 suppresses inappropriate HR-dependent processing of stalled forks and favors the RAD6 repair pathway (Marini & Krejci, 2010).
Figure 1.5 | Posttranslational modifications of PCNA govern translesion synthesis. During unstressed replication PCNA retains posttranslational neutral status. In response to template lesions, mono and poly ubiquitination of PCNA in response to replication stress or DNA damage lead to error-prone or error-free bypass of the damage respectively. Rad6/Rad18 mediate monoubiquitination and Ubc13-Mms2/Rad5 catalyse polyubiquitination of the sliding clamp.
In eukaryotes, in the absence of checkpoint functions or in situations where the checkpoint fails to stabilize the arrested replication forks when DNA polymerase function is compromised, forks collapse and HR is required for fork restart. Unlike bacteria, eukaryotic cells lack a PriA-like system to reload the replisomes on DNA when forks collapse. Viability in certain *S. cerevisiae* replication mutants (*mec1-srf, pol30* and *rad27*) has been shown to require recombination proteins (Merrill & Holm, 1998; Merrill & Holm, 1999; Symington, 1998). Rad52 and Mre11 form foci in response to HU treatment in checkpoint deficient cells, where the replisome dissociates from the nascent DNA strand (Lisby et al., 2004). Similarly in *S. pombe* HR is required for viability in *rad2* (FEN1) null cells (Murray et al., 1994) and Rad52 foci are seen in response to HU in *cds1* null cells, defective in the intra S phase checkpoint (Irmisch et al., 2009). Checkpoint deficient *S. pombe* cells cannot complete the replication and accumulate aberrant structures at the site of fork collapse. The aberrant structures are suppressed in a *cds1 rad51* double knockout background but the viability is not restored consistent with homologous recombination being necessary for replication restart of collapsed forks (Meister et al., 2005). In *Xenopus* extracts, GINS complex and Polɛ reloading at forks collapsed at ssDNA lesions depends on Rad51 and MRN complex (Hashimoto et al., 2012). The exact pathways by which HR rescues the collapsed forks remain elusive. Break induced replication (BIR) has been suggested as a possible non-reciprocal recombination pathway to restart replication of one-ended broken forks. BIR depends on all the factors involved in passive replication with the exception of ORC and Cdc6 (Llorente et al., 2008; Sakofsky et al., 2012). BIR could also be induced via cleavage of a collapsed fork to form a one-ended DSB by nucleases such as Mus81 but
studies on HR-dependent replication restart at programmed polar RFBs have not found any evidence for such an intermediate (Lambert et al., 2010; Mizuno et al., 2013; Larsen et al., 2014). However, these studies on programmed polar RFBs in both yeasts have revealed the influence of HR-dependent pathways on restoring collapsed replication forks and completing DNA replication.

Programmed polar RFBs in the budding yeast rDNA have been extensively studied to understand the fate of stalled forks during unperturbed replication. RFB repeats arrest replication forks in a unidirectional manner to avoid the collisions between replication and transcription machineries. Fork arrest requires Fob1 and arrested forks are then stabilized via a checkpoint independent function of Tof1 and Csm3 (Calzada et al., 2005). A recombination hotspot (HOT1) is also located within the rDNA repeats and this overlaps with the RFBs. The regulation of recombination in the rDNA locus is exerted through the actions of Fob1, the transcription silencing protein Sir2 and cohesin (Kobayashi & Horiuchi, 1996; Tsang & Carr, 2008). Equal sister chromatid recombination events, which are dependent on Fob1 and transcription, are promoted by HOT1 to maintain the rDNA homeostasis.

HR also plays an important role in mating type switching of fission yeast. The mat locus consists of the transcriptionally active mat1 and two silent donor cassettes mat2P and mat3M. The polar RTS1 barrier optimizes the switching process by preventing the RFs moving through the locus in the telomere to centromere direction, which could interfere with efficient switching process at mat1. In switchable cells a Swi1/Swi3 and Swi7-dependent polar imprint is made on the lagging strand on the centromere side of mat1 during the first round of replication. In the second round of replication this imprint on the leading strand in one of the daughter cells leads to formation of a one-ended DSB, the 3’ end of which invades the homologous region in
mat2 or mat3 and initiates gene conversion. The second strand is then synthesized using the first strand as template. This results in formation of two daughter cells one of which has switched mating type (Klar, 2007). In the absence of the donor loci the one-ended DSB must be repaired using the sister chromatid and this is dependent on HR and Mus81 to restart the fork (Roseaulin et al., 2008).

Recent studies in fission yeast have employed the mating type locus polar replication termination sequence (RTS1) to investigate the events involved in initiating replication restart when forks are arrested at a specific site in every cell cycle. When integrated at an ectopic locus, RTS1 was shown to induce Rad52- and Rad51-dependent recombination between direct repeats by 50 fold. Whereas, in the presence of the RecQ helicase Rqh1 (WT), the observed recombination events were characterized as conversion events, deletion of Rqh1 resulted in deletion type events suggesting a DSB-free initiation of recombination at RTS1 (Ahn et al., 2005). In another fork stalling system developed in Carr’s laboratory, two inverted repeats of RTS1 flanking a marker at an ectopic locus caused rapid collapse of replication forks, survival of which was dependent on recombination proteins (Lambert et al., 2005). This led the authors to propose that the replication rescue in this system occurs via HR without a DSB intermediate as in contrast to the one-ended DSB at the mating type locus (Roseaulin et al., 2008) a DSB could not be seen even in backgrounds where such a break would not be repaired (Lambert et al., 2005; Lambert et al., 2010; Mizuno et al., 2009). In the following sections I briefly summaries the results of these studies.
1.11 – RTS1 FUNCTIONS

As previously explained, RTS1 is necessary for efficient mating-type switching, and was the first replication fork barrier implicated to have a role in cellular differentiation (Dalgaard & Klar, 2001). RTS1 contains two cis-acting elements known as region A and region B, which cooperate for efficient replication termination. Region A is a ~64-bp purine-rich region of %73 GA content, and region B contains four copies of a ~60-bp motif. Similar to fork pausing at rDNA RFBs, Swi1 and Swi3 are required for replication termination at RTS1. Fork stalling at RTS1 also requires DNA binding proteins Rtf1 and Rtf2. Rtf2 binds the purine-rich region and enhances the efficiency of the barrier but is not necessary for its function, and the rtf2Δ phenotype can be suppressed by Rtf1 overexpression. Rtf1 binds the repeat motifs and this interaction is essential for the RTS1 barrier activity (Codlin & Dalgaard, 2003). Rtf1 consist of two myb/SANT domains one of which interacts with both regions A and B of RTS1, whereas the second domain does not show great DNA binding affinity. The C-terminal tail of Rtf1 is required for replication arrest at RTS1, and mediates Rtf1 self-dimerization/polymerization. A model for replication termination at RTS1 was proposed where four Rtf1 molecules bind the four repeats within the region B of RTS1. More Rtf1 is recruited to region A via Rtf1 C-terminal domain self polymerization activity resulting in an efficient polar fork stalling at RTS1 (Eydmann et al., 2008) (see Figure 1.6).
Figure 1.6 | Model for Rtf1 mediated replication fork arrest at RTS1. Four Rtf1 molecules bind the four repeats of the RTS1 (region B). C-terminal self polymerisation activity of Rtf1 results in recruitment and interaction of more Rtf1 with region A of the RTS1. This results in efficient polar fork stalling activity of the barrier. Rtf2, Swi1, and Swi3 also interact with the barrier.
1.12 – RTS1, FORK COLLAPSE AND RESTART

To create a difficult-to-replicate region and study RF restart, Lambert et al. (2005) created a system, named RuraR, by integrating inverted repeats of RTS1 separated by a 1.8kb sequence containing the ura4 gene at the ura4 locus on ChrIII of fission yeast (Figure 1.7). The direction of replication is centromere to telomere at the ura4 locus due to the presence of the efficient double origin ARS3004/3005 centromere proximal to the locus. To regulate fork stalling at ura4 locus, the Pnmt41 inducible promoter was used to control Rtf1 expression (Lambert et al., 2005). Induction of fork stalling in this system resulted in gene inversion, site-specific GCRs, and HR-protein dependent cell viability. Recombination proteins were recruited to the site of fork stalling and GCRs were recombination dependent. Moreover, GCRs were not dependent on checkpoint functions, suggesting the rapid collapse of the replisome at RTS1.

In a later variant of the stalling system developed by Mizuno et al. (2009), two inverted repeats of the ura4 gene flanked by two RTS1 inverted repeats (named RuiuR) were integrated at ura4 locus creating a small palindrome. The palindrome was interrupted by insertion of a 14-bp spacer sequence (Figure 1.7). Induction of fork stalling in the palindrome system led to loss of viability due to HR-dependent formation of acentric and dicentric palindromic chromosomes. These were also observed at a lower frequency in the inverted repeat (RuraR) system. It should be noted that these palindromic chromosomes are sister chromatid fusions (see Figure 1.8) and, while the acentric fails to attach to the mitotic spindle, the dicentric alignes correctly but forms a chromosome bridge at anaphase.

In both systems no evidence of DSBs was detected even in strains defective for factors required for processing DSBs (Lambert et al., 2010; Mizuno et al., 2009). This
evidence led to the model of a DSB independent mechanism of HR fork restart: a strand invasion event facilitated by recruitment of HR proteins to the nascent strand at the site of the collapsed fork. Strand invasion into the correct template initiates HR-dependent fork restart. However, inaccurate strand invasion into the wrong template leads to non-allelic HR (NAHR), which results in GCRs (Lambert et al., 2010; Mizuno et al., 2009) (This model is discussed in more detail in Chapter four. See Figure 4.7 for models). The increased frequency of GCRs in the palindrome (RuiuR) system was hypothesized to be due to branch migration of the invading strand in the palindrome to form a HJ at the center of the palindrome. This branch migration would not be possible in the inverted repeat (RuraR) system (Lambert et al., 2010). To prevent the predicted branch migration, a series of constructs were made where a portion of the centromere-proximal ura4 gene in the palindrome system was replaced with an exogenous sequence (named RPalR), and to prevent the non-allelic HR restart the telomere-proximal RTS1 sequence of RPalR was replaced with Ter2/3 rDNA RFBs (named TPalR) (see Figure 1.7). The RPalR system was predicted to reduce the GCR levels and TPalR system to generate no GCRs. However, induction of fork stalling resulted in GCRs in both systems. This data suggested an alternative pathway of GCR production where the collapsed RF is restarted accurately by HR on the correct template but the restarted fork reverses the orientation of replication (U-turns) when replicating the palindrome center (Mizuno et al., 2013). Thus two mechanisms leading to the generation of GCRs have been defined: firstly template exchange or non-allelic homologous recombination (NAHR) where the wrong template is used during restart, and secondly the restarted fork would restart correctly on the right template but this fork in non-canonical and error-prone with a high propensity to U-turn at inverted repeats (see Figure 4.7 for models). Both mechanisms generate intermediates that have to be resolved. NAHR dependent restart
generates HJs and the U-turn of the restarted fork generates HJ-like intermediates, the resolution of which results in formation of dicentric and acentric chromosomes. The error-prone nature of HR-restarted forks was confirmed by the Lambert lab in a separate study. Iraqui et al. (2012) showed that the restarted fork was prone to replication slippage events leading to small insertions and deletions. Therefore, although HR is required for replication restart and completion of replication, HR-dependent restart can lead to replication-induced genomic instability. Genomic instability is a hallmark of cancer, human hereditary diseases and many human syndromes (Aguilera & Garcia-Muse, 2013; Branzei & Foiani, 2010; Petermann & Helleday, 2010; Weinert et al., 2009).
Figure 1.7 | RuraR and RuiuR fork stalling systems. Schematic of chromosome III (Chr.III) of *S. pombe*. Fork stalling systems are integrated into the *ura4* locus on Chr.III telomere proximal to efficient early firing ARS3004/3005. In RuraR inverted repeats of *RTS1* flank the *ura4* gene (blue arrows indicate the orientation). In RuiuR inverted repeats of the *ura4* gene are flanked by inverted repeats of the *RTS1* creating a small 5.3kb palindromic sequence. The centre of the palindrome is interrupted with 14bp of unrelated sequences. In RpalR the centromere proximal portion of *ura4* is replaced with unrelated sequences. In TpalR the telomere proximal *RTS1* is replaced with rDNA Ter2/3 polar RFBs. Induction of Rtf1 expression results in activation of *RTS1* barrier activity.
Figure 1.8 | HR-dependent replication restart in inverted repeats generates GCRs. Diagram showing the generation of GCRs after HR-dependent replication restart in inverted repeats. These are sister chromatid fusions resulting in reciprocal palindromic acentric and capped chromosomes. The acentric fails to attach to the mitotic spindle but the centromere on the dicentric (capped chromosome) alines correctly at mitosis due to the retention of sister chromatid cohesion but forms a bridge at anaphase.
1.13 – AIMS OF THE PROJECT

A limitation of the replication stalling systems used in Carr, Murray, and Lambert labs was that induction of replication stalling was controlled by Rtf1 expression from the \textit{nmt} promoter and this takes 16-24 hours for full induction (Basi \textit{et al.}, 1993). The \textit{urg1} uracil-regulatable promoter originally reported by the Bahler lab (Watt, 2008) and subsequently optimised in the Carr lab (Watson \textit{et al.}, 2011; Watson \textit{et al.}, 2013) provides a budding yeast GAL-like system in fission yeast for rapid induction of genes of interest within a single cell cycle. Using this inducible system to control the expression of Rtf1 enables us to study the generation and consequences of fork arrest at a specific locus in a single cell cycle. The aim of this project is to monitor the generation of chromosome rearrangements upon induction of Rtf1 and fork arrest in synchronous cultures and to identify the intermediates formed by the restarted fork U-turn and to correlate the timing of events with checkpoint activation.
CHAPTER 2 – MATERIALS AND METHODS

2.1 – MEDIA USED IN THIS STUDY

2.1.1 – S. POMBE MEDIA

The media used were either of liquid or solid state. Supplements were added to the media as required at 100mg/l.

YE media:
0.5% w/v (5g/l) yeast extract 3.0% w/v (30g/l) glucose

YES media:
0.5% w/v (5g/l) yeast extract 3.0% w/v (30g/l) glucose
2.5g/l Difco Bacto Agar

Phloxin YES: 20mg/l Phloxin B (Sigma) was added to YES.

Edinburgh Minimal Media (EMM2)

50ml/l 20× EMM2 salts
25ml/l 20% NH4Cl
25ml/l 0.4M Na2HPO4
12.5ml/l 40% Glucose
1ml/l 1000× Vitamins
0.1ml/l 10000× Trace elements
### 20× EMM2 salts

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<tr>
<th>Concentration (g/l)</th>
<th>Substance</th>
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<tr>
<td>61.20</td>
<td>Potassium hydrogen phthallate</td>
</tr>
<tr>
<td>20.00</td>
<td>KCl</td>
</tr>
<tr>
<td>21.40</td>
<td>MgCl2.6H2O</td>
</tr>
<tr>
<td>0.20</td>
<td>Na2 SO4</td>
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<td>0.26</td>
<td>CaCl2.2H2O</td>
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### 1000× Vitamins

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<tr>
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<td>Pantothenic acid</td>
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<tr>
<td>10.0</td>
<td>Nicotinic acid</td>
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<tr>
<td>10.0</td>
<td>Inositol</td>
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<tr>
<td>0.01</td>
<td>d-Biotin x</td>
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### 10000× Trace elements

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<tr>
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<td>MnSO4</td>
</tr>
<tr>
<td>4.0</td>
<td>ZnSO4.7H2O</td>
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<tr>
<td>2.0</td>
<td>FeCl3.6H2O</td>
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<td>1.5</td>
<td>Na2 MoO4 1.0g/l KI</td>
</tr>
<tr>
<td>0.4</td>
<td>CuSO4.5H2O</td>
</tr>
<tr>
<td>10.0</td>
<td>Citric acid</td>
</tr>
</tbody>
</table>

Where necessary supplemented with: adenine, histidine, leucine, thiamine, uracil at final concentration of 100mg/l. The medium was filter sterilized after making.
2.1.2 – *E. coli* Media

Media used were either of solid or liquid state. Supplements were added to sterile liquid or molten agar prior to use.

**L-Broth:**

- 10g/l NaCl
- 10g/l tryptone
- 5g/l yeast extract
- 1g/l glucose
- 30mg/l thymine

**SOC medium:**

- 20g/l tryptone
- 5g/l yeast extract
- 0.5g/l NaCl

The medium was autoclaved and 10ml of filter-sterilised 1M MgCl₂, and 10ml of filter-sterilised 1M MgSO₄ was added to it prior to use.

2.2 – Chemicals used for selection

Table 2.1 | Chemicals used for selection in this study

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>50mg/l</td>
</tr>
</tbody>
</table>
Geneticin disulphite | 200mg/l  
Hygromycin | 200-400mg/l  
Kanamycin | 200mg/l

### 2.3 – List of the strains used in this study

Table 2.2 | List of the strains used in this study

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<tr>
<th>Strain</th>
<th>Genotype</th>
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<td>YSM012</td>
<td>$h^- \text{ura4D-18 urg1}::Purg1_{\text{NR}}::\text{kan rtf1}::\text{nat}$</td>
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2.4 – S. POMBE TECHNIQUES

2.4.1 – RANDOM SPORE ANALYSIS OF S. POMBE CROSSES

To cross the strains, freshly grown cells of appropriate strains were mixed together on ELN plates and incubated at appropriate temperature (based on the cross) for two to three days. The ascus formation was monitored under the microscope and a full loop of cells was resuspended in 1ml of water containing 1µl/ml Helix Pomatia Juice (BioSepra #213472). The mixture was incubated over night at room temperature. ~500 spores were then plated on YEA plates.

2.4.2 – YEAST TRANSFORMATION

Cells were grown in YE over night to a density of $5 \times 10^6$/ml. 2 x $10^8$ cells were collected for each transformation and washed in 50ml water. Cells were then washed in 5ml of LiAc-TE (0.1M lithium acetate [$pH$ 7.5], 10mM Tris-HCl [$pH$ 7.5], 1mM EDTA). Cells were resuspended in LiAc-TE at $2 \times 10^9$ cells/ml. 10µl of DNA or 1µl plasmid DNA and 2µl of salmon sperm DNA were added to 100µl of the cell suspension. Mixture was incubated for 10 minutes at room temperature. 260µl of 40% PEG/LiAc-TE (PEG4000 was dissolved in LiAc-TE) was added to the mixture. Cells were incubated for 30-60 at 30°C then 43µl of DMSO was added. Cells were heat-shocked at 42°C for 5 minutes and then washed in 1ml of sterile water. Samples were resuspended in 100µl of sterile water and plated onto appropriate selection plates.
2.4.3 – RECOMBINATION MEDIATED CASSETTE EXCHANGE

The appropriate PAW8 plasmid was transformed into the desired *S. pombe* base strain constructed as described in Watson *et al.* (2008). Transformant were selected for (leu<sup>+</sup> cells). Transformants were then grown in EMM2 containing leucine (so that cells lose the plasmid) and other necessary supplements. About 500 cells were then plated onto minimal plates containing leucine and other necessary supplements. The resulting colonies were then replica plated onto –leu plates to check for the absence of the plasmid and YEA plates containing the appropriate antibiotic selection to check for the loss of the antibiotic resistance gene in the base strain hence the success of the cassette exchange.

2.4.4 – GENOMIC DNA EXTRACTION

Cells were grown in 50ml YE to an OD of 1.0 and were resuspended in 1ml of SP1 buffer (1.2M sorbitol, 50mM citric acid, 50mM Na2HPO4, 40mM EDTA [pH 5.6]) containing 1mg/ml Lyticase. Cells were incubated at 37°C until the cell wall was digested (10-30 minutes). Cells were spun at 3000rpm and the pellet was resuspended in 450µl of 5× TE (0.05M Tris-HCl, 0.005M EDTA [pH 7.5]). 50µl of 10% SDS was added and the mixture was incubated for 5 minutes at room temperature. 150µl of 5M KAc was added and samples were incubated on ice for 10 minutes. Samples were spun at 13000rpm for 10 minutes and the supernatant was transferred to a fresh tube. 1 volume isopropanol was added to the supernatant and the mixture was spun at 14000 for 10 minutes at 4°C. The supernatant was aspirated and the pellet was washed with 0.5ml of 70% ethanol. The mixture was spun and the pellet was resuspended in 250µl of 5x
TE. 10µl of 10mg/ml RNase in 5× TE was added and samples were incubated for 20 minutes at 37°C. 4µl of 10% SDS and 20µl of 10m5/ml proteinase K were added to the mixture and samples were incubated at 55°C for an hour. For DNA extraction 500µl chloroform: isooamyl alcohol was added, samples were spun at 13000rpm for 5 minutes at room temperature, and the upper phase was transferred to a new tube. This step was repeated once more. Then 1/10 volume 3M sodium acetate and 1 volume isopropanol was added and samples were incubated on ice for 10 minutes. Samples were then spun at 14000rpm for 15 minutes at 4°C and washed twice with 500µl of 70% ethanol. The pellet was then resuspended in 100µl of 1× TE.

2.4.5 – PREPARATION OF AGAROSE-EMBEDDED DNA

Cells were grown to mid-log-phase and 1.5 × 10⁸ cells were harvested and treated with 1% volume sodium azide and 10% volume 0.5M EDTA [pH 8] and kept on ice for ten minutes. Cells were then spun at 3.5k rpm and washed with 50ml of sterile water. Samples were then resuspended in 1ml of CSE buffer (20mM citrate/phosphate [pH 5.6], 40mM EDTA, 1.2M sorbitol). 250µl of 3000unit/ml lyticase solution in CSE was added to samples which were then incubated at 37°C for 10-30 minutes. Once samples were digested, they were spun at 1000g and resuspended in 150µl of TSE buffer (10mM Tris-HCl [pH 7.5], 45mM EDTA, 0.9M sorbitol). Samples were incubated at 37°C for three minutes. 200µl of pre-warmed 0.8% agarose (Lonza InCert® Agarose) in TSE was added to samples and then the samples were loaded into plug molds. The plug molds were incubated on ice for 5 minutes. Then the agarose plugs were extruded into 5ml of lysis buffer 1 (50mM Tris-HCl [pH 7.5], 250mM EDTA, 1% SDS) and incubated at 50°C for 90 minutes. Then the agarose plugs were removed into lysis
buffer 2 (1% lauryl sarcosine, 500mM EDTA [pH 9.5]), 125µl of 20mg/ml proteinase K was added, and samples were incubated at 55°C for 24 hours. Then another 125µl of 20mg/ml proteinase K was added and samples were incubates at 55°C for 24 hours. Samples were stored at 4°C.

2.4.6 – Digestion of agarose-embedded DNA

1/3 of DNA plugs were washed with ice-cold TE for 30 minutes. This was repeated twice more. Samples were then incubated on ice in 1ml of appropriate digestion buffer. After removal of the buffer, 400µl of the buffer and 100 units of the appropriate enzyme were added and samples were incubated at 37°C for four hours.

2.4.7 – Electrophoresis of digested agarose-embedded DNA

A solution of agarose in 1× TBE of appropriate concentration was prepared and kept at 55°C for 30 mins. Meanwhile, the digested agarose-embedded DNA was washed three times in 1× TBE for 10 minutes. The agarose plugs were then placed on a comb and fixed on the comb using few drops of melted agarose. In the cold room, melted agarose was poured in a tray and the comb (containing the agarose plugs) was carefully placed on the tray. The agarose was left to set for 30 minutes. The gel was then run at 50V for appropriate length of time (depending on the size of the fragment to be analysed) at room temperature. The samples were then transferred onto a nylon membrane.
2.4.8 – PULSED-FIELD GEL ELECTROPHORESIS (PFGE)

0.8% agarose (Biorad Certified Megabase Agarose) was melted in 1× TAE buffer and kept at 55°C. Half of a DNA plug was equilibrated three times with 1× TAE for 10 minutes. The agarose plugs were then placed on a comb and fixed on the comb using few drops of melted agarose. The melted agarose was poured in the appropriate gel tray and the comb was placed. Once the gel was set, it was placed on the Biorad CHEF-DRIII tank and run using below settings:

Cooling module 14°C

Power supply [Biorad CHEF-DRIII]

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2.4.9 – TWO DIMENSIONAL DNA GEL ELECTROPHORESIS (2DGE)

Cells were grown to mid-log-phase and 1.25 × 10^9 cells were harvested and treated with 1% volume sodium azide and 10% volume 0.5M EDTA [pH 8] and kept on ice. Cells were pelleted and washed with 20ml of ice-cold water and transferred into a 50ml falcon tube. Cells were pelleted at 3.5K rpm at 4°C for three minutes and the supernatant was completely removed. Liquid nitrogen was used to snap freeze the samples. The frozen sample was thawed on ice and resuspended in 2.5ml of CSE (20mM Citrate/Phosphate [pH 5.6], 40mM EDTA, 1.2M Sorbitol). 500μl of
300 units/ml lyticase in CSE was added to the samples. The cell suspension was incubed at 37°C for 10-15 minutes. Samples were transferred on ice and pelleted at 1000g at 4°C. The supernatant was aspirated and the pellet was resuspended in 300μl of CSE. The suspension was incubated at 37°C for three minutes. 400μl of pre-warmed 1% agarose in CSE was mixed with each sample. The mixture was then loaded into a plug mould. The plug molds were then incubated on ice for 10 minutes. The DNA plugs were then extruded into 10ml of PK buffer (1% lauryl sarcosine, 25mM EDTA [pH 8]) containing 0.5ml of 20mg/ml Proteinase K solution and incubated at 50°C for 30 minutes. This step was repeated twice more after removal of the old PK buffer each time. The buffer was then removed. Another 10ml of buffer PK containing 0.5 ml of 20mg/ml Proteinase K was added and the samples were incubated at 50°C over night. Five plugs per sample were incubated in 50ml of ice-cold 50× TE (10mM Tris-HCl [pH 7.5], 50mM EDTA) for three hours. The buffer was then changed for fresh ice-cold 50× TE and samples were incubated at 4°C over night. The plugs were then washed three times in 50ml of ice-cold 1× TE (10mM Tris-HCl [pH 7.5], 1mM EDTA). Plugs were transferred into 2ml test tubes and incubated with 1ml of 2× appropriate NEB digestion buffer for 30 minutes on ice. The buffer was then changed to 1ml of 1× NEB buffer and samples were incubated another 30 minutes on ice. The buffer was then replaced with 0.4ml of fresh 1× NEB buffer and 100 units of the desired restriction enzyme were added to the tube. The samples were incubated at 37°C for two hours. Samples were incubated at 70°C for 10 minutes and then at 37°C for five minutes. Another 100 units of the restriction enzyme was added and samples were incubated at 37°C for one hour. 10μl of 10mg/ml RNase and 10μl of beta-Agarase I were added and the samples were incubated at 37°C for one hour. Samples were then spun at 13K rpm at 4°C for one minute. The supernatant was collected and 90μl of 3M sodium acetate and 1ml of
isopropanol were added. Samples were kept at 4°C overnight. Samples were pelleted
and washed with ice-cold 70% ethanol. Pellets were dried at room temperature for 10
minutes and then resuspended in 20μl of TE. 5μl of 20× loading dye (0.83%
Bromophenol Blue, 0.83% Xylene Cyanol FF, 50% Glycerol) was added and samples
were loaded on an agarose gel prepared with 0.35% agarose in 1× TBE. The gel was run
at 50V for an appropriate length of time depending on the size of the DNA fragment to
be analyzed. The gel was then stained using 22.5μl of ethidium bromide in 750ml of the
used running buffer. The first dimension run was then cut out of the gel and placed in
the second dimension gel tray. Pre-melted 0.9% agarose in 1× TBE containing 10.5μl of
10mg/ml ethidium bromide was then poured and incubated at 4°C until the gel was set.
The gel was then run at 200V in 2l of 1× TBE containing 70μl of 10mg/ml ethidium
bromide. The DNA was then transferred onto a nylon membrane by capillary transfer
(see Southern blot section) and stored at 4°C.

2.4.10 – SOUTHERN BLOT

The genomic DNA was then digested using the appropriate enzyme. To do this, the
appropriate amount of each sample was digested in 5% volume of the appropriate
digestion enzyme and 10% volume appropriate 10x enzyme buffer at 37°C for 60
minutes. The samples were then run on a long agarose gel of appropriate concentration
(depending on the size of the fragment) in 1× TBE at 50V.
The gel was then incubated for 20 minutes in depurinating solution (0.25M HCl) in a
shaker. Then the gel was washed in denaturing solution (1.5M NaCl and 0.5M NaOH)
for 30 minutes on a shaker. Then gel was washed in neutralizing solution (1M Tris and
1.5M NaCl). The gel was then transferred to a membrane employing 10× SSC buffer
(1.5M NaCl, 0.15M sodium citrate [pH 7]) and capillarity force over night. The membrane was then washed in 2× SSC buffer for 5 minutes on a shaker. The membrane was air dried on a piece of filter paper and then the DNA was cross-linked to the membrane using UV light at 1200J/m2. The membrane was stored at 4°C.

For Hybridising probe to the membrane, first the membrane was washed in dH2O for 5 minutes. Then 80ml of preheated 65°C hybridising solution I (6× SSC, 1x Denhardt [100x: 2% Ficoll 400, 300mM NaCl, 2% polyvinylpyrrolidone, 2% BSA], 1% sarcosyl, 0.1% BSA) was added to the hydrated membrane in a tube. The tube then was placed in hybridising oven for one hour at 65°C. Meanwhile, 1µl of 50ng/µl probe was added to 44µl dH2O. The solution was boiled in a water bath for 5 minutes and then placed on ice. In the radioactivity room, the labelling mix and 5µl of 35P-αdCTP were added to the DNA and the mixture was incubated at 37°C for 15 minutes. The labeled probe was then spun in a pre-spun G50 column at 3000rpm for 1 minute and incubated at 100°C for 5 minutes. Then the mixture was kept on ice. Then the probe was added to 20ml preheated 65°C hybridising solution II (6× SSC, 1x Denhardt, 1% sarcosyl, 200µl 10mg/ml salmon sperm DNA). Then hybridising solution I was replaced with hybridising solution II and the tube was put back in the oven at 65°C over night. Then the membrane was washed with 50ml preheated 65°C buffer I (2× SSC, 1% SDS) in the oven for 10 minutes and then with 450ml of buffer I on a shaker for 15 minutes. In the following step the membrane was washed twice, each time with 500ml of 42°C buffer II (0.1× SSC, 0.1% SDS) on a shaker for 15 minutes. The membrane was then air dried on tissue and wrapped in cling film and placed in a phosphoimager cassette over night. The membrane was scanned to obtain the Southern blotting results.
2.4.11 – TCA WHOLE CELL PROTEIN EXTRACTION

5 × 10⁷ cells of logarithmically growing cells were pelleted and washed with 50ml of dH₂O. Cells were then resuspended in 200μl of 20% trichloroacetic acid (TCA) solution. Acid washed glass beads were added to the samples. Cells were lysed using a ryboliser (FastPrep24, MP) at 6.5 m/s for 30 seconds. This step was repeated 2-3 times. The samples were then collected by puncturing the tubes and centrifugation into new tubes. Samples were pelleted at 14K at 4°C for 10 minutes and the supernatant was removed. Samples were resuspended in 200μl of 1× TCA sample buffer, boiled for five minutes, and stored at -20°C.

1× TCA Sample Buffer:

1 volume 4× SDS sample buffer
1 volume 1 M Tris, pH 8
2 volume dH₂O
2.5% β-mercaptoethanol

4× SDS Sample Buffer

250mM Tris-base [pH 6.8]
20% Glycerol
0.004 g/ml (w/v) Bromphenol blue
0.08 g/ml (w/v) SDS
2.4.12 – WESTERN BLOT

Appropriate amounts of resolving and stacking polyacrylamide (ProtoGel 30%, 37.5:1 Acrylamide to Bisacrylamide) solutions were made as described in Sambrook et al. (1989) and gels were prepared. Samples were run through the stacking gel at 80V and through the resolving gel at 100V using 1x running buffer (0.025M Tris base, 0.25M Glycine, 0.1% SDS). Samples were then transferred onto a nitrocellulose membrane in transfer buffer (20mM Tris, 20% Methanol, 750mM Glycine) at room temperature for 90 minutes. The membrane was then blocked using 3% milk solution (Marvel dried skimmed milk in PBS (Phosphate buffered saline)) at 4°C over night. The appropriate dilution of the primary antibody was added to PBS solution contacting 3% milk and 0.1% Tween20. The membrane was incubated at room temperature for one hour. The membrane was washed 3x in 0.1% Tween20 in PBS for ten minutes. The appropriate dilution of the secondary antibody was added to membrane submerged in PBS containing 3% milk and 0.1% Tween20 and incubated for one hour at room temperature. The membrane was washed 3x in 0.1% Tween20 in PBS for ten minutes. The membrane was then dried using a paper tissue. ECL plus western blot detection reagents were added to the membrane and the reaction was detected using an X-ray film in the dark room. The film was developed with a Xograph Imaging Systems Compact X4.

2.4.13 – WESTERN BLOT USING PHOS-TAG

To detect the Cds1 phosphoshift, Phos-tag was used in the gel mixture as described below:
7.5% resolving gel mix was prepared containing final concentration of 20µM Phostag, and 40µmM MnCl$_2$. Stacking gel was prepared as described in previous section. The gel was run at 15mA. Wet-transfer onto a cellulose membrane was performed at 300mA for two hours. The membrane was blocked using 3% milk in PBS, 0.1% tween mixture for one hour at room temperature. The membrane was incubated in 0.5% milk in PBS containing 1:2000 dilution of the Cds1-antibody at room temperature over night. The membrane was washed three times in PBS solution containing 1% tween for 20 minutes. The membrane was incubated in 1:2500 dilution of the secondary antibody in 0.5% milk in PBS solution for one hour. The membrane was then washed another three times in PBS solution containing 1% tween for 20 minutes. ECL plus western blot detection reagents were added to the membrane and the reaction was detected using an X-ray film in the dark room. The film was developed with a Xograph Imaging Systems Compact X4.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HA</td>
<td>Mouse monoclonal</td>
<td>Santa Cruz, F7 sc- 7392</td>
<td>1:5000</td>
</tr>
<tr>
<td>Anti-Cdc2</td>
<td>Rabbit monoclonal</td>
<td>Santa Cruz, sc-53</td>
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</tr>
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<td>Abcam, ab17353</td>
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</tr>
<tr>
<td>Anti-Cds1</td>
<td>Rabbit polyclonal</td>
<td>Provided by Y. Daikagou</td>
<td>1:2000</td>
</tr>
<tr>
<td>Rabbit anti-mouse HRP</td>
<td>Rabbit polyclonal</td>
<td>Dakocytomation, P0260</td>
<td>1:2500</td>
</tr>
<tr>
<td>Swine anti-rabbit HRP</td>
<td>Rabbit polyclonal</td>
<td>Dakocytomation, P0217</td>
<td>1:2500</td>
</tr>
</tbody>
</table>
2.4.14 – FACS ANALYSIS

5ml of cells of density of $1 \times 10^7$ were added to 500µl of pre-chilled 200mM EDTA [$pH$ 8] and 50µl of 10% sodium azide was added to samples. Samples were spun at 3K rpm for three minutes. Cells were resuspended in 1ml of dH2O and spun at 13K for one minute. Samples were then resuspended in 1ml of 70% ethanol and stored at 4°C. 500µl of each sample was then spun at 13K for one minute. Samples were then washed with 500µl of 50mM sodium citrate [$pH$ 7] and resuspended in 500µl of sodium citrate. 50µl of 10mg/ml RNAse was added and samples were incubated at 37°C for three hours. 10µl of 500µg/ml propidium iodide was added to FACS tubes and 200µl of each sample was added. 1ml of FACS buffer was added to each sample which was then vortexed and applied to the FACS machine.

2.5 – E. coli TECHNIQUES

2.5.1 – DH5α COMPETENT E. coli TRANSFORMATION

Cells were thawed on ice for 20 minutes. 100ng of DNA was added to 100µl aliquot of DH5α cells. Samples were then incubated on ice for 20 minutes. Cells were heat-shocked at 42°C for 30 seconds. Then 1ml of LB was added to the cells and samples were incubated at 37°C for an hour shaking at 250rpm. The cells were pelleted at 5500rpm for 1 minute and plated on LB agar containing the appropriate antibiotic.
2.5.2 – Plasmid Maxipreps and Minipreps

For maxipreps and minipreps cells were grown at 37°C over night in 100ml or 10ml of LB containing the appropriate antibody respectively. Cells then were harvested by centrifugation at 6000rpm for 5 minutes at 4°C. The DNA was then extracted following QIAGEN maxi and mini plasmid purification handbook. The concentration of the plasmid obtained was then measured against a marker and samples were stored at -20°C.

2.6 – General Techniques

2.6.1 – Ethanol Precipitation

2.5 volume of 100% ethanol and 1/10 volume of 3M NaOAc were added to the DNA samples and the mixture was vortexed and placed on ice for 10 minutes. Samples were then centrifuged at 14000rpm for 15 minutes. The supernatant was aspirated and the samples were washed twice using 0.5ml 70% ethanol (centrifuged at 14000rpm for 5 minutes). The pellet was then air dried and then resuspended in the appropriate volume of 1× TE.
2.6.2 – DNA ELECTROPHORESIS

Gels were poured at 0.8% agarose in 0.5x TE containing 0.5μg/mg ethidium bromide. Samples were mixed with loading dye and were loaded on the gel. The gel was run in 0.5x TE for 45 minutes at 100V. The DNA was then visualised under UV light.

2.6.3 – DNA GEL PURIFICATION

Samples were loaded on 8% low melting point agarose (Invitrogen # 16520) in 0.5x TE gel. The gel was run and then was visualized in exposure to 350nm UV light. The appropriate band was cut out and the DNA was extracted following Qiagen gel extraction protocol.

2.6.4 – RESTRICTION ENZYME DIGEST

To carry out the digestions, 5% volume of desired restriction enzyme and 10% volume of the appropriate 10x restriction enzyme buffer were added to the DNA sample. The mixture then was incubated at 37°C for 30-60 minutes. The success of the digestion was then checked by running the sample on a gel.

2.6.5 – DNA LIGATION

To set up DNA ligation mixtures, DNA concentrations of the insert and vector was measured using NanoDrop™ 1000.
A three to one ratio of insert ends to vector ends were (50-100ng of vector DNA depending on the size) added to below general reaction mixture to make a final volume of 10µl. T4 DNA ligase (NEB #M0202S) was used for ligation reactions.

10x T4 Buffer: 1µl
ATP: 1µl
DTT: 1µl
T4 Ligase: 1µl

2.6.6 – Removal of the 3’ overhangs from DNA ends

T4 DNA polymerase (NEB #M0203S) was used to remove the 3’ overhangs from the ends of DNA and form blunt ends. 1 unit of T4 DNA polymerase per each microgram of DNA was added to DNA solution in 1x NEB buffer II and presence of dNTPs. Samples were then incubated at 12°C for 15 minutes. To stop the reaction, excess of EDTA (>10mM) was added and samples were incubated at 75°C for 20 minutes.

2.6.7 – Removal of the 5’ phosphate group from DNA ends

Antarctic Phosphatase (NEB #M0289S) was used to remove the 5’-P group from DNA ends. To do this, 10% volume Antarctic Phosphatase buffer and 5% volume Antarctic Phosphatase were added and samples were incubated at 37°C for 30-60 minutes. The reaction was then stopped by incubating the samples at 65°C for 10 minutes.
CHAPTER 3 – OPTIMISATION OF FORKSTALLING

INDUCTION AND CELL CYCLE SYNCHRONISATION

3.1 – INTRODUCTION

Replication fork stalling at inverted repeats of the replication termination site, RTS1, placed in an inverted orientation at a unique locus in the fission yeast genome induces HR-dependent generation of GCRs and the accumulation of dicentric and acentric chromosomes (Lambert et al., 2005; Lambert et al., 2010; Mizuno et al., 2009; Mizuno et al., 2013). Replication stalling is dependent on Rtf1, which binds RTS1 and in these studies Rtf1 expression was under the control of the thiamine repressible nmt promoter, which takes 16 hours to fully induce (Basi et al., 1993). Since the S. pombe cell cycle is completed within two to three hours, it can be estimated that the reported level of rearrangements in the previous studies was a steady state level accumulated over three generations (samples were analyzed after 24 hours of induction Pnmt to allow maximum induction levels of Rtf1, since Rf1 levels reach maximum within 16hrs of induction, it can be estimated that rearrangements accumulate over three generations (~8hrs)). The induction time of nmt promoter is a significant disadvantage when the aim is to investigate the timing of rearrangements in a single cell cycle considering the time required for the nmt induction and S. pombe cell cycle. Therefore an alternative method of rapidly inducing Rtf1 using the urg1 promoter was optimized as described below.

Watt et al. (2008) characterized the promoter of the urg1 gene and showed that the transcript levels peak approximately 30 minutes after addition of uracil to medium.
Work in the Carr laboratory showed that while induction kinetics were not maintained if the *urg1* promoter was moved from its native locus, the replacement of the native *urg1* open reading frame (ORF) with ectopic ORFs results in similar induction kinetics of these ectopic ORFs to that of *urg1* (Watson *et al.*, 2011). This led to the development of a Cre recombinase and lox recombination based recombination-mediated cassette exchange (RMCE) system (Watson *et al.*, 2008) at the *urg1* locus to facilitate rapid exchange of ORFs at this locus (Watson *et al.*, 2011). This provided an inducible system enabling rapid induction of Rtf1 to initiate the fork stalling in a single cell cycle. The initial inducible system to regulate Rtf1 is shown in Figure 3.1 (adapted from Watson *et al.* (2011) and further optimization described in this chapter).

Synchronous cell cultures facilitate the biochemical assessment of the timing of the rearrangements and checkpoint responses to fork stalling. Fission yeast cells can be synchronized by making use of temperature sensitive cell cycle mutants, drugs, or selection based on cell size. While synchronizing by size selection (i.e. centrifugal elutriation, lactose gradients) remain the most physiological, centrifugal elutriation can only be carried out on a single strain at a time and lactose gradients do not yield adequate number of cells required for biochemical assessments. Use of temperature sensitive cell cycle mutants facilitates the side by side comparison of multiple strains. The *cdc25-22* allele arrests the cell cycle at G2-M boundary at temperatures above 35°C, and the *cdc10-M17* allele can be used to arrest the cell cycle in G1. However, temperatures above 35°C induce heat shock response in *S. pombe*, cells are very sick at 37°C and die at 38°C (Forsburg & Rhind, 2006).
Figure 3.1 | Kinetics of P\textsubscript{urg1} driven induction of Rtf1. A- Diagram showing the control of expression of Rtf1 from the \textit{urg1} promoter and subsequent activation of fork stalling. B- mRNA levels of P\textsubscript{urg1} driven expression of \textit{urg1} and \textit{rtf1} expressed from the \textit{urg1} and \textit{rtf1} loci upon addition of uracil to medium. C- Western blot showing Urg1, and Rtf1 protein levels after expression under the control of P\textsubscript{urg1} at the \textit{urg1} locus. Note that the levels of Rtf1 protein are much higher when expressed from the \textit{urg1} locus compared to \textit{nmt1} induction at the native locus, even though the mRNAs are expressed with a similar range. Please note that all data presented in this figure are provided by A. Watson.
The Yanagida laboratory first characterized the nuclear division arrest (nda) genes in a screen for cell division cycle mutants (Toda et al., 1983; Umesono et al., 1983), and subsequently showed that nada3+ gene encodes β-tubulin (Hiraoka et al., 1984). At restrictive temperatures (<20°C), nada3-KM311 cells fail to form functional β-tubulin and the mitotic spindle, and uniformly arrest nuclear division in prometaphase with condensed chromosomes. However, this nuclear division arrest is highly reversible upon temperature shift to permissive temperatures (>30°C) and the mutant cells resume the rest of the mitosis synchronously (Hiraoka et al., 1984). Nuclear division arrest in nada3-KM311 is brought about by the action of the spindle assembly checkpoint (SAC), which prevents the initiation of anaphase until all the kinetochores are stably captured by the spindle. In the presence of unattached kinetochores the SAC is ‘on’ and anaphase is inhibited. This inhibition is alleviated and the SAC is satisfied once all the kinetochores are stably attached to microtubules (Nezi & Musacchio, 2009). 

In this chapter I describe the development of a rapidly inducible fork stalling system in synchronous S. pombe cultures. This system was then used to characterize the kinetics of RF restart-dependent GCRs as detailed in subsequent chapters. 

I therefore utilized a cold sensitive mutant, nada3-KM311, to arrest the cell cycle in mitosis. The other advantage of using nada3-KM311 to synchronize cells is that, unlike cdc25-22 and cdc10-M17 mutants, the profile of the origin firing in the subsequent S phase is similar to that of an unperturbed S phase (Y. Daigaku, personal communication).
Figure 3.2 | Strains constructed for this study. Diagram showing the basic genetic make up of the strains used in this study. Please note that additional alleles were used in strains when appropriate (e.g. *chk1-3HA* to investigate the checkpoint response).


3.2 – Regulation of \textit{nda3-KM311} Block and Release

Hiraoka \textit{et al.} (1984) obtained the optimal synchrony using \textit{nda3-KM311} to block cell cycle in prometaphase by shifting the temperature down to 20°C for ten hours, and shifting the temperature up to 36°C to release the cells from the cell cycle block. They also showed that cultures of densities higher that 5 \times 10^6 cells ml\textsuperscript{-1} do not achieve the same efficiency of synchrony after the block and release (Hiraoka \textit{et al.}, 1984). In order to reduce the time required to achieve good synchrony, a series experimental conditions were tested to identify the condition conferring an optimal synchronization.

Furthermore, to avoid the induction of a heat response, a releasing temperature of 30°C was chosen. The strain \textit{h\textsuperscript{+}} \textit{smt0 ade6-704 nda3-KM311} (nda3 control) was grown to early log-phase (3 \times 10^6 cells ml\textsuperscript{-1}) and arrested at either 20°C or 16°C for four, five, or six hours. To release from the block, the temperature was shifted up to 30°C and cell cycle progression was monitored by septation index. To score cell cycle progression, cells were fixed in methanol at 15 minute intervals after release, stained with DAPI and Calcofluor to detect DNA and septum respectively, and scored based on having either, one nucleus (G2), two separated nuclei (mitotic), and two nuclei with septum (septated). Cells showing chromosome mis-segregation, chromosome bridges, or where the septum bisected the nucleus were scored as ‘cut’ phenotype (Figure 3.3). As discussed by Hiraoka \textit{et al.} (1984), cells blocked at 20°C with blocking times under ten hours did not show desired synchrony (only data from a five-hour arrest are shown as an example, Figure 3.6 A). However, cells blocked at 16°C for six hours showed highly synchronous cell cycle progression with a septation peak of \approx80\% at 60 minutes after the release. In \textit{S. pombe} S phase coincides with the formation of the septum (Figure 1.1). Extended analysis of the cell cycle progression after release of \textit{nda3}-
KM311 cells blocked at 16°C for six hours showed the synchronous cell cycle progression with septation peaking at 60 minute in the first cell cycle and at 240 minute in the second (Figure 3.3B).
nda3-KM311 cells show synchronous cell cycle progression after block and release. A- Graphs showing the cell cycle progression of nda3-KM311 cells arrested at different restrictive temperatures and released at 30°C. To examine the optimal cell cycle synchrony conditions, cells of *h- smt0 ade6-704 nda3-KM311* were grown to a concentration of 3x10⁶ cells ml⁻¹ at 30°C and arrested at either 16°C or 20°C. Cells were released from the block by temperature shift to 30°C, and cell cycle progression was monitored by analysing septation index. DNA was stained with DAPI and calcofluor was used to visualize the septum. Mitotic cells are binucleate and S phase coincides with the formation of septum. Aberrant mitotic ‘cut’ cells show chromosome bridges and lagging chromosomes. Optimal cell cycle synchrony is achieved when cells are arrested at 16°C for six hours (bottom left). Analysis of septation index shows a peak of septation of 80% under these conditions. B- Cell cycle progression of nda3-KM311 after 6-hour block at 16°C and release at 30°C. The peak of first S phase takes place 60 minutes after the release and the second S phase at 240 minutes.
Figure 3.3

A

Arrested at 16°C for 4 hrs

Arrested at 16°C for 5 hrs

Arrested at 16°C for 6 hrs

Arrested at 20°C for 5 hrs

M: mitotic cells
S: S phase
C: cut cells

B

% of cells

0 30 60 90 120 150 180 210 240 270 300 330 360 390
3.3 – **Regulation of P<sub>urg1</sub> Driven Expression of Rtf1**

3.3.1 – **Cryptic start-stop codons**

Previous work in the Carr laboratory indicated that control of Rtf1 expression by the urg1 promoter was somewhat leaky (Watson et al., 2008). This resulted in the induction of fork stalling even in the ‘Off’ state. In an attempt to reduce the ‘Off’ state levels of rearrangements when using the urg1 promoter to drive Rtf1 expression, mutations were made that introduced an AUG codon in the 5′-UTR of the mRNA to reduce the translational efficiency (Kozak, 2002), and strains containing either one or two cryptic start-stop (CSS) codons upstream of the Rtf1 ORF were created. The levels of rearrangements were assessed in a replication stall system where inverted RTS1 stall sites flank inverted repeats of ura4<sup>+</sup> and form part of a palindrome (Figure 3.4A). This system, termed RuuiR, generates the maximum rearrangements, with 20% of cells dying after Rtf1 induction using the nmt1 promoter (Mizuno et al., 2009). The RuuiR strain was crossed into the urg1 base strain (h- smt0 ura4-D18 leu-32 urg1_NR::HPH rtf1::natMX6 nda3-KM311) and the rtf1 cassette with the cryptic start-stop codons integrated by recombination mediated cassette exchange (RMCE) (see Figure 3.2 for schematic of resulting strain). Restriction fragment length analysis was performed to determine the background level of rearrangements without induction. Cells were grown to mid-log phase and harvested. DNA was extracted in agarose plugs and restriction enzyme digestion carried out using BglII. DNA species were separated using gel electrophoresis and detected by Southern blot analysis.
Figure 3.4 | Rearrangements in Purg1 ‘Off’ cultures. A- Diagram showing the BglII fragments and expected sizes before and after rearrangements at the RuiuR locus. Probe ‘Cen’ and ‘Tel’ detect the initial locus and dicentric and acentric fragments, respectively, after rearrangements. B- Representative Southern blot showing the rearrangement levels in two independent isolates of RuiuR Purg1_NR 1XCSS:rtf1 nda3-KM311 or RuiuR Purg1_NR 2XCSS:rtf1 nda3-KM311 when Purg1 is NOT induced. Genomic DNA was prepared in plugs and digested with BglII and restriction products were separated by gel electrophoresis. Probe Cen was used to detect the dicentric fragment. C- Quantification of the data in B shows 30±5% of the DNA in the rearranged form. For quantification of the dicentric signal, the average intensity of the dicentric band calculated by ImageQuant™ was calculated as a percentage of the total intensities of the dicentric and original signals.
In the absence of rearrangements a ~11kb ‘original’ band was detected, and after rearrangement a ~16kb dicentric band (Figure 3.4A), and a ~6kb acentric band are expected. Quantification of these bands provides a measure of the “Off” rates of expression. Probe Cen, homologous to sequences centromere proximal to RuiuR was used to detect the dicentric fragment in the Southern blots. Figure 3.4B and C shows the results of this experiment and the quantification of the Southern blot signals. Use of 5’ UTR CSS sites did not result in any reduction in the ‘Off’ levels of Rtf1 (Figure 3.4). The background level of rearrangements was >20% in all RuiuR $P_{arg1_{NR}}$ 1XCSS/2XCSS:rtf1 nda3-KM311 strains analyzed. This level of rearrangements at the ‘Off’ state was too high to be of use in further analysis.

### 3.3.2 – Destabilisation of Transcript using DSR Elements

In an attempt to reduce the stability of the transcript I next tested the use of DSR elements. Harigaya et al. (2006) characterized a mechanism for selective removal of meiosis specific mRNAs in vegetatively growing *S. pombe* cells. In this process, the YTH domain protein Mmi1 directs meiosis specific transcripts, characterized by the presence of 3’-end determinant of selective removal (DSR) sequences, to nuclear exosomes for degradation. The 157bp DSR element of *spo5* was identified (Harigaya et al., 2006) and it was shown that the DSR elements contain tandem repeats of U(U/C)AAAC motif (Chen et al., 2011; Yamashita et al., 2012). To reduce the ‘Off’ levels of Rtf1 expression the *spo5* DSR element was cloned into the 3’UTR of *rtf1* in the previously described PAW8ENdeI plasmid (Watson et al., 2008) harboring GFP-tagged *rtf1:eGFP*. The resulting *rtf1:eGFP-DSR* was used to create the RuiuR
\(P_{\text{argl}_{\text{NR}}}:rtf1:eGFP-\text{DSR} \text{ nda3-KM311}\) strain by RMCE. In parallel, to test the possible effects of the GFP tag on rates of Rtf1 stability and degradation, an untagged rtf1-DSR cassette was used to create \(RuuR P_{\text{argl}_{\text{NR}}}:rtf1-\text{DSR nda3-KM311}\). Restriction fragment length analysis was utilized to examine the induced and background levels of rearrangements. Cells of the resulting strains were prepared in two rounds of liquid precultures in EMM2 minimal medium lacking uracil and then grown to early log-phase. Uracil at 0.25mg/ml was added to induce the ‘On’ cultures and cells were harvested after 180 minutes (~one cell cycle in EMM2 minimal medium). DNA was extracted in agarose plugs and digested with \(Bgl\)II. DNA gel electrophoresis was performed and dicentric fragments were detected using probe Cen in Southern blots (Figure 3.5). DNA samples extracted from the original \(RuuR nmt41::rtf1\) strain (described in Mizuno et al. (2009)) were used as controls. The cells of both \(RuuR P_{\text{argl}_{\text{NR}}}:rtf1:eGFP-\text{DSR}\) (Figure 3.5A) and \(RuuR P_{\text{argl}_{\text{NR}}}:rtf1-\text{DSR}\) (Figure 3.5B) showed significant reductions in the background levels of rearrangements (0.4%-1%) in the ‘Off’ state, and the levels of rearrangements were comparable to that of \(RuuR P_{nm41::rtf1}\) cells when the \(nmt\) promoter was repressed. Induction of the \(argl\) promoter resulted in a 5-10% accumulation of dicentrics after three hours in both \(RuuR P_{\text{argl}_{\text{NR}}}:rtf1:eGFP-\text{DSR}\) Figure 3.5A) and \(RuuR P_{\text{argl}_{\text{NR}}}:rtf1-\text{DSR}\) cultures (Figure 3.5B). This showed that the GFP tag did not affect the Rtf1 protein levels in either of the induced or un-induced situations. These data show that utilizing DSR elements to regulate \(argl\) promoter transcripts, results in a reduction of protein levels in both induced and un-induced states. This was published (Watson AT, Daigaku Y, Mohebi S, Etheridge TJ, Chahwan C, Murray JM, Carr AM. PlosOne, 2013).
Figure 3.5 (following page) | DSR element reduces Rtf1-dependent chromosomal rearrangements. A- Induction of Rtf1 using *urg1* promoter in the RuuR system results in the formation of dicentric chromosomes. Lanes labelled as ‘nmt rtf1’ are the original *nmt41* driven *rtf1* RuuR strains used as controls. *urg1 rtf1-GFP-DSR 1-7* are seven independent isolates of *h- smt0 RuuR leu-32 P_{urg1, NR}::rtf1:eGFP-DSR rtf1::natMX6 nda3-KM311*. ON is 3hrs after the addition of uracil to induce *P_{urg1}* and OFF are the equivalent uninduced cultures. Genomic DNA was extracted in plugs and digested with *BglII*. Probe Cen was used to detect the dicentric fragment. B- Quantification of the data in A shows that using the DSR element reduces *P_{urg1}* background levels to a similar level to that of *P_{nmt41}* driven expression of Rtf1. Induction of *P_{urg1}* resulted in ~10 fold accumulation of dicentrics after 3hrs. C- Induction of *P_{urg1}* results in formation of dicentrics in strains where *rtf1* does not have the GFP tag. Lanes labelled as ‘nmt rtf1’ are the original *nmt41* driven *rtf1* RuuR strains as controls. *urg1 rtf1-DSR 1-7* are seven independent isolates of *h- smt0 RuuR leu-32 P_{urg1, NR}::rtf1:DSR rtf1::natMX6 nda3-KM311*. D- Quantification of data in C shows that similarly to the GFP tagged *rtf1* strains using DSR sequences reduces the ‘OFF’ levels of rearrangements in *P_{urg1}* strains to a level comparable to that of the *nmt1* promoter.
Figure 3.5

A

B

C

D

Dicentric

Initial

16Kb

11Kb

B

25

20

15

10

5

0

nmt

rtf1

ON

1

2

3

4

5

6

7

8

ntt

rtf1

OFF

urg1::rtf1-GFP:DSR - ON

urg1::rtf1-DSR - OFF

0

10

5

15

20

25

30

35

nmt

rtf1

ON

1

2

3

4

5

6

7

8

ntt

rtf1

OFF

urg1::rtf1-DSR - ON

urg1::rtf1-DSR - OFF
3.3.3 – Characterisation of GCRs formed after induction of \( P_{URG1} \)

In order to confirm that the use of the DSR element to regulate Rtf1 levels results in efficient fork stalling at \( RTS1 \), the level of GCRs was determined after induction for three hours. Since one cell cycle takes approximately three hours in minimal media at 30°C and the majority of \( S. pombe \) cells in a logarithmically growing culture are in G2, most cells should have gone through a single S phase under these conditions. Pulsed field gel electrophoresis (PFGE) was used to investigate formation of acentric species upon induction of \( urg1 \) promoter for one cell cycle. To examine the accumulation of intact acentric chromosomes, independent isolates of \( RuiuR P_{urg1,NR::rtf1-DSR} nda3-KM311 \) were grown to early log phase and induced for three hours. To rule out an effect of the \( nda3-KM311 \) background rearrangements were also characterized in an \( nda3^+ \) background (\( RuiuR P_{urg1,NR::rtf1-DSR} \)). Levels of acentric formation were compared to those after induction of \( nmt41 \) driven Rtf1 after 24 hours (\( RuiuR P_{nmt41::rtf1} \)) (Figure 3.6). Cells were harvested and DNA was extracted in agarose plugs. Probe ‘Tel’ homologous to telomeric sequences was used to detect the acentics. Analysis of the PFGE data showed that induction of the \( urg1 \) promoter results in accumulation of the acentric species after three hours (Figure 3.6). The ‘off’ levels of rearrangements in \( P_{URG1} \) strains were similar to the \( P_{nmt} \) control strain. Moreover, the \( nda3-KM311 \) allele did not affect the rates of induction and background levels of rearrangements as shown by comparison to the \( urg1 \) strain with wild type \( nda3^+ \) (second and eleventh lanes). These results implied that Rtf1 expression under the \( urg1 \) promoter is efficient and results in accumulation of acentric chromosomes.
Figure 3.6 | Acentric chromosomes accumulate upon induction of $P_{urg1}$.  
A- EtBr stained pulsed field gel (top) and corresponding Southern blot (bottom) showing the chromosomal rearrangements in the RuiuR system after induction of $P_{urg1}$::$rtf1$-DSR for 3hrs. I, II, and III on the Etbr stained gel indicate the three chromosomes. Chromosome III is detected at ∼3.5Mb and the acentric chromosome at ∼1.5Mb using Probe Tel. Lanes labelled as nmt-$rtf1$ (original $P_{nmt}$ strain) and urg1-DSR are used as controls. nda3-Urg1-DSR 1-7 are the same strains as used in Figure 3.5 (C). Note the sizes of chromosome III and the acentric chromosome vary with rDNA repeat copy number as the rDNA occupies two regions at either end of chromosome III.

B- Quantification of data in A shows accumulation of acentric 3hrs after the addition of uracil. The ‘OFF’ levels remain similar to that of nmt promoter. nda3-KM311 allele does not affect the kinetics of $P_{urg1}$ induction.
3.3.4 – Characterisation of replication intermediates formed after P_{urg1} driven expression of Rtf1

To further confirm the efficiency of the fork stalling at RTS1 using the urg1 promoter, two-dimensional gel electrophoresis (2DGE) was carried out after induction of Rtf1. Branched DNA molecules are known to migrate anomalously in agarose gels compared to linear fragments of equal mass (Bell & Byers, 1983). In 2DGE, DNA species are separated according to restriction fragment size in the first dimension, and based on their shape in the second dimension. To achieve this, the first dimension is run at low voltage in low percentage agarose. This allows the low mobility branched molecules (RIs) to run close to their true molecular weight, and this separates DNA molecules according to mass. The second dimension is run at high voltage using a gel of high agarose concentration and in the presence of ethidium bromide. These conditions exaggerate the difference between the mobility of molecules of different shapes (i.e. linear vs branched). This results in separation based on shape, which enables the analysis of branched replication intermediates such as bubbles or Y arcs (Bell & Byers, 1983; Brewer & Fangman, 1987). Experimental evidence obtained from replication of the 2μM plasmid revealed the patterns produced by RIs (Figure 3.7B) in two-dimensional gels (Brewer & Fangman, 1987). Retardation of passively replicated Y shaped molecules forms a Y arc, due to the differences in deviation of their three dimensional shape from the linear molecules. A small Y migrates close to the monomer and a large Y further away on the downward section of the arc. A half replicated molecule is made of three equal branches and this deviates the most from the linear and therefore is retarded the most in the second dimension to form the apex of the Y arc. Replication bubbles are produced by the movement of two diverging RFs initiated
within the fragment. These open structures migrate most slowly. Double Ys are produced by the approach of two converging forks initiated outside the fragment and run in a line closer to the linear than Y-shaped molecules. Spots on an arc result from the accumulation of similar shape and sized molecules, as when replication is paused at a specific site. Finally, X-shaped molecules such as HJs run as a spike upward from the end of the Y arc to the end of the double Y arc.

To visualize replication arrest in asynchronous cultures, cells of h- smt0 RuiuR leu-32 P$_{urg1\_NR::rtf1}$-DSR rtf1::natMX6 nda3-KM311 were grown to early log phase and the ‘On’ cultures were induced. Cells were harvested at the ‘Off’ state, and three, four and six hours after induction. DNA was extracted from the cells in agarose plugs, digested with AseI (Figure 3.7A) and 2DGE carried out. A probe with homology to sequences centromeric to RuiuR (Cen) was then used to identify replication intermediates in the Southern blot. 2DGE analysis of the ‘Off’ state (figure 3.7E) showed formation of a Y arc signal indicating the passive progression of replication in the region. The faint spot on the Y arc showed an accumulation of replication intermediates of a particular size (8-9kb) consistent with a slight degree of pausing at RTS1 due to the leakiness of urg1 promoter.
Figure 3.7 (following page) | Fork stalling is efficient when P_{urg1} drives Rtf1 expression. A- cartoon showing the original and dicentric AseI fragments at Ru1uR and the expected sizes. B- Schematic illustrating the different intermediates separated by 2DGE. C, D- Diagrams showing the expected replication intermediates at Ru1uR in ‘OFF’ and ‘ON’ cultures respectively. E- Southern blots showing the 2DGE analysis of replication intermediates of asynchronous Ru1uR cultures. Cells were grown to early log phase and uracil was added to induce Pur1. Cells were harvested at 0 minutes (‘OFF’, top left), 3hrs (top right), 4hrs (bottom left), and 6hrs (bottom right) after the addition of uracil. When fork stalling is ‘OFF’ signals corresponding to the 7kb monomer spot (blue arrow) and passively replicating ‘Y’ arc (black arrow) are visible. A signal corresponding to a slight degree of pausing is at the expected size of 8-9kb on the ‘Y’ arc (red arrow). Three hours after induction, two signals at 8-9.5kb on the ‘Y’ arc (blue arrow) and 9.5kb on the double ‘Y’ arc (red arrow) corresponding to a single pause at RTSI and double arrest respectively are detected. Recombination dependent intermediates are visible at 11-13kb (green arrow). Four hours after induction a clear pause signal, the nature of which will be discussed in the next chapter, is detectable (orange arrow). Six hours after induction the intensity of the 7.4kb monomer spot increases due to the accumulation of the dicentric (blue arrow). F- lower exposure of 6 hr blot to show dicentric monomer spot (blue arrow).
Figure 3.7

(A) Diagram illustrating the genetic arrangement and rearrangement processes.

(B) Diagram showing the 'Bubble' and 'Fork Arrest' processes.

(C) Diagram illustrating the 'Pause 'Off'' process.

(D) Diagram depicting the 'Pause 'On'' process.

(E) Snapshots showing the effects of RuluR on DNA structures at different times.

(F) Close-up of the DNA structures from (E).
However, when \( \text{P} \text{urg1} \) was induced, after three hours of induction the ‘Y’ arc signal was not detectable and a signal corresponding to the size expected for single fork arrests at \( \text{RTS1} \) sites was observed. The double pause at both \( \text{RTS1} \) sites was detected as a ~9kb spot on the double Y arc. A signal migrated at ~11-13kb corresponding to recombination intermediates (Lambert \textit{et al.}, 2010) formed at the apex of the Y arc. Moreover, the intensity of the 7.4kb monomer spot signal increased, which indicates the accumulation of rearranged dicentric species. Four hours after induction of Rtf1 an additional signal was detectable at 10-11kb. The nature of the molecules producing this signal is discussed in the next chapter. Six hours after induction of Rtf1 a further increase in the rearranged monomer signal was observed indicating the accumulation of dicentric products over time. The intensity of the observed ‘pausing’ signal (~90%) was comparable to previously published data (Lambert \textit{et al.}, 2010), demonstrating the efficient expression of Rtf1 and stalling when \( \text{urg1} \) promoter and DSR element are used to control Rtf1 expression.

\[3.3.5\] \textbf{Characterisation of kinetics of fork stalling}

To test whether the DSR element affected the kinetics of induction of fork stalling, 2DGE was carried out in synchronous cultures of \( \text{P}_\text{urgl}_{\text{NR}}::\text{rtfl-DSR nada3-KM311} \) and as control (No DSR) strain \( \text{P}_\text{urgl}_{\text{NR}}::\text{rtfl nada3-KM311} \) (Figure 3.8). Cells of both strains were grown to early log phase and synchronised as described in 3.1. \( \text{P}_\text{urgl} \) was induced before the release and samples were harvested at 0, 60, 75, and 90 minutes after the release. DNA was extracted, digested with \( \text{AseI} \), and 2DGE was performed. Probe ‘Cen’ was used to identify the replication intermediates.
Figure 3.8

Use of the DSR element does not affect the fork stalling efficiency. Southern blots showing the 2DGE analysis of replication intermediates in synchronised RuiuR cells. Cells were grown to early log phase and synchronised. Uracil was added before releasing the cells and samples were taken at time points 0, 60, 75, and 90 minutes.

A- Diagrams showing the expected replication intermediates at RuiuR when fork stalling is induced. B- Southern blots showing the replication intermediates of the control strain without the DSR element. At t=0, synchronised cells are still in mitosis, therefore only a monomer signal is detected. At 60 minutes after the release, S phase is initiated and single and double pause signals at RTS1 sites are visible. A faint trace of ‘Y’ arc is also detectable. At 75 minutes recombination intermediates are detected consistent with HR restart. These intermediates persist and are still detectable at 90 minutes, as is the faint ‘Y’ arc. C- Southern blots showing the replication intermediates of the DSR strain used in Fig 3.7. The results show the detection of similar intermediates to that of the control strain at each time point. The quantification of the pause signals at t=60 showed similar intensity of pause spots between the control (A) and the DSR (B) strains (87% and 90% respectively).
The analysis of the replication intermediates observed in synchronous cultures of both the control strain (no DSR element to reduce the background Rtf1 levels) and the DSR strain used in previous experiments showed detection of only the monomere signal at timepoint 0 after the release. This is expected as the \( nda3-KM311 \) cells arrest the cell cycle in prometaphase at restrictive temperature and resume the rest of mitosis upon shifting the temperature to the permissive temperature. One hour after the release, signals corresponding to the single and double pause at \( RTSI \) sites, and a faint ‘Y’ arc are detectable in both the control and the strain harboring the DSR sequence at intensities of 87% and 90% respectively. In order to quantify the arrest efficiency, the intensity of the pausing signals was presented as a percentage of the total intensities of replication intermediates observed. By 75 minutes after the release, both control and the DSR strain showed a signal corresponding to recombination-dependent replication intermediates correlating with the initiation of HR-dependent fork restart at \( RTSI \) sites. Moreover the faint trace of the ‘Y’ arc was detectable in both strains. Analysis of the intermediates observed at 90 minutes after the release showed the accumulation of similar intermediates to that of 75 minutes. These results indicated that the efficiency of the fork stalling at \( RTSI \) was not affected when the DSR element is employed to control the Rtf1 levels, and the overall reduction of Rtf1 levels did not affect the kinetics of barrier activity at \( RTSI \) when compared to the strain without the DSR element.

\[ \text{3.4 – DISCUSSION} \]

Development of an inducible replication stalling systems such as RuiuR provided a unique opportunity to investigate the role of HR in the restart of collapsed replication
forks. The results of genetic and biochemical studies in our laboratories demonstrated the importance of HR in the restart of replication at RTS1. However, studies of the timing of HR-restart were not technically achievable due to the extended time required for full induction of the medium strength nmt41 promoter. Identification of the uracil inducible urg1 promoter offered a plausible inducible system similar to P_GAL of S. cerevisiae. Although P_urg1 showed similar dynamic range of ‘On’ and ‘Off’ states of protein levels to that of nmt1 promoter, its basal level of transcription was relatively high and Rtf1 protein levels at ‘Off’ state were too high and resulted in GCRs in the Ru11R system before induction. In this chapter I showed that regulation of rtf1 RNA stability using the spo5 DSR element results in a reduction of the basal transcription levels controlled by the urg1 promoter to a level similar to that of P_nmt1. Despite the fact that using the DSR element results in a general reduction of protein levels in both ‘On’ and ‘Off’ states of the promoter (Watson et al., 2013), induction resulted in expression of enough Rtf1 molecules to drive efficient fork stalling at RTS1 and, importantly, with similar kinetics to that of the strain with no DSR element (demonstrated by PFGE and 2DGE in synchronous and asynchronous cultures).

Several conditions of block and release of nda3-KM311 allele were examined in the aim of identifying the best synchronization conditions. I reported the optimal synchrony conditions and demonstrated the synchronous cell cycle progression by following septation index. Optimization of rapid induction of Rtf1 from urg1 promoter, and nda3-KM311 cell cycle synchrony provided a versatile tool to investigate the timing of events leading to GCRs in fork stalling systems, and cell cycle regulation in response to these events.
CHAPTER 4 – CHARACTERISATION OF HR-DEPENDENT REPLICATION RESTART IN A SINGLE CELL CYCLE

4.1 – INTRODUCTION

Replication restart in a palindrome has been shown to generate GCRs at a high frequency (Mizuno et al., 2009). Two DSB free mechanisms have been identified for the generation of acentrics and dicentrics after HR-dependent replication restart at RTS1. In the NAHR-dependent model of rearrangements strand invasion of the nacent 3’ strand at the stite of the collapsed fork into the wrong template leads to GCRs. Strand invasion into the correct strand restarts replication at RTS1 but the restarted fork is non-cannonical and U-turns at the centre of the palindrome and this results in GCRs (Lambert et al., 2010; Mizuno et al., 2013). The data leading to these hypotheses were the result of steady state of rearrangements over three generations, as the nmt promoter was used to control Rtf1 expression and cultures were analysed three generations after full induction of the nmt promoter. In order to support the models that rearrangements occur upon replication restart, and to identify the replication intermediates corresponding to the two mechanisms of generation of GCRs, the timing of rearrangements and replication intermediates were investigated in a single cell cycle using the rapid induction of the fork stalling system (RuiuR) that I optimized in synchronous cultures (as described in Chapter three).
4.2 – CHARACTERISATION OF CELL CYCLE PROGRESSION UPON INDUCTON OF FORK STALLING

To characterize cell cycle progression upon induction of fork stalling in the RuiuR system, the strain *RuiuR P_{arg1, NR::rtf1-DSR nda3-KM311}* was grown to early log phase. The cell cycle was blocked in mitosis by incubating at 16°C, and uracil was added to induce replication pausing at *RTS1* sites in the ‘On’ culture one hour before the release by shifting the temperature up to 30°C. Figure 4.1A shows a graphic explaining the experimental set up. Samples for septation index and flow cytometry (FACS) analysis were harvested every 15 minutes after the release. Septation index was scored as explained in section 3.1 and FACS analysis was performed. Flow cytometry is a powerful tool for measuring DNA content and monitoring cell cycle distribution. In FACS analysis, haploid G1 cells show a 1C DNA content, whereas G2 cells are of 2C DNA content. In *S. pombe* G2 phase compromises 70% of the cell cycle. Cells complete M, G1 and enter S phase prior to cell division. Therefore, mitotic and G1 cells are also of 2C DNA content. This causes a predominantly a 2C DNA content in an asynchronous culture. S phase is characterised by the area under an intermediate peak between 2C and 4C DNA content as the two daughter cells, which are still joined together and so counted as a single unit, move transiently to a 4C content and back to 2C as they separate (Sabatinos & Forsburg, 2009).

Scoring septation index showed that in both ‘Off’ and ‘On’ cultures the first peak of septation occurs at 60 minutes after the release. Both cultures proceeded to mitosis at ~215 minutes after the release with the second peak of septation occurring ~240 minutes after the release. Since S phase in fission yeast is coincident with formation of septum, this indicated that S phase occurred at 60 and 240 min after
release and that the second S phase was not delayed in the induced culture. Consistent with this, the analysis of FACS data in both induced and uninduced RuiuR cultures (Figure 4.1C) revealed that there was no significant difference in progression of cell cycle between the induced and uninduced cultures (judged by shift of the 2C peak towards a 4C peak) with the first S phase initiated at 60 minutes and completed by 120±15 minutes after the release, and the second occurring at 240 minutes.

Cell cycle progression in both induced and uninduced cultures was similar to that of the nda3-KM311 control strain (no palindrome, see Figure 3.3B). This indicated no delay into mitosis after induction of fork stalling in the induced culture compared to the ‘Off’ and no palindrome controls. However a population of ‘cut’ cells where observed during the second M phase after the release in the ‘On’ culture (Figure 4.1B lower panel) and chromosome bridges were observed during mitosis, showing that GCRs had occurred (Figure 4.1D). These data showed that the generation of chromosomal rearrangements after HR-dependent restart at RTS1 does not lead to a mitotic delay, nor to a delay to S phase in the next cell cycle.
Figure 4.1 (following page) | HR-dependent restart does not lead to mitotic delay. A- cartoon illustrating the experimental set up. Cells were synchronized in mitosis by nda3-KM311 block and samples were taken at intervals after the release for appropriate experimentation. B- Septation and mitotic indices showing the cell cycle progression profile of ‘Off’ (top) and ‘On’ cultures. Coloured bar indicates the different stages of the cell cycle. DNA was stained with DAPI and calcofluor was used to visualize the septum. Mitotic cells are binucleate and S phase coincides with the formation of septum. Aberrant mitotic cells with chromosome bridges and lagging chromosomes or where the septum bisects the DNA were scored as ‘cut’. C- Flow cytometry profiles of DNA content of the ‘Off’ (top) and ‘On (bottom). Asterisks indicate the shift corresponding to S phase. D- Examples of chromosome bridges observed during the second mitosis after the release.
Figure 4.1

A

Experimental design:

Arrest cells in M at 18\(^\circ\)C  Induce Rtf1  Release cells at 30\(^\circ\)C

---

B

Pause Off

- M
- S
- C

---

Pause On

- M
- S
- C

---

M: mitotic cells  S: S phase  C: cut cells

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C

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D

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4.3 – DICENTRIC CHROMOSOMES ACCUMULATE IN G2

To characterize the timing of chromosomal rearrangements after the restart, restriction fragment analysis was performed on DNA samples obtained at intervals from either induced, or uninduced synchronous cultures of RuiuR Pargl::rtf1-DSR nada3-KM311. An rtf1Δ (no stall) strain (RuiuR Pargl::hphMX6 nada3-KM311 rtf1::natMX6) was used as control. Samples from an uninduced culture were used to determine the background level of rearrangements due to slight leakiness of Pargl. Strains were grown to early log phase and the cell cycle was blocked in mitosis by shifting the temperature to 16°C for six hours. Uracil was added to the ‘On’ culture prior to the release and cells were released by shifting the temperature to 30°C. Samples were taken at intervals and genomic DNA was extracted in agarose plugs. DNA was then digested with AseI (see Figure 3.7 for a schematic of AseI restriction sites in RuiuR after rearrangement) and gel electrophoresis was performed. A 7kb band is expected in the absence of rearrangements, the dicentric fragment migrates at 7.4kb, and the acentric fragment at 6.6kb band. Probe ‘Cen’ homologous to sequences centromere proximal to RuiuR was used to detect the initial and dicentric fragments in the Southern blot analysis. Figure 4.2A shows the result of this experiment and quantification of the bands observed in the Southern blot.
Figure 4.2 (following page) | Dicentric chromosomes accumulate in G2. A- (left hand side)- Representative Southern blot showing the timing of formation of rearrangements in the cell cycle. In all ‘On’, ‘Off’, and ‘no stall’, cells were grown to early log phase and synchronized. The time course started as cells were released and samples were collected at indicated intervals. DNA was prepared in agarose plugs and restriction fragment length analysis was performed using AseI. A- (right hand side)- Quantification of the bands observed in the Southern blot. B- (left hand side)- Representative Southern blot showing the restriction fragment length analysis using BglII. The experiment was repeated as in A and DNA samples were digested using BglII. B (right hand side)- Quantification of the data in Southern blot. To quantify the signals observed in the Southern blots, the intensity of each band calculated using ImageQuant™ was presented as a percentage of the total intensity of signals of each individual lane. Cartoons illustrating BglII and AseI restriction sites and rearrangements at RiuuR are presented above the blots. In both time courses the intensity of the dicentric fragment increases from 150 min (early G2) but slow migrating RI are seen in S and decline in G2.
As expected, in the *rtf1Δ* samples only the 7kb original *AseI* fragment was observed consistent with GCRs being dependent on HR restart of arrested fork at *RTS1*. The analysis of ‘Off’ samples taken at 60 minutes intervals over the first cell cycle showed both the 7kb original band and the 7.4kb dicentric band, which remained constant throughout the time course experiment. This indicated the background levels of rearrangements due to leakiness of the *urg1* promoter. In the ‘On’ samples, where the stalling was induced, the 7kb original and the 7.4kb dicentric bands are present throughout the time course. However, quantification of the dicentric signal showed that the dicentric fragment increased from early G2 (150 minutes after the release) leading to 15.8% of rearrangements by late G2. Moreover, a signal of slow migrating replication/recombination intermediates was detected at the start of S phase (60 minutes after the release). The intensity of this signal peaked at 105 minutes after the release and dropped upon the completion of S phase (~135 minutes after the release). The RI species peaked during the S phase and were resolved as the dicentric signal accumulated, consistent with the conversion of one form to the other.

This experiment was repeated on both ‘On’ and ‘Off’ cultures (Figure 4.2B) and restriction fragment length analysis was performed as explained above using *BglII* for better separation of original and rearranged bands (cartoon and expected sizes of RuiuR showing *BglII* restriction sites is presented in Figure 4.2B and 3.4). Consistent with the previous experiment, quantification of the signals seen in the Southern blot showed similar kinetics of formation and disappearance of bands. Dicentric chromosomes accumulated in G2 as the slow migrating intermediates were resolved and the ‘Off’ levels of rearrangements remained constant throughout the time course experiment.
4.4 – HOMOLOGOUS RECOMBINATION DEPENDENT RESTART OCCURS IN S PHASE

To characterise the slow migrating intermediates formed during the S phase (Figure 4.2), 2DGE was employed. Cells of *RuiuR* *P*$_{arg1\_NR}$:*rfl\_DSR nda3\_KM311* and the no stall control strain *RuiuR* *P*$_{arg1\_NR}$:*hphMX6 nda3\_KM311 rfl\_natMX6* were grown to early log phase and synchronised in M. Fork stalling was induced, or not, one hour before the release and the cells released from the block. Samples were collected for 2DGE analysis at one hour intervals for ‘Off’ and ‘no stall’ controls and in the induced culture at 15 minute intervals during the first S phase and 30 minutes intervals over G2 covering up to the second S phase after the release (Figure 4.3). DNA was extracted, digested with *Ase*I, and 2DGE was performed. Probe ‘Cen’ homologous to sequences centromere proximal to RuiuR was used to detect RIs. In the *rft1\_Delta* ‘no stall’ control culture (Figure 4.3A) at time point 0 after the release, where cells were in mitosis, only the monomer spot is detected. Analysis of samples from the rest of the time course showed passive replication of the RuiuR fragment as judged by the formation of a native Y arc at 60 minutes. The Y arc is hardly detectable by 180 minutes after the release (G2). Similarly, in the ‘Off’ culture (Figure 4.3B) only the monomer is detectable at time point 0. Analysis of later samples showed the formation of the Y arc at 60 minutes and this signal declined by 180 minutes after the release (G2). In addition, a pause signal on the Y arc corresponding to the accumulation of Y structures at *RTSI* was detected at time points 60 and 120 minutes, consistent with the slight degree of leakiness of *P*$_{arg1\_NR}$. 

Figure 4.3 (following page) | Homologous recombination restarts replication in S phase. 2DGE analysis of RuiuR replication intermediates detected by a probe homologous to sequences centromere proximal to RuiuR. Cells were grown to early log phase and synchronized. Fork stalling was induced, or not, 60 minutes before the release and the time course was started as cells were released. Samples were collected at indicated intervals. DNA was prepared and digested with AseI. A- Schematic illustrating different intermediates separated by 2DGE. B & C- Diagrams showing the expected replication intermediates in RuiuR system in ‘OFF’ and ‘ON’ states respectively. D- 2DGE analysis of ‘no stall’ control. The black arrow indicates the Y arc and the light blue arrow indicates the monomer spot. E- 2DGE analysis of the ‘Off’ state of fork stalling. The red arrow indicates the arrest at RTS1. F- 2DGE analysis of the ‘On’ culture at intervals through a single cell cycle. The red arrow indicates the pause at RTS1 and the dark blue indicates the double pause at RTS1 sites. The green and yellow arrows indicate the HR-dependent intermediates and the purple arrow indicates a novel spot in the second cell cycle.
Figure 4.3

(A) Bubble → Y arc → X Spike → Double Y → Linear → Monomer

(B) Pause 'Off'

(C) Pause 'On'

(D) RuiuR Δ-0, RuiuR Δ-60, RuiuR Δ-120, RuiuR Δ-180

(E) RuiuR Off-0, RuiuR Off-60, RuiuR Off-120, RuiuR Off-180

In the ‘On’ culture, initiation of S phase is evident 45 minutes after the release, as judged by a faint short Y arc signal and the accumulation of Y structures at a pause spot migrating at a size consistent with arrest at centromere proximal RTS1 pause site. As discussed in Chapter one, active replication origins ars3004/5 are 3kb centromere proximal to RuuR, whereas the telomere proximal origin, ars3003, is 40kb away. The pause signal on the Y arc reaches maximum intensity 60 minutes after the release (see Figure 3.7 and 3.8 for comparison) with the double pause at both centromere and telomere RTS1 sites visible on the double Y arc. At 75 minutes after the release, two additional signals were detected, one at the apex of the Y arc, and one on the X spike. These intermediates have been shown by Lambert et al. to be dependent on HR and thus are consistent with HR-dependent replication restart (Lambert et al., 2010). These HR dependent intermediates persist throughout the S phase and decrease in intensity in G2 (from t=150 minute). Replication intermediates observed in G2 and M (time point 180 when 80-90% of cells were in late G2) were comparable to that of the ‘Off’ culture: the intensity of the arrest signal and the HR-dependent intermediates dropped to a minimum. However in the S phase of the second cell cycle (240 minute after the release) the spots corresponding to the pause at RTS1 sites and HR restart reappear. An additional spot specific to the second cell cycle was also detected, the nature of which is discussed in the next section. These results indicated that HR-dependent restart of the fork occurred in S phase 15 minutes after replication stalling, and this led to formation of HR intermediates that were detected for approximately 45 minutes until declining in G2.
4.5 – DICENTRIC CHROMOSOMES REPLICATE IN THE SECOND CELL CYCLE

In order to visualize and differentiate between any potential acentric and dicentric specific replication intermediates, the membranes that contained the samples shown in (Figure 4.3) were reprobed with a probe (Probe Tel) homologous to sequences telomere proximal to RuiuR. Figure 4.4 shows the expected sizes of AseI fragment of RuiuR when rearranged to acentric and dicentric chromosomes and a cartoon to show intermediates detected by both probes after 2DGE. The major direction of replication through the locus is centromere to telomere as most forks will arrive first from the nearby centromere-proximal origin (ars3004/5). Both probes detect the telomere and centromere proximal single arrested forks. However, the intensity of these pause signals will depend on the probe. As detailed in Figure 4.4 C and D when using Probe Tel the intensity of the telomere proximal arrest is expected to be greater than that of the centromere proximal arrest as it will hybridise to two regions of homology on the Y-shaped telomere-proximal arrest molecule compared to one region of homology on a centromere-proximal arrest molecule.
**Figure 4.4**

**A** Cartoon showing the *AseI* fragment *RuiuR* and expected sizes when rearranged to acentric and dicentric chromosome.

**B** Schematic of replication intermediates of *AseI* RuiuR detected with Probe Tel (left) homologous to sequences telomere proximal to RuiuR, and Probe Cen (right) homologous to sequences centromere proximal to RuiuR.

**C** Cartoon illustrating single replication arrest at telomere proximal *RTS1* in RuiuR.

**D** Cartoon illustrating single replication arrest at centromere proximal *RTS1* in RuiuR. Note that both Probe Cen and Tel detect the both telomere and centromere proximal single arrested forks. However when using Probe Tel the intensity of the telomere proximal arrest is expected to be greater than that of the centromere proximal arrest and vice versa. The major direction of replication through the locus is centromere to telomere as most forks will arrive first from the nearby centromere proximal origin.
Figure 4.5A shows the results of re-probing the membranes presented in Figure 4.3 with the telomeric probe (Probe Tel). At t=0 the monomer spot signal detected with Probe Tel corresponds to the 7kb original AseI fragment with an additional minor spot of 6.6kb corresponding to the rearranged acentric fragment, consistent with the background level of rearrangements (Figure 4.5A). Due to re-probing the membrane the signal intensities were weaker but at 75 minutes after release single and double pause sites and HR-dependent intermediates, indicative of fork arrest and restart were detected. These intermediates decline in G2 similarly to Figure 4.3, and reappeared again in the second cell cycle (time point 240).

To differentiate between acentric and dicentric specific intermediates, the images obtained from the experiment shown in Figure 4.3 (probed with Probe Cen) were recolored in magenta and the images obtained from probing with Probe Tel were recolored in green. The resulting images were merged (Figure 4.5B). Replication intermediates in common between both probes were detectable in white, P Tel specific (corresponding to acentric) in green, and P Cen specific (corresponding to dicentric) in magenta. As expected from the size, the 6.6kb monomer spot was detected only by Probe Tel. In addition, the monomer proximal tail of the single pause at RTS1 on the Y arc was detected in green corresponding to the pausing at telomere proximal RTS1 (time point 75-120). While this pause is also be detected by P Cen it is more intense with the P Tel probe as this would hybridize to two regions on the Y arc (see Figure 4.4 C and D).

The double pause at both RTS1 sites was detectable in white. However the second cell cycle specific signal detected in time point 240 of Figure 4.3C was detected in magenta. While this could be due to the weak Probe Tel signal, it is likely that this is specific to Probe Cen as a similar Probe Cen-specific spot is seen after Pnmt41 induction.
of Rtf1 arrest (K. Mizuno, personal communication). The size of this replication intermediate is ~9-10kb consistent with the expected size of the arrest at RTS1 on the dicentric chromosome (monomer size: 7.4kb), therefore this signal likely was formed due to the replication pausing at RTS1 site during replication of the dicentric chromosome in the second cell cycle after the release.
Figure 4.5 (following page) | The dicentric replicates in the second S phase. A- 2DGE analysis of RuiuR replication intermediates detected by a probe (Probe Tel) telomere proximal to RuiuR. The membranes from experiment shown in Figure 4.3 were reprobed with Probe Tel. B- Overlays of acentric and dicentric specific signals. Images obtained from figure 4.3 were recoloured in magenta (dicentric) and pictures from 4.5A in green (acentric), and merged. The spots common between acentric and dicentric light up in white, Probe Tel specific (acentric) in green, and Probe Cen specific (dicentric) in magenta. The white arrow in time point 240 indicates the second cell cycle dicentric-specific novel spot corresponding to replication of the dicentric in the second S phase.
Figure 4.5

A

<table>
<thead>
<tr>
<th>Probe Tel</th>
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<tbody>
<tr>
<td>RuiuR On-0</td>
</tr>
<tr>
<td>RuiuR On-75</td>
</tr>
<tr>
<td>RuiuR On-150</td>
</tr>
</tbody>
</table>

B

| RuiuR On-0 | RuiuR On-30 | RuiuR On-45 | RuiuR On-60 |
| RuiuR On-75 | RuiuR On-90 | RuiuR On-105 | RuiuR On-120 |
| RuiuR On-150 | RuiuR On-180 | RuiuR On-210 | RuiuR On-240 |
4.6 – Kinetics of HR-dependent restart during S phase

To further characterize the timing of replication restart at RTS1, a time course with samples taken five-minute intervals during the S phase was performed. The strain RuiuR $P_{arg1-NR}:rtf1-DSR nda3-KM311$ was grown to early log phase and the cell cycle was arrested at 16°C for six hours. Rtf1 expression was induced one hour before the release. Cells were released by shifting the temperature to 30°C and samples were collected at five-minute intervals. DNA was extracted and digested with AseI. 2DGE was performed and Probe Cen homologous to centromere proximal sequences to RuiuR was used to detect replication intermediates in the Southern blots (Figure 4.6). Consistent with the result of the experiment shown in section 4.4, only the monomer spot was detected in mitosis (time point 0). Similarly to the previous time course, initiation of S phase was marked by faint pausing at RTS1 45 minutes after the release and the intensity of the arrest signal at RTS1 reaches maximum intensity by 60 minutes after the release. Note that the intermediates detectable are equivalent at the same time points in Figure 4.3 (e.g. compare 75, 90 and 105 minutes) showing the reproducibility of cell cycle progression between experiments. At t=65, the HR-dependent spot at the apex of the Y arc was detected but the larger HR-dependent molecules seen on the X spike were not detected until 75 minutes after the release. These replication intermediates persist throughout the S phase with decreasing intensity in G2.
**Figure 4.6**

**Kinetics of HR-dependent restart in S phase.** 2DGE analysis of replication intermediates at RuuiR. Probe Cen was used to detect signals in Southern blots. Cells were grown to early log phase and synchronised in mitosis. Fork stalling was induced 60 mins before the release and the time course started as the cells were released. Samples were collected at indicated time points. The green arrow ($t=65$) indicates the HR-dependent intermediates at the apex of the Y arc. The yellow arrow ($t=75$) indicates the HR-dependent intermediates on the X spike.
4.7 – DISCUSSION

In this chapter, the timing of HR-dependent restart of collapsed replication forks at RTS1 in the RuiuR system was investigated. The analysis of cell cycle progression showed that both ‘On’ where replication arrest is induced at RuiuR and control ‘Off’ cultures progress through the cell cycle with similar kinetics with no delay into the entry to mitosis or S phase in the next cell cycle. However, chromosome mis-segregation was seen in at mitosis in the induced culture due to the formation of dicentric chromosomes. Investigation into the timing of HR-restart-dependent chromosomal rearrangements showed the accumulation of the dicentric species in ~15-20% of the cells in G2 when the replication barrier was active. This accumulation coincided with a decrease in the intensity of slow migrating intermediates. Analysis of these slow migrating forms showed that these corresponded to replication arrest and HR-dependent restart intermediates. Strikingly, restart occurred in S phase within 15 minutes of replication arrest, and the resulting HR intermediates were dealt with before mitosis.

Two mechanisms leading to GCRs in the fork stalling system were previously identified: non-allelic homologous recombination (NAHR), and the U-turn of the restarted fork (Lambert et al., 2010; Mizuno et al., 2013). NAHR occurs when the Rad51-coated 3’ nascent strand behind the collapsed fork invades into the wrong homologous template leading to formation of a D-loop and the subsequent HJ(s) (Figure 4.7A). Depending on the orientation of the resolution of the HJ(s) either the parental chromosome is restored (non-cross over) or dicentric and acentic chromosomes are formed (cross over). The U-turn of the restarted fork, however, occurs when replication restarts via strand invasion on the correct template but the restarted fork is non-canonical and error-prone, and U-turns at the center of the palindrome resulting in
formation of the acentric and dicentric chromosomes (Figure 4.7B). Lambert et al. (2010) identified the HR-dependent replication intermediates resulting from NAHR by 2DGE in the RuraR fork stalling system, where rearrangements are only NAHR-dependent. Thus ectopic recombination events provide a marker for replication restart and it should be noted that replication restart on the correct template is not detectable by 2DGE. Lambert et al. defined two HR-dependent intermediates: a joint molecule spot was detected at the apex of the Y arc, which by semi-quantitative PCR only contained one junction between the RTS1 repeats and thus corresponds to a D-loop, and the signal observed on the X spike which contained HJs. In the palindrome system RuiuR, however, rearrangements are dependent on both NAHR and U-turn of the restarted fork (Mizuno et al., 2013). The U-turn would be predicted to form a closed fork structure that would be converted to an HJ-like structure by the converging fork. After AseI digestion the expected sizes for D-loop and U-turn intermediates are 10.5kb, and 10.3-10.7kb respectively. Therefore, both intermediates are predicted to run at the apex of the Y arc and are not distinguishable.

Analysis of replication intermediates after replication fork arrest at RuiuR showed the HR-dependent restart of the replication at RTS1 15 minutes after the fork collapse, judged by detection of the HR-dependent replication intermediates at both the apex of the Y arc and on the X spike. However, closer investigation of the timing of restart revealed a temporal separation between the time that the signal at the apex of the Y arc and the signal on the X spike were detected.
Figure 4.7 | HR dependent mechanisms of replication restart at RTS1. 

A - Cartoon showing the Asel fragment of RuiuR. B - Model demonstrating the NAHR restart of the replication. After fork arrest, association of HR proteins forms a recombinogenic 3’ end, which can invade either at the correct or incorrect locus. An invasion into the incorrect locus leads to formation of D-loop (10.5kb) and subsequent HJ formation (>14kb). Please note that a second end capture is also possible leading to formation of two HJs (not shown). Dashed lines indicate HR-restarted replication. C - Model for HR-dependent error-prone restart of replication. A recombinogenic 3’ end formed at the arrest site at RTS1 invades at the correct locus. The restarted fork is non-canonical and error-prone, and executes a U-turn (10.7kb) at the centre of the palindrome leading to GCRs. The dashed lines indicate the HR-dependent restart. Expected sizes of replication intermediates are noted below the corresponding cartoons.
This could suggest that conversion of a D-loop or a closed fork structure to HJ-like intermediates depends on the time required for the replication arrest and restart of the second fork. Alternatively, the delay between detection of closed fork/D-loop structures and the X shaped molecules could be an indicative of a temporal separation between the two pathways of the HR-dependent rearrangement. In both mechanisms of restart HJs or HJ like structures are formed, resolution of which results in GCRs. In the next chapter the relative contribution of the two pathways to the RI seen on replication restart is investigated.

In summary, these data determined the timing in a single cell cycle of replication arrest, HR-dependent replication restart and formation of GCRs upon induction of fork stalling. The timing of restart as measured by the detection of HR-dependent intermediates is consistent with the timing of replication of the intervening *ura4* sequences as measured by DNA copy number change (I. Miyabe, personal communication).
CHAPTER 5 – CHARACTERISATION OF REPLICATION INTERMEDIATES CORRESPONDING TO RESTART MECHANISMS

5.1 – INTRODUCTION

Two mechanisms have been identified for HR-dependent formation of acentric and dicentric chromosomes, NAHR template switch and the U-turn of the restarted fork (Lambert et al., 2010; Mizuno et al., 2013) (See figure 4.7). NAHR-dependent GCRs were generated in the RuraR fork stalling system where two inverted repeats of the RTS1 are separated by 1.8kb ura4+ gene. Introduction of an inverted repeat of the ura4+ gene to create a 5.3kb palindrome containing inverted repeats of the RTS1 sequence (RuiuR) resulted in a significant increase in the rates of GCR generation. This was originally thought to be due to the branch migration of the invading strand to create an HJ at the center of palindrome in the RuiuR system. However, GCRs were observed in TpalR system where ~500bp of the centromere proximal ura4+ in RuiuR was truncated to eliminate branch migration, and the telomere proximal RTS1 was replaced with TER2/3 rDNA fork barriers to eliminate the possibility of faulty restart by NAHR (see Figure 1.7). It must be noted that fork restart at the rDNA barrier is not dependent on either Rtf1 or HR proteins and simply pauses the converging fork allowing more time for HR-dependent restart of collapsed fork at RTS1 (Kings et al., 2004; Mizuno et al., 2013). Thus the GCRs generated in the TpalR system must be due the error-prone
nature of the restarted fork, which U-turns at the center of the inverted repeat. With the aim of identifying the timing of GCRs and replication intermediates corresponding to the U-turn event, experiments were set out to carry out over one cell cycle in TpalR system, as in this system which lacks a second RTS1 NAHR is abolished. The RuraR system in which a U-turn event is not possible and rearrangements are due to NAHR was used to differentiate between the two HR-dependent chromosomal rearrangement mechanisms.

5.2 – TIMING OF RESTART DEPENDENT REARRANGEMENTS IN THE CELL CYCLE IN TPALR SYSTEM

In order to characterize the timing of chromosomal rearrangements in the TpalR fork stalling system, the strain TpalR P_urg1_NR:rtf1-DSR nda3-KM311 was grown to early log phase. Cell cycle was arrested at 16°C for six hours and fork stalling was induced by addition of uracil to medium one hour before the release. Cell cycle was release by shifting the temperature to 30°C and samples for time course experiment were taken at intervals. DNA was extracted in agarose plugs and restriction fragment length analysis was performed using BglII. This was expected to give rise to a ~10kb initial band, a ~14kb band when DNA is rearranged to the dicentric form, and a ~5kb band corresponding to the acentric fragment (see Figure 5.1A for map and expected sizes of the BglII fragments at TpalR). Probe Cen homologous to sequences centromere proximal to TpalR was used to detect dicentric chromosomes in the Southern blots (Figure 5.1B). Consistent with the observations in the RuiuR construct, the 10kb original and the 14kb dicentric bands were observed and the intensity of the dicentric
band increased with a similar timing in G2 indicating the accumulation of dicentric chromosomes. Slow migrating replication intermediates were detectable by the start of the S phase (t=60), and declined in early G2 as dicentric chromosomes started to accumulate. Replication intermediates reappeared upon entry into the S phase of the second cell cycle after the release (time point 210-240). This data confirmed that the chromosomal rearrangements caused by the U-turn of the non-canonical restarted fork occur within a single cell cycle, and that the replication intermediates formed due to HR-dependent replication restart are dealt with before entry to mitosis.
Figure 5.1 (following page) | HR-dependent restart induces GCRs in TpalR in a single cell cycle. **A**- diagram showing the *Bgl*II sites at TpalR (left) and table showing the expected fragment sizes (right). **B**- Graph showing the mitotic and septation indices of the induced TpalR culture. Samples were collected at 15 minute intervals after the release and cells were fixed in methanol. DNA was stained with DAPI and septum with calcofluor. Cells with two separate nuclei were scored as mitotic (M), cells with two nuclei and septum as septated (S), and cells showing chromosome mis-segregation, bisection of DNA by septum, or lagging chromosomes as ‘cut’ (C). **C**- Southern blot from a representative time course showing the timing of the chromosomal rearrangements over a single cell cycle. Cells were grown to early log phase and synchronised in mitosis. Fork stalling was induced one hour before release and the time course started upon the release at 30°C. Fragment length analysis was performed using *Bgl*II and probe Cen homologous to sequences centromere proximal to TpalR was used to detect the locus.
**Figure 5.1**

**A**

Diagram showing the TpaIR region with markers such as Telomere, Ter2/3, RRS1, and Centromere, and a BgIII digestion chart indicating initial, dicentric, and acentric fragments with sizes 9.9, 14.4, and 5.4 kb. The diagram includes pTel, pCen, and um4.

**B**

Graph showing three lines labeled M, S, and C, with time points at 0, 60, 120, 180, and 240, and stages M, S, G2, and C.

**C**

Image of BgIII digested probe Cen and RI, Dicentric, and Initial bands with time points at 0, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, and 240, labeled ON.
5.3 – Analysis of Replication Intermediates of TpalR

In order to characterise the replication intermediates seen during the S phase in Figure 5.1, 2DGE was carried out in synchronous cell cultures. TpalR P_{urg1, NR}:rtf1-DSR nda3-KM311 cells were grown to early log phase and synchronised in mitosis. Fork stalling was induced, or not, one hour before the release and the time course started as cells were released. Samples were taken at one-hour intervals in the uninduced culture and at 15-minute intervals over the S phase and 30-minute intervals over G2, covering up to the second cell cycle after the release in the induced culture. DNA was extracted in agarose plugs and digested with AseI (see Figure 5.2A for AseI sites in TpalR). 2DGE was performed and Probe Cen homologous to sequences centromere proximal to TpalR was used to detect replication intermediates in Southern blots. Figure 5.2B shows the result of this experiment in the ‘Off’ culture. At t=0 where cells were still in mitosis, only the 5.8kb monomer was detected. Passive replication indicated by formation of the Y arc was seen at time points 60 and 120 and this signal was gone at time point 180 after the release. A faint signal corresponding to arrest at RTS1 was detected consistent with a slight degree of leakiness of P_{urg1}. Similarly to the experiments described in the previous chapter, in the induced culture (Figure 5.2C), at t=0 only the monomer was detected and initiation of the S phase was seen at 45 minutes after the release judged by detection of the Y arc and faint pausing at RTS1 (~7kb). 60 minutes after the release, fork pausing at TER2/3 was detected at ~6.5kb and full arrest at RTS1 at ~7-8kb, indicating that replication fork arrest occurs with similar kinetics to the RuiuR system. Double arrest signal at both RTS1 and TER2/3 was detected on the double Y line at ~7.5-8.5kb. A signal was detected at the apex of Y arc corresponding to the predicted size of a U-turn intermediate (~8.9kb). Moreover, an extended double Y signal
reaching sizes bigger than 12kb was detected. This could be due to convergence of forks restarting at the Ter2/3 barriers with the U-turn at the centre of the palindrome. In time points 75-135 the intensity of the fork arrest signals drops significantly indicating that most of the cells in the culture restart replication in this region. However a signal was observed on the X spike corresponding to accumulation of X structures and this signal was gone by 150 minutes after the release. At late G2/m (t=180) the replication intermediates were comparable to that observed in the ‘Off’ cultures, indicating that the replication intermediates formed upon HR-dependent restart at RTS1 are dealt with before entry to the next cell cycle. In the S phase of the second cell cycle (t=240) the signals corresponding to the fork arrests and U-turn and HJ like intermediates reappeared. These data indicated that intermediates consistent with the U-turn of the restarted replication fork arise in the S phase, with similar timing to intermediates in the RuiuR system. X shaped molecules then accumulated, which disappeared in G2 prior to entry into the next cell cycle. This strongly suggests that the U turn of the restarted fork gives rise to a closed fork structure that is detectable as a defined spot on the Y arc. This is converted to double Y and X structures by the incoming fork. These intermediates are resolved in G2 into acentric and dicentric chromosomes, consistent with the resolution of HJ-like structures.
HR-dependent restart occurs in S phase. 2DGE analysis of TpalR replication intermediates detected by a probe homologous to sequences centromere proximal to TpalR. Cells were grown to early log phase and synchronized. Fork stalling was induced, or not, 60 minutes before the release and the time course was started as cells were released. Samples were collected at indicated intervals. DNA was prepared in agarose plugs and digested using \textit{Ase}I. \textbf{A}- Cartoon showing TpalR and \textit{Ase}I restriction sites (left) and table showing the expected sizes (right) \textbf{B}- 2DGE analysis of the ‘Off’ state. The black arrow indicates the Y arc and the blue arrow indicates the monomer spot. \textbf{C}- 2DGE analysis of the ‘On’ culture. The red arrow indicates the arrest at \textit{RTS1}, the green arrow shows the pause at \textit{TER2/3}, and the orange indicates the double pause at \textit{RTS1} and \textit{TER2/3} sites. The purple arrow indicates the U-turn intermediate and the yellow arrow indicates the signal corresponding to accumulation of X shaped molecules.
Figure 5.2

A

![Diagram showing Telomere, A Ter2/3, RTS1, ARS3004, and Centromere with distances labeled in kb (2.7 kb, 3.1 kb, 1.8 kb, 1.2 kb).]

B

- TpalR Off-0
- TpalR Off-60
- TpalR Off-120
- TpalR Off-180

C

- TpalR On-0
- TpalR On-45
- TpalR On-60
- TpalR On-75
- TpalR On-90
- TpalR On-105
- TpalR On-120
- TpalR On-135
- TpalR On-150
- TpalR On-180
- TpalR On-210
- TpalR On-240

Asel digestion [kb]

Initial

| 5.8 | 6.2 | 5.4 |

Dicentric Acentric
5.4 – Analysis of Replication Intermediates of RuraR

In order to visualize the replication intermediates formed upon induction of fork stalling in the RuraR construct where a U-turn event is not possible, 2DGE was performed on samples obtained from synchronous cultures. Strain RuraR P_{urg1::rtf1-DSR nda3-KM311} was grown to early log phase and cell cycle was synchronised. Fork stalling was induced, or not, one hour before the release and samples were taken at intervals upon the release. DNA was extracted in agarose plugs and digested with AseI (see Figure 5.3A for a map of RuraR and AseI sites). 2DGE was performed and Probe Cen homologous to sequences centromere proximal to RuraR was used to detect replication intermediates in the Southern blots (Figure 5.3). Analysis of replication intermediates of the ‘Off’ state (Figure 5.3B) indicated passive progression of replication throughout the cell cycle and a slight degree of fork arrest at centromere proximal RTS1 showing the leakiness of urg1 promoter. In the induced culture (Figure 5.3C), at t=0 after the release, where cells were completing mitosis, only the ~5.2kb monomer spot was detected. With similar timing to the previous systems initiation of S phase was evident at 45 minutes judged by the formation of Y arc and a faint arrest signal at centromere proximal RTS1 at ~6.5-7kb, and detection of Y arc. 60 minutes after the release signals corresponding to telomere proximal (~6kb), centromere proximal, and double arrest (~7.5-8kb) at both RTS1 sequences of RuraR were detected. While a signal corresponding to the D-loop intermediate was not detectable a faint X spike signal corresponding to HR-dependent intermediates was detected, and these intermediates accumulated by 90 minutes but were resolved by 150 minutes after the release (G2). In late G2 (t=180) only the monomer and a faint trace of arrest at centromere proximal RTS1 were detected. In the S phase of the second cell cycle (t=240) signals corresponding to fork arrests and HJ
intermediates reappeared. These data indicated that NAHR occurred in S phase and resolution of the resulting HR intermediates in G2, with a similar timing to resolution of intermediates due to the U turn of the restarted fork, before entry into the next cell cycle.
Figure 5.3 (following page) | Replication intermediates due to NAHR at RuraR. 2DGE analysis of RuraR replication intermediates detected by a probe homologous to centromere proximal sequences to RuraR. Cells were grown to early log phase and synchronized. Fork stalling was induced, or not, 60 minutes before the release and the time course was started as cells were released. Samples were collected at indicated intervals. DNA was prepared in agarose plugs and digested using AseI. A- Cartoon showing RuraR and AseI restriction sites (left) and table showing the expected sizes of rearrangements (right) B- 2DGE analysis of the ‘Off’ state. The black arrow indicates the Y arc and the blue arrow indicates the monomer spot. C- 2DGE analysis of the ‘On’ culture. The red arrow indicates the arrest at centromere proximal RTS1, the green arrow shows the arrest at telomere proximal RTS1, and the orange indicates the double pause at both RTS1 sequences of RuraR. The purple arrow indicates the signal corresponding to HR intermediates.
Figure 5.3

A

B

C
5.5 – Further Characterization of Putative U-Turn Intermediate

The U-turn model of chromosome rearrangement at RTS1 predicts the formation of a closed fork-like structure, which would migrate on the Y arc dependent on the restriction enzyme digest. In order to test that the signal observed at the apex of the Y arc in the TPalR system corresponds to the U-turn 2DGE was performed on DNA fragment digested with a different restriction enzyme (BamHI). It was predicted that this would result in migration of the U-turn signal on the downward large Y arc according to the restriction fragment length (Figure 5.4).

Cells were grown to early log phase and synchronized in mitosis. Fork stalling was induced, or not, 60 minutes before the release and samples were collected 90 minutes after the release. DNA was extracted and digested using BamHI. Fork stalling at RTS1 is predicted to form ~12kb pause spot on the small Y arc. The U-turn intermediate should run at ~14kb on the large Y arc. The analysis is complicated due to technical problems associated with running such large fragments on 2DGE. It was found that migration of the non-linear molecules was retarded and molecules did not run according to their true size. This can be seen by comparison of the dicentric monomer (blue arrow) and the pause signal (red arrow) which are both expected to run at ~12kb on the linear and Y arc respectively. However, if it is assumed that all non-linear molecules are retarded to similar extent and the faint spot detected on the large Y arc only in the ‘On’ culture (green arrow) would correspond to the U-turn intermediate. This supports the prediction that a closed fork structure would migrate on the Y arc according to size. To optimize and confirm this analysis both agarose gel concentration and running voltage of the first and second dimensions need to be reduced.
Figure 5.4 | Further characterization of putative U-turn intermediate. 2DGE analysis of TpalR replication intermediates detected by a probe homologous to centromere proximal sequences of TpalR. Cells were grown to early log phase and synchronized in mitosis. Fork stalling was induced, or not, 60 minutes before the release and samples were collected 90 minutes after the release. DNA was extracted in agarose plugs and digested using BamHI. A- Cartoon showing TpalR and BamHI restriction sites (left) and table showing the expected sizes of rearrangements (right) B- diagrams showing the predicted replication intermediates of TpalR when pausing is ‘Off” (left) and ‘On’ (right). Rearranged dicentric chromosomes are expected at ~12kb on the linear. Fork stalling signal at RTS1 is expected at ~12kb on the small Y arc. A U-turn intermediate is expected at ~14kb on the large Y arc. C- 2DGE analysis of the ‘Off” state (left). The black arrow indicates the Y arc. 2DGE analysis of the ‘On” culture (right). The red arrow indicates the arrest at RTS1, blue arrow indicates the dicentric monomer spot, and the green arrow indicates a spot corresponding to a U-turn intermediate on the large Y arc. The white dashed line traces the Y arc. Although the rearranged dicentric chromosomes and the pause signal are both expected to run at ~12kb on the linear and Y arc respectively, the migration of the non-linear molecules was retarded and not according to their true size. However, it can be assumed that all non-linear molecules are retarded to a similar extent and thus the spot detected on the large Y arc corresponds to the U-turn intermediate.
5.6 – Discussion

Similarly to the findings in the RuiuR fork stalling system reported in previous chapter, here I show that in the TpalR fork stalling system, where restart by NAHR is not possible due to the absence of a homologous telomere proximal RTS1 sequence, dicentric chromosomes start to accumulate in G2 phase of the cell cycle (1D gel). This accumulation occurred simultaneously with disappearance of slow migrating replication intermediates. Analysis of these intermediates showed HR-dependent restart of the collapsed fork in S phase, resulting in accumulation of HR intermediates that were resolved in G2 before entry into the next cell cycle. A replication intermediate corresponding to the expected size of a U-turn event was identified at the apex of Y arc. The U-turn model of chromosomal rearrangement at RTS1 predicts formation of a closed fork like structure, which would migrate on the Y arc dependent on the restriction enzyme digest. Preliminary experiments using an alternative restriction digest support this but further experiments are required to optimize the 2DGE conditions and confirm these results.

Analysis of the replication intermediates in the RuraR system, where rearrangements occurs via the NAHR mechanism, showed the occurrence of the NAHR in the S phase, with the resulting HR intermediates being resolved in G2 prior to entry into the next cell cycle. A signal corresponding to a D-loop intermediate could not be detected. This may be due to the expected size of an D-loop intermediate (~6.2kb) as this would run with the signal corresponding to the fork arrest at centromere proximal RTS1. In addition, Lambert et al. (2010) used cross-linking agents to stabilize replication intermediates, and Benzoylated Naphthoylated DEAE–Cellulose (BND) to enrich for replication intermediates from steady state cultures. BND binds ssDNA in
high salt conditions. When DNA was prepared without crosslinking the D-loop signal was not detectable, suggesting the transient and labile nature of this intermediate. In contrast, the data presented in this chapter showed the detection of an intermediate corresponding to a U-turn event in the TpalR system, consistent with the closed fork-like structure being a more stable intermediate.

In both the TpalR and RuraR systems and the palindrome system RuiuR (see previous chapter) double Y or X shaped intermediates were detectable from 90 min. In TpalR these predominantly ran on the double Y arc, in RuraR were predominantly on the X spike and consistent with both types of rearrangements occurring in RuiuR on both arcs in this system. In all cases these intermediates decreased in G2 consistent with resolution of all intermediates occurring before mitosis. These data confirmed the occurrence of the HR-dependent restart in S phase and resolution of the resulting intermediates in G2 resulting in an accumulation of dicentric chromosomes. Moreover, considering the labile nature of a D-loop intermediate in this system and the more stable nature of a U-turn intermediate, it can be concluded that the intermediate detected at the apex of Y arc in RuiuR system (Chapter 4) likely corresponds to a U-turn event.
CHAPTER 6 – ANALYSIS OF CHECKPOINT RESPONSES TO GROSS CHROMOSOMAL REARRANGEMENTS

6.1 – INTRODUCTION

In previous chapters I determined the timing of replication restart of the collapsed fork and showed the generation of gross chromosomal rearrangements. Similar to the RuraR and TpalR fork stalling systems, in the RuiuR fork stalling system HR-dependent restart of the collapsed fork occurs in S phase, and this results in the generation of HR intermediates. These HR intermediates accumulated in S phase and were resolved in G2 (approximately 55 minutes later), coinciding with an increase in gross chromosomal rearrangements and accumulation of dicentric and acentric chromosomes. However, analysis of cell cycle progression showed no cell cycle delay and cells progressed into mitosis where chromosome bridges were formed at anaphase. This implied that the HR intermediates and subsequent GCRs were not detected by checkpoint machineries. Therefore I set out to directly investigate the checkpoint responses to replication restart dependent HR intermediates and GCRs. Given that in the RuiuR system GCRs were seen in ~15% of the population and since cross overs leading to acentric and dicentric chromosomes result from 50% of HJs, dependent on the plane of the resolution, it can be estimated that ~30% of cells in the population contains recombination intermediates that are resolved in G2. Thus this provides an ideal system to investigate whether HR intermediates at a single locus are detectable by the DNA integrity checkpoints.
As described in Chapter one, in fission yeast the ATR homologue Rad3 is activated in response to both replication stress and DNA damage, whereas the role of ATM homologue Tel1 is minimal in the checkpoint response. However, Rad3 dependent phosphorylation of effector kinases Cds1 and Chk1 can be used as a marker for activation of DNA replication checkpoint and DNA damage checkpoint respectively.

6.2 – A SINGLE ARRESTED FORK IS NOT SUFFICIENT FOR GLOBAL ACTIVATION OF DNA REPLICATION CHECKPOINT

To investigate the DNA replication checkpoint response to collapsed forks at an specific locus in the S. pombe genome, RuiuR Purg1::nr::rtf1-DSR nda3-KM311 cells were grown to early log phase and synchronised in mitosis. Fork stalling was induced before the release and time course samples were harvested at 15-minute intervals after release covering up to the end of the second cell cycle. In S pombe, depleting the nucleotide pool by HU treatment leads to global replication stress and activation of the replication checkpoint. As controls an asynchronous culture and cells exposed to HU for three hours were used. Whole cell protein was extracted and western blot analysis used to detect phosphorylation of Cds1 (Figure 6.1).

To test the sensitivity of the western blot assay, the phosphorylation shift of Cds1 was investigated in dilutions of protein extract obtained from the asynchronous cells treated with 10mM HU for three hours (+HU), with the -HU control from untreated asynchronous cells (Figure 6.1A). The phosphorylation shift of the Cds1 was detectable in all dilutions of the +HU samples (down to 1% +HU). To detect the possible activation of DNA replication checkpoint to forks collapsed at a specific locous
within the genome, phosphorylation shift of Cds1 was investigated in protein extracts obtained from cells harvested at indicated time points in Figure 6.1B. In the HU treated control the mobility shift of pCds1 was detected indicating the activation of the DNA replication checkpoint (Figure 6.1B). However, no pCds1 shift was detected in either the first or the second S phase after the induction of fork stalling. As the majority (>90%) of replication forks arrest at RTS1 in RuiuR (Lambert et al., 2010), it can be concluded that a fork arrest at a single locus does not lead to global activation of the DNA replication checkpoint.
Figure 6.1 | Replication restart at single locus does not globally activate the DNA replication checkpoint. A- Western blot showing the detection of pCds1 shift in HU treated cells (+HU) and serial dilutions of HU treated extracts with the untreated asynchronous sample (-HU) indicated as percentage of the HU treated control in the diluted sample. pCds1 is detectable in dilutions down to 1% of the HU treated sample. B- Representative western blot showing the pCds1 shift after Rtf1 induction. The lane labeled +HU indicates asynchronous control cells treated with 10mM HU for three hours. Cds1 was detected by anti-Cds1 antibody. Asterisk indicates a none-specific band that acts as a loading control. The colored bar indicated the different stages of the cell cycle.
6.3 – DNA DAMAGE CHECKPOINT IS ACTIVATED IN THE SECOND CELL CYCLE AFTER FORK STALLING

To analyse the DNA damage checkpoint response to HR intermediates and GCRs, \textit{RiuuR P_{arg1}_{NR}:rtf1-DSR chk1:3HA nda3-KM311} cells were grown to early log phase. Cell cycle synchronised in mitosis and Rtf1 induction was induced, or not, one hour before the release. The cell cycle block was alleviated and samples were harvested at 15-minute intervals for two cell cycles. Whole cell protein extracts were prepared and analysed by western blotting (Figure 6.2). The time course was also carried out on a ‘no stall’ \textit{rtf1Δ} strain (\textit{RiuuR P_{arg1}_{NR}:HPH chk1:3HA nda3-KM311 rtf1::natMX6}).

Asynchronous cells and cells treated with 200 Gy were used as controls to detect the phosphoshift of pChk1. Chk1 phosphorylation was detectable in neither the ‘no stall’ nor the ‘Off’ cultures when compared to pCk1 shift of ionising radiation treated control.

In the induced culture, no pChk1 was detected in the first cell cycle but the mobility shift of pChk1 was visible in G2 of the second cell cycle (time point 285 onwards). This indicates that replication fork collapse at \textit{RTS1}, HR restart dependent events that lead to formation of HR intermediates and subsequent accumulation of GCRs in the G2 after fork stalling do not activate the DNA damage checkpoint in the first cell cycle.

However, the checkpoint was activated in the second G2 implying the dependency of this activation on passage through mitosis and cell division.
Figure 6.2 | DNA damage checkpoint is activated in the second cell cycle after induction of Rtf1. Representative western blot showing the pChk1 shift after Rtf1 induction in induced (top panel), uninduced (middle panel), and ‘no stall’ (bottom panel) cultures. The lane labeled $\gamma$ indicates asynchronous control cells treated with 200 Gy. (Cdc2 loading control is presented in Figure 6.4). Chk1-HA was detected by anti-HA antibody. The coloured bar indicates the different stages of the cell cycle.
6.4 – DNA DAMAGE CHECKPOINT ACTIVATION IS DEPENDENT ON PASSAGE THROUGH MITOSIS

HR-dependent restart of collapsed forks at RTS1, and the resulting acentric and dicentric chromosomes did not trigger a checkpoint response in the first cell cycle. The DNA damage checkpoint, however, was activated in the G2 of second cell cycle after induction of fork stalling. This implied checkpoint activation was due to the generation of DNA damage after segregation of the dicentric chromosome at mitosis. This chromosome would align correctly at metaphase but form a chromosome bridge at anaphase. Breakage of the bridge by molecular forces or by septum formation at cytokinesis would generate a one-ended DSB in the next cell cycle. To test whether checkpoint activation was dependent on passage through mitosis, the second mitosis was disrupted using thiabenzadole (TBZ), a microtubule depolymerising drug. Cells from strain RuiuR Parg1_NR::rtf1-DSR chk1:3HA nda3-KM311 were grown to early log phase and synchronised in mitosis. Fork stalling was induced, or not, one hour before the release and samples were collected at 15-minute intervals after the release. TBZ was added to half of the induced culture 120 minutes after the release (G2). Whole cell protein extracts were prepared and western blot analysis was carried out. Asynchronous cells irradiated with 200 Gy were used as control to detect mobility shift of pChk1. Cell cycle progression was monitored by scoring mitotic and septation indices (Figure 6.3). Analysis of cell cycle progression showed normal progression of the cell cycle in the induced culture but partial inhibition of the second cell cycle in the induced culture treated with TBZ (Figure 6.3A). Note that TBZ treatment does not block progression of all cells into the next cell cycle. Investigation of Chk1 phosphorylation (Figure 6.3B) showed the absence of pChk1 shift in the uninduced culture but in the induced culture
without TBZ, the Chk1 phosphorylation shift was detected as expected in the second G2. However, the induced culture treated with TBZ failed to trigger a full checkpoint response (Quantified in Figure 6.3C). These data showed that while HR intermediates and GCRs did not trigger the DNA integrity checkpoints in the first cell cycle, the checkpoint is activated dependent on passage through mitosis. These data is consistent with the induction of DNA damage during mitosis and/or cytokinesis triggering a checkpoint response in the second cell cycle.
Figure 6.3 | Checkpoint activation is dependent on passage through mitosis.

A- Graph showing the cell cycle progression of TBZ treated and untreated induced cultures. m and s denote the percentage of mitotic and septated cells after release from the mitotic block. B- Western blot analysis showing the mobility shift of pChk1 in induced – TBZ (top panel), induced +TBZ (middle panel), and uninduced -TBZ cultures. Coloured bar indicates different stages of the cell cycle. C- Quantification of Chk1 phosphorylation at indicated time points. D- Enlargement of indicated time points from B.
6.5 – DNA DAMAGE RESPONSE DOES NOT LEAD TO H2A PHOSPHORYLATION

As discussed in Chapter one, in response to DNA damage histone H2A is phosphorylated in an ATM/ATR dependent manner, and this assists recruitment of various downstream checkpoint proteins and checkpoint signal amplification. To examine the phosphorylation state of H2A after induction of fork stalling, RuiuR  

\[ P_{arg1 NR}::rtf1-DSR \text{ chk1:3HA nda3-KM311} \]  
cells were grown to early log phase and synchronised in mitosis. Rtf1 was induced, or not, 60 minutes before the release and samples were collected at intervals after the release. In parallel, the time course experiment was performed on an rtf1A ‘no stall’ strain. Whole cell protein extracts were prepared and western blot analysis was performed. Anti-pH2A antibody was used to detect pH2A (Figure 6.4). IR treated asynchronous cells were used as a control to detect H2A phosphorylation, and Cdc2 was used as a loading control. In neither ‘On’, ‘Off’, nor ‘no stall’ cultures was pH2A detectable in the first or the second cell cycles after the release. Therefore, a single site of DNA damage is not sufficient to induce global phosphorylation of H2A detectable by western blot analysis.
Figure 6.4 | A single DNA break does not lead to detectable levels of phosphorylation of H2A. Western blots showing the phosphorylation state of H2A (top panel) and Cdc2 loading control (bottom panel) in ‘On’ A, ‘Off’ B, and ‘no stall’ C cultures. The lane labeled with $\gamma$ indicates the IR (200 Gy) treated asynchronous control. The colored bar indicates different stages of the cell cycle. Asterisk indicates non-specific band. pH2A was detected with anti-pH2A antibody. Cdc2 was detected with anti-Cdc2 antibody.
6.6 – Discussion

In this chapter the checkpoint response to events following restart of an arrested replication fork at a specific site in genome was investigated. In previous chapters I showed that induction of fork stalling in RuiuR system leads to fork arrest in S phase. Replication is restarted shortly after pausing and requires homologous recombination (Lambert et al., 2005), but this restart occurs at the expense of generating in appropriate recombination intermediates that are resolved to form acentric and dicentric chromosomes in G2. However analysis of cell cycle progression showed no mitotic delay in response to HR intermediates or GCRs. Since the dicentric is a sister chromatid fusion with a single functional centromere, it is predicted that the centromere would align correctly at mitosis satisfying the SAC, but generate a chromosome bridge at anaphase. Breakage of the bridge by either spindle forces or breakage by the septum would lead to the daughter cells inheriting a single ended DSB.

GCR levels are not affected by loss of checkpoint proteins Rad3, Cds1, and Chk1 in the RuraR system (Tsang et al., 2014) or in the RuiuR palindrome system (K. Mizuno, personal communication). However, a slight loss of viability was seen in rad3Δ mutants consistent with a requirement for Rad3 to limit resection behind the collapsed fork (Tsang et al., 2014). To establish the status of checkpoint activation in response to fork collapse and HR restart, the phosphorylation state of effector kinases of both intra-S (Cds1) and DNA damage (Chk1) checkpoint was investigated. Induction of fork stalling did not lead to activation of the replication checkpoint in either the first, or the second cell cycle after Rtf1 induction. This indicated that a single collapsed fork in not sufficient to globally activate the intra-S checkpoint. The lack of checkpoint activation in the second S phase after release could be due to the timing of breakage as
septum formation is coincident with second S phase. This would imply that breakage occurs as a result of cytokinesis rather than mechanical forces. Alternatively, the DSBs formed by mechanical breakage or cytokinesis are not processed to expose ssDNA during S phase. The DNA damage checkpoint was not activated in the first cell cycle after fork stalling induction but was activated in the second G2 dependent on passage through mitosis. This is consistent with processing at the DSB in G2 being dependent on CDK levels. No global phosphorylation of H2A was detected demonstrating that one DSB is not sufficient to detect H2A phosphorylation by western blot analysis. It should be noted that up to 15% of cells in the second cell cycle would contain a DSB but >90% of cells in the first cell cycle arrest and restart replication leading to ~30% containing HR intermediates which resolve into acentric and dicentric chromosomes in 15% of cells. Thus, the DNA damage checkpoint activation in the second cell cycle provides a control for the lack of activation in the first cell cycle.
CHAPTER 7 – DISCUSSION AND CONCLUSIONS

7.1 – OVERVIEW

Preserving genome integrity is of vital importance to cellular life. The genetic material is under constant insult from indigenous and exogenous agents resulting in replication arrest. Therefore, eukaryotic cells have evolved failsafe mechanisms, known as checkpoints, to ensure the detection and appropriate response to replication stress. The replication checkpoint helps to ensure the faithful completion of replication after replication stress by activating dormant origin firing, delaying cell cycle progression, maintaining arrested RFs in replication competent state, and preventing processing of arrested RFs, which can lead to genomic instability (Branzei & Foianai, 2005; Lambert et al., 2007). Arrested replication forks can be rescued by dormant origin firing and converging replication forks, however in regions with low origin density or regions of unidirectional replication, replication restart mechanisms become essential for the completion of replication. Homologous recombination plays an important role in restart of arrested replication forks but at the expense of non-allelic homologous recombination (NAHR) resulting in loss of heterozygosity, gross chromosomal rearrangements (GCRs) and chromosomal instability (Carr & Lambert, 2013). Several lines of evidence have demonstrated the link between replication stress and human hereditary diseases, tumourigenesis and cancer (Magdalou et al., 2014; Mirkin, 2007). Burrell et al. (2013) showed that replication stress was the main cause of chromosome instability in colorectal cancer. In human early embryonic development, replication arrest and restart
at small CGG repeats of FMR1 locus causes an alteration in the direction of replication leading to repeat expansion. This results in the absence of FMR1-encoded protein and leads to Fragile X syndrome (FXS), the most common form of hereditary mental disability in males (Gerhardt et al., 2014).

The stochastic nature of replication stress has posed an obstacle to defining the role of checkpoints in response to individual replication problems and HR restart of replication forks. In this study I investigated the timing of HR-dependent replication restart and the checkpoints response to events following replication fork collapse at a specific locus in the genome of fission yeast.

To study site-specific replication restart a polar replication fork barrier enforced by Rtf1 binding to RTS1 sequences was employed. Induction of Rtf1 results in fork collapse at RTS1 in S phase. HR is required for restart (Lambert et al., 2005) but this is error prone and generates GCRs due to NAHR (Lambert et al., 2010) and the U-turn of the restarted started fork between small inverted repeats (Mizuno et al., 2013). In this study I developed a system to analyse events in a single defined cell cycle following replication collapse and restart. Cell cycle synchrony using the cold sensitive beta tubulin mutant, nda3KM311, and rapid induction of Rtf1 expression using ura1 promoter were optimised. Using this system, I showed that replication arrest at RTS1 occurred in S phase. Collapsed replication forks were restarted by HR in S phase, at the expense of formation of HR intermediates that required resolving in 30% of the population. The nature of the intermediates resulting from the two alternative mechanisms generating GCRs was investigated. In both cases HR intermediates were resolved in G2 before entry into mitosis, coinciding with the accumulation of GCRs in ~15% of the population. Investigation into the activation of the DNA integrity checkpoints showed that replication arrest, HR-dependent restart, and accumulation of
GCRs do not globally activate either the DNA replication or DNA damage checkpoints in the first cell cycle. However, the DNA damage checkpoint was activated in G2 of the second cell cycle.

7.2 – Replication Intermediates

In the fork stalling systems, forks collapse at RTS1 and subsequent HR-dependent restart drives accumulation of HR joint molecules, which I showed to peak in S phase and decline in G2. Previous work in the Carr, Lambert, and Murray laboratories identified two mechanisms of formation of GCRs in the RTS1 fork stalling systems, NAHR and U-turn of the restarted fork (Lambert et al., 2010; Mizuno et al., 2013). The HJ-like intermediates observed in approximately ~30% of the population (estimated based on the level of cross-over products observed) in RuiuR are the result of both mechanisms. Mizuno et al. (2013) showed that in the RuiuR system the majority of dicentric formation is due to error-prone nature of the restarted fork executing a U-turn at the centre of the palindrome.

In NAHR the first detectable HR intermediate is the D-loop (Lambert et al, 2010) (see Figure 4.3). In the alternative mechanism the non-canonical and error-prone restarted replication fork U-turns at an inverted repeat. This is predicted to form a closed fork structure. Since both D-loop and U turn are predicted to run at the same place in the RuiuR system I used two separate systems to distinguish U-turn and NAHR events: TpalR, where NAHR is abolished, and the original Lambert system RuraR, where there is no palindrome and only NAHR is possible. While the D-loop was not detectable (likely due to the fact that crosslinking was not employed to prevent branch
migration), I identified a stable replication intermediate corresponding to the U-turn event on both RuiuR and TpalR (Chapters four & five). This migrated on the Y arc dependent on size consistent with it being a closed Y structure. This is the first characterisation of this structure. Future work would be to optimise the amount of this intermediate with a view to visualisation by EM.

7.3 – Holliday Junctions

Restart by NAHR is predicted to give rise to intermediates containing a single HJ or in the case of a second end capture double HJs (dHJs). (The model for the generation of single HJs is given in Figure 4.7. For the dHJ model see Lambert et al., 2010). These HJ intermediates were visible in the RuraR system as a descending X spike and were present from S to early G2 (75-135 minutes).

The error-prone model of replication restart at RTS1 (U-turn) also predicts the formation of HJ-like intermediates upon convergence of the second restarted fork (Mizuno et al., 2013). The exact nature of this structure is unknown but it was visible in both RuiuR and TpalR systems as an extension to the double Y structure terminating at the top of the X spike (consistent with it being an HJ-like structure). Given the constraints of sister chromatid cohesion it could be argued that, unlike the HJs formed by NAHR, this structure might not be recognised and resolved until under tension in mitosis (see Figure 7.1) but these intermediates declined in G2 with a similar kinetics to the HJs seen in the RuraR system. These data indicated that the U-turn of the restarted fork results in the formation of HJ-like intermediates, which are processed in G2.
Figure 7.1 | U-turn of the HR-restarted fork. Model illustrating the error-prone HR-dependent restart of the collapsed fork at RTS1. This model predicts formation of a closed fork structure, which is converted into a HJ-like structure.
7.4 – Resolution of Intermediates

Restart by NAHR is predicted to give rise to intermediates containing a single HJ or in the case of a second end capture double HJs (dHJs). The error-prone model of replication restart at RTS1 (U-turn) also leads to intermediates that resemble a single HJ (Mizuno et al., 2013). All these intermediates require further processing by dissolvase or resolvase complexes.

dHJs can be dissolved through the action of the RecQ helicase in concert with Top3 (Wu & Hickson, 2003). In S. pombe this activity is carried out by Rqh1 in collaboration with topoisomeraseIII. However, Lambert et al. (2010) argued that Rqh1 was dispensable for processing of dHR intermediates formed by NAHR due to their inability to branch migrate in the RuraR system. Consistent with this both the level of HR intermediates and viability were not affected in a rqh1 null background (Lambert et al., 2010). Single HJs are not dissolvable and must be resolved. This is the case for single HJs resulting from NAHR and for U-turn intermediates that are postulated to resemble a single HJ-like structure.

In bacteria, RuvC has been shown to have selective resolvase activity for double stranded four-way HR joint molecules (Benson & West, 1994; Takahagi et al., 1994). A RuvC homologue has not been identified in eukaryotic cells but structure specific nucleases have been proposed to resolve HR intermediates (Shwartz & Heyer, 2011). Three resolvase complexes have been identified in eukaryotes, Sp/ScSlx1-Slx4, hSLX1-SLX4, ScYen1/hGEN1, and SpMus81-EME1, ScMus81-Mms4, hMUS81-EME1. Unlike budding yeast and higher eukaryotes, no Yen1/GEN1 homologue has been identified in fission yeast. The structure-specific nuclease activity of Slx1-Slx4 has been demonstrated on an array of branched molecules owing to Slx1 enzymatic activity
(Coulon et al., 2004; Fekairi et al., 2009; Fricke & Brill, 2003; Munoz et al., 2009; Svendsen et al., 2009), but deletion of core Rad52 proteins does not restore viability of \( sgs1\Delta \), \( sgs1\Delta slx1/slx4\Delta \) backgrounds indicating that Slx1-Slx4 does not act on processing of HR intermediates (Bastin-Shanower et al., 2003). Rather the complex is required for maintenance of rDNA homeostasis (Coulon et al., 2006; Kaliraman & Brill 2002).

Unlike Slx1-Slx4, loss of viability of \( rqh1\Delta mus81\Delta \) is restored by deletion of HR genes (Boddy et al., 2000) indicating the role of Mus81 in processing of HR intermediates in the absence of dissolvase in fission yeast. To eliminate the inappropriate processing of stalled replication forks, the nuclease activity of Mus81-Eme1 complex is cell cycle regulated. In budding yeast Mus81-Mms4 is activated in G2 by Cdc5-dependent phosphorylation of Mms4 (Gallo-Fernández et al., 2012; Matos et al., 2011). In fission yeast, Eme1 is phosphorylated in a cell cycle-dependent manner by Cdc2 (CDK), and this primes Eme1 for Rad3-dependent super phosphorylation which results in activation of the complex (Dehe et al., 2013). Moreover in \( S. pombe \), in response to replication stress, Mus81 is negatively regulated by Cds1 and excluded from chromatin (Boddy et al., 2000; Froget et al., 2008; Kai et al., 2005). The cell cycle dependent regulation of Mus81-Eme1 complex could explain the persistent nature of the HR intermediates observed after replication restart in fork stalling systems until G2. HR-dependent restart leads to formation of the HR joint molecules that are resolved in G2 when Mus81-Eme1 complex is activated. This could be tested either by creation of a Mus81 ‘shut off’ strain, in which Mus81 expression is controlled by \( nmt41 \) repressible promoter, or using auxin inducible degron (AID) system, which involves a plant specific protein degradation mechanism depending on response to auxin, and a conserved poly ubiquitination pathway (Kanke et al., 2011).
In higher eukaryotes, the MUS81-EME1 complex exists throughout the cell cycle but requires interaction with SLX1-SLX4 complex for its HJ resolution activity. This interaction is cell cycle regulated and occurs predominantly at prometaphase. This is proposed to be due to a preference for HJ dissolution by BLM, and to prevent inappropriate processing of HJs by MUS81 in early stages of the cell cycle (Castor et al., 2013; Garner et al., 2013; Wyatt et al., 2013). In *S. pombe*, Mus81-Eme1 activity is also cell cycle regulated through CDK-dependent phosphorylation of Eme1. However this phosphorylation peaks after S phase and the complex is most active in G2. This is thought to minimise inappropriate Mus81 processing to prevent genomic instability in S phase, and to ensure resolution of intermediates that eluded Rqh1 processing before mitosis. This also highlights the dependency of fission yeast on Mus81 for HJ processing, likely due to the lack of a Yen1/GEN1 homologue (Boddy et al., 2001; Dehe et al., 2013).

### 7.5 – Checkpoint activation

Investigation of the checkpoint responses (Chapter six) to replication stalling and HR-dependent restart, showed no checkpoint activation in the first cell cycle after fork stalling when HR intermediates were formed and resolved into isochromosomes, but the DNA damage checkpoint was activated in the second cell cycle. The checkpoint sensors ATM and ATR respond to extended regions of stretches of RPA coated ssDNA. ssDNA is formed during S phase due to uncoupling of replicative helicase and polymerase at stalled forks and in G2 by processing of DSBs. Replication arrest at the *RTS1* DNA protein barrier is thought to act via blocking the replicative helicase.
(Eydmann et al., 2008) and thus ssDNA is not exposed by uncoupling of replicative holoenzyme. Rather fork collapse leads to fork regression generating a 3’ end, which coated by Rad51, can invade into the original template independently of a DSB (Lambert et al., 2010; Mizuno et al., 2009). Tsang et al. (2014) showed that Rad3 (ATR) checkpoint sensor limited the resection behind the collapsed fork at RTS1 but did not affect the HR-dependent restart or GCRs. Consistent with lack of ssDNA formation during the block and restart at RTS1 I showed that the intra S checkpoint was not globally activated in the first cell cycle after the release as judged by Cds1 phosphorylation. Thus the intra S phase checkpoint acts locally to restart replication at the site of damage rather than globally to inhibit mitosis.

I showed the dependency of DNA damage checkpoint activation on passage through mitosis in the second cell cycle after induction of fork stalling. This suggests occurrence of a DSB during mitosis or cytokinesis. In human cells this would activate a checkpoint in G1 (Giunta et al., 2010) but in the S. pombe system used here cells proceeded into S phase without delay. This may be due to the fact that S phase is coincident with septation and therefore a break at cytokinesis may not occur before the cells are in S phase. The DNA replication checkpoint prevents end resection during the S phase (Boddy et al., 2000; Hu et al., 2012; Sogo et al., 2002; Tsang et al., 2014). DNA end resection of DSBs has been shown to be CDK dependent and therefore limited in G1 and S. Collaboration of the MRN complex with Ctp1 (ScSae2/hCtPI) is required for initiation of resection at DSBs (Garcia et al., 2011; Symington & Gautier, 2011). CDK-dependent phosphorylation of Sae2/CtPI has been shown to promote resection at DSBs (Huertas et al., 2008; Huertas & Jackson, 2009). In fission yeast, Ctp1 expression is cell cycle regulated with no to low abundance in G1-S and highest expression in G2 (Limbo et al., 2007). It has been shown in budding yeast that once
resection is initiated by MRN-CtpI, Exo1 in concert with Sgs1-Dna2 carries out long-range resection (Gravel et al., 2008; Mimitou & Symington, 2008). Exo1 is phosphorylated by CDK in S/G2 and this promotes its end resecting ability (Tomimatsu et al., 2014). Also, CDK-dependent phosphorylation of Dna2 has been shown to promote its recruitment to the site of DSB, and resection (Chen et al., 2011). In the system used here activation of the DNA damage checkpoint in G2 implies that the cell cycle-dependent regulation of resection of the DSB formed by dicentric breakage delays processing until G2 of the second cell cycle after fork stalling induction, exposing ssDNA and subsequently activating the DNA damage checkpoint.

7.6 – MODEL

The data presented in this study leads to the following model (Figure 7.2) in which replication collapse at RTS1 is restarted by HR mechanisms in S phase leading to accumulation of HR intermediates. The absence of ssDNA in these structures results in no signalling to the intra S or DNA damage checkpoints. The accumulated HR intermediates are resolved in G2, consistent with when Mus81/Eme1 becomes active, leading to the accumulation of GCRs. Dicentric chromosomes align correctly on spindle satisfying the spindle assembly checkpoint and anaphase is triggered. This results in formation of chromosome bridges that break producing a DSB during mitosis and/or cytokinesis and leading to the daughter cells inheriting a one-ended DSB. Resection is prevented by the intra S checkpoint but the DSB is subject to processing in G2 when CDK levels rise resulting in the accumulation of RPA coated ssDNA. The exposed ssDNA activates the DNA damage checkpoint in the second cell cycle.
Figure 7.2 (following page) | Model of checkpoint activation in response to replication fork collapse and restart. Activation of fork barrier activity leads to fork collapse at RTS1. Replication is restarted in a DSB-free homologous recombination dependent manner in S phase, without activating the intra S checkpoint due to lack of ssDNA. However, this restart event is error prone and leads to accumulation of HR intermediates in S phase. These HR intermediates are resolved in G2 giving rise to acentric and dicentric formation. Rearranged dicentric chromosomes are captured by spindle and broken by formation of septum (cytokinesis, coincident with second S phase). The intra S checkpoint prevents resection of this DSB in the second S phase. However, high CDK activity leads to DSB resection in G2, exposing ssDNA. This in turn, activates the DNA damage checkpoint (Chk1) in the second cell cycle after induction of fork stalling.
Figure 7.2

Checkpoints Activity

- **G1**
  - Intra S checkpoint (Rad3/Cds1) prevents resection
  - No global Cds1 activation

- **S**
  - DNA integrity checkpoint (Chk1) not activated (no ssDNA)
  - No delay to mitosis
  - Spindle checkpoint satisfied (kinetochore occupancy/tension)
  - No anaphase delay

- **G2**
  - Dicentric forms anaphase bridge
  - Chromosome broken at cytokinesis
  - One ended DSB

- **M**

- **G1**
  - Intra S checkpoint (Rad3/Cds1) prevents resection
  - No global Cds1 activation

- **S**
  - G2 resection (Cdk dependent)
  - ss DNA
  - DNA damage checkpoint (Chk1) activated
7.7 – CONCLUDING REMARKS

These results are of importance as defects in DNA replication have been shown to greatly increase chromosomal instability, and chromosomal instability is hallmark of cancer development (Branzei & Foiani, 2007). Common fragile sites (CFSs), first described as gaps induced in human metaphase chromosomes under mild replication stress, are known to have difficult to replicate sequences prone to form secondary structures, harbour large genes, and exhibit low origin density (Franchitto, 2013).

Several studies have shown replication stress dependent expression of CFSs, leading to chromosomal instability and cancer development (Le Beau et al., 1998; Hellman et al., 2000; Mitsui et al., 2010; Ozeri-Galai et al., 2011; Palakodeti et al., 2004; Palakodeti et al., 2010; Ried et al., 2000; Wang et al., 1999). Our fork stalling system offers a versatile tool to study replication restart, resembling the characteristics of CFSs by creating a difficult to replicate region, and providing further evidence of role of replication stress in expression of CFSs. The data presented in this study supports a model of genomic instability where inappropriate HR events initiated by fork collapse and restart generate intermediates which are not detected by checkpoints, and this leads to gross chromosomal rearrangements. Passage of these GCRs through mitosis leads to chromosome bridges and acentric chromosomes, generating DSBs and driving further chromosomal instability.
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APPENDIX
Optimisation of the *Schizosaccharomyces pombe urg1* Expression System

Adam T. Watson, Yasukazu Daigaku, Saed Mohebi, Thomas J. Etheridge, Charly Chahwan, Johanne M. Murray, Antony M. Carr*

Genome Damage and Stability Centre, School of Life Sciences, University of Sussex, Brighton, East Sussex, United Kingdom

**Abstract**

The ability to study protein function *in vivo* often relies on systems that regulate the presence and absence of the protein of interest. Two limitations for previously described transcriptional control systems that are used to regulate protein expression in *fission yeast* are: the time taken for inducing conditions to initiate transcription and the ability to achieve very low basal transcription in the “OFF-state”. In previous work, we described a Cre recombination-mediated system that allows the rapid and efficient regulation of any gene of interest by the *urg1* promoter, which has a dynamic range of approximately 75-fold and which is induced within 30-60 minutes of uracil addition. In this report we describe easy-to-use and versatile modules that can be exploited to significantly tune down *P_{urg1} “OFF-levels”* while maintaining an equivalent dynamic range. We also provide plasmids and tools for combining *P_{urg1}* transcriptional control with the auxin degron tag to help maintain a null-like phenotype. We demonstrate the utility of this system by improved regulation of HO-dependent site-specific DSB formation, by the regulation Rtf1-dependent replication fork arrest and by controlling Rhp18*-dependent post replication repair.

**Introduction**

The study of protein function *in vivo* is greatly aided by systems that deplete the protein of interest. Whether or not depletion of a protein is biologically significant (causes a phenotype) will depend on the amount of protein required for its function. The amount of cellular protein is the result of multiple levels of regulation, including transcription rate, mRNA stability, translational efficiency and protein turnover. The study of gene function may require control over one or more of these processes.

In the fission yeast *Schizosaccharomyces pombe* transcription rate has traditionally been controlled using modified constitutive or inducible promoters of varying strength. The promoter of the alcohol dehydrogenase (*adh1*) gene, it’s weak derivative *adh1*-15 and it’s much weaker derivative *adh1*-01, are typical examples of a widely used constitutive promoter [1,2,3]. The most widely used inducible promoter used in S. pombe is derived from the *nmt1* (no message in thiamine) gene [4]. The *nmt1* promoter has the added advantage of intermediate promoter strengths that are achieved through mutation of the TATA box, generating intermediate (*nmt41*) and low (*nmt01*) strength versions [5].

While these *nmt*-derived promoters offer a choice of transcription levels, they all take 12-16 hours to show induction and 15–21 hours to reach maximum induction levels once thiamine is removed. This is a significant disadvantage considering that the fission yeast cell cycle is completed within 2–3 hours. More recently, Watt et al. (2000) characterised the promoter of the *urg1* gene, where *urg1* transcript levels peak 30 minutes after the addition of uracil [6]. However, attempts to reproduce this ectopically resulted in a significant increase in “OFF-state” transcription, severely limiting the dynamic range and thus its utility. We recently demonstrated that induction kinetics driven by the *urg1* promoter (*P_{urg1})*, and the dynamic range, are maintained when ectopic open reading frames (ORFs) replace the native *urg1* ORF [7]. To facilitate rapid and simple manipulation of *urg1* locus, a Cre recombinase and lox recombination site-based Recombination-Mediated Cassette Exchange (RMCE) system was developed [7]. This facilitates rapid and efficient exchange of sequences to place any chosen ORF under control of the endogenous *P_{urg1} for schematic, see Figure 1A).

While it is now possible to regulate transcription of any gene at the *urg1* locus in response to uracil addition, several disadvantages remain: first, while the dynamic range of ~75 fold is good, the basal level of proteins regulated by *P_{urg1} remain significant. Thus, the minimal repressed (“OFF”) level of protein is often too high to visualise a phenotype equivalent to a null mutation. Second, the induced (“ON”) level is correspondingly also high, which may cause problems when studying proteins who’s endogenous levels are comparatively low. Third, while *P_{urg1} transcription resists to basal levels within 30–60 minutes of uracil removal, the protein being studied will decay with kinetics that are determined by the stability of the protein produced. We thus sought mechanisms to further regulate *P_{urg1} dependent* transcript levels and to facilitate removal of the induced protein following transcription shut-off.

In *S. pombe*, a mechanism for the selective removal of meiosis-specific mRNAs in mitotic cells has been characterised. The
removal involves the YTH-domain containing protein Mmi1 [8] which binds meiosis-specific mRNAs containing Determinant of Selective Removal (DSR) sequences, usually located at the 3' end of the transcript. Mmi1 greatly increases transcript turnover by directing DSR-containing transcripts to nuclear exosomes for degradation [8,9]. By deletion analysis, the DSR elements of ssm4...
**Results**

The Mmi1/DSR mRNA degradation system reduces protein levels expressed from the urg1 locus

An advantage of the *S. pombe* mmi1 (no message in thiamine) inducible promoter system is the ability to attenuate promoter activity levels through progressive deletion of the TATA-box sequence [5]. However, deletion of a potential urg1 promoter TATA-box sequence identified by 5' RACE [7] did not significantly reduce promoter activity (data not shown). We therefore decided to exploit the recently characterised mechanism that selectively removes meiosis-specific mRNAs from mitotic cells in *S. pombe*. The mechanism involves Mmi1 binding to a target region—the DSR (Determinant of Selective Removal)—and guiding the mRNA for degradation via the nuclear exosomes [8,9]. The hexanucleotide motif U(U/C)AAAC is highly enriched in the DSR and tandem repeats of this motif function as an artificial DSR in heterologous gene systems [10,11].

We modified our published urg1 promoter system [7] to contain either the 157bp DSR element derived from the *S. pombe* spo5 gene or various numbers of repeats of the DSR core motif: TTAAC. To achieve this, we modified the Cre-expression plasmid pAW8E/ValI [7] by inserting either the 157bp spo5/DSR element or between 1 and 8 copies (referred to as 1XDSR through 8XDSR) of the DSR core motif adjacent to the pAW8E/ValI multiple cloning site such that, in the corresponding mRNA, it will be 3' of the ORF. To maintain identical motif spacing to that previously characterised [10], the repeat motifs were separated by six base pairs copied randomly from bacteriophage lambda DNA. We next introduced the open reading frame (ORF) of the yeast codon-optimised green fluorescent protein (yEGFP) between the MCS and the DSR sequences. When integrated at the urg1 locus, these constructs will express GFP as the ATG initiation codon contained within the *NdeI* restriction enzyme site (CATAAG), present in pAW8E/ValI MCS, is in-frame with the yEGFP ORF. These pAW8E/ValI-CyYEGFP-DSR plasmids were then used to create, via Cre-mediated RMCE, a series of yeast strains where expression of yEGFP was controlled at the urg1 locus by the P\_mgi locus (we designate the modified urg1 promoter, which remains at the urg1 locus, but contains a loxP recombination site at −37, P\_mgi locus to distinguish it from the endogenous promoter). The transcripts resulting from the P\_mgi locus in these cells contain, in their 3' untranslated regions, various forms of the DSR (Figure 2A). A control strain containing no DSR sequences (NO DSR) and another carrying 8 copies of the mutated core motif GTAAAAC (8XmDSR) were also constructed. This mutated core motif has been shown to largely ablate DSR activity [10].

To examine how efficiently the DSR/Mmi1 RNA degradation pathway reduced yEGFP protein levels, the amount of yEGFP in extracts prepared from log-phase cells in the absence (P\_mgi/ off) or presence (P\_mgi/ on) of uracil were analysed by immunoblotting (Figure 2B). The P\_mgi/2yEGFP-NO DSR cells accumulated high levels of yEGFP protein 120mins after induction (lane 13) but a clear signal was also observable in un-induced P\_mgi/2yEGFP-NO DSR cells when compared to control AW501 (urg1\_I) cells (lanes 2 and 1 respectively). This demonstrates the rapid induction of the urg1 system and also the “leakiness” of the repressed urg1 promoter. One and two copies of the DSR core motif (TATAAC) show no significant reduction of yEGFP protein levels either in un-induced (lanes 4 and 5) or induced cells (lanes 13 and 16). However, increasing the number of core motifs to three (P\_mgi/3yEGFP-3XDSR cells) resulted in an observable reduction in protein levels for both un-induced (lane 6) and induced (lane 17) situations. Further reductions in yEGFP expression levels were observed with
Figure 2. Determinant of Selective Removal (DSR) sequences can reduce protein levels expressed from Purg1lox (A). Schematic of the Purg1lox locus following RCME to introduce yEGFP under the control of the urg1 promoter. The resulting transcript encodes the ORF followed immediately after the stop codon by one of a variety of DSR sequences. These act to target the transcript to the nuclear exosome. (B). The strains created with the yEGFP ORF with and without DSR sequences present in the 3’ UTR: AW640 (NO DSR), AW702 (8XmDSR), AW726 (1XDSR), AW728 (2XDSR), AW730 (3XDSR), AW732 (4XDSR), AW694 (5XDSR), AW698 (6XDSR), AW700 (7XDSR), AW638 (spo5DSR) (see also Table 1). The strains were cultured in EMM+Lt o56106 cells at 30°C (uracil absent - Purg1lox OFF). Uracil was added at 0.25 mg/ml and cells grown for 120 minutes (Purg1lox ON). Total protein extracts from un-induced Purg1lox OFF cells (lanes 2–12) and induced Purg1lox ON cells (lanes 13–23) were separated by SDS PAGE prior to Western blotting using anti-GFP to detect yEGFP (upper panels) and anti-tubulin for a loading control (lower panel). Lane 1 = WT control urg1+ strain (AW501). (C) Comparison of the induced Purg1lox-yEGFP levels in B. Induced cell total protein extracts were used to estimate
the fold-decrease in protein levels after induction. The NO DSR sample (lane 13 panel B) was serially diluted using SDS sample buffer and analysed (lanes 2 to 7) alongside undiluted induced 3XDSR (AW730 - lane 8), 4XDSR (AW732 - lane 9), 6XDSR (AW696 - lane 10), 8XDSR (AW700 - lane 11) and spo5DSR (AW638 - lane 12) samples. Lane 13 = undiluted un-induced NO DSR sample (the same as lane 2 panel B). (D) The kinetics of yEGFP accumulation is unaffected when using a DSR. Time-course showing yEGFP protein levels in NO DSR (AW640 - lanes 2 to 8) and spo5DSR (AW638 - lanes 10 to 16) cells after addition of uracil at 0.25 mg/ml to induce Purg1{lox}. Analysis of yEGFP levels by western blot as described in B. Samples taken at time-points shown (mins). Lanes 1 and 9 = control urg1{lox} strain (AW501). (E) Over-expression of mRNAs containing DSR sequences does not affect cell growth/viability. Strains shown were serially diluted 10-fold in water and spotted on EMM+L plates supplemented with uracil at concentration shown. Pictures were taken after 3 days at 30°C. doi:10.1371/journal.pone.0083800.g002

increasing number of DSR repeats (lanes 18 to 22) indicating increased RNA processing. Purg1{lox}yEGFP-9mXDSR cells, our negative control which harbours eight copies of the mutated (GTAAAC) motif, accumulated significantly levels of yEGFP protein (lanes 3 and 14) although there was a modest decrease when compared to the NO DSR control. This implies the mutated motif retains some function. In our positive control cells, Purg1{lox}yEGFP-spo5DSR, the amount of yEGFP was significantly reduced following induction by uracil addition. In the un-induced cell samples the detection limit of our yEGFP probe was significantly reduced following induction by uracil addition for these cells we require a more sensitive method to determine Purg1{lox} repressed protein levels (see below). Induced Purg1{lox}yEGFP-spo5DSR cells showed similar yEGFP levels to those observed for Purg1{lox}yEGFP-6XDSR cells (lanes 20 and 23). The relationship between the number of core TTAAAC repeats and DSR activity was not, however, linear (Figure 2B). This may reflect the mechanism whereby Mmi1 binds the DSR containing transcript.

To estimate the fold decrease in induced protein levels we used the samples from Figure 2B for further western blot analysis. The induced Purg1{lox}yEGFP-NO DSR sample was serially diluted in SDS-sample buffer 5-, 10-, 25-, 50- and 100-fold (lanes 2 to 7) and immunoblotted along with undiluted induced Purg1{lox}yEGFP-3XDSR, -4XDSR, -6XDSR, -8XDSR and -spo5DSR cell samples (lanes 8-12, Figure 2C). Three tandem repeats of the DSR core decrease levels approximately 5-fold when compared to cells containing no DSR sequences. Cells carrying 4, 6 and 8 core repeat motifs reduce levels further (10-, 25- and 50-fold lower respectively). In Purg1{lox}yEGFP-spo5DSR cells, the level is reduced approximately 25-fold compared to the NO DSR control. Importantly, the yEGFP level in NO DSR repressed cells (lane 15) lies between the induced levels seen for cells containing 8 DSR repeats (lane 11) and the spo5DSR (lane 12). Thus, by exploiting the constitutive degradation of transcripts through the introduction of different DSR constructs we can choose "ON" (+ uracil) and "OFF" (+ uracil) levels of protein across a significantly better dynamic range when compared to the constitutive Purg1{lox} RMCE system.

The rapid induction of the Purg1{lox} is the major advantage of the expression system [7]. To establish if this rapid induction is maintained when DSR regulatory sequences are used, we performed an induction time-course and determined yEGFP protein levels by western blotting (Figure 2D). While the spo5DSR sequences reduced the total level of yEGFP, the kinetics of yEGFP accumulation in Purg1{lox}yEGFP-NO DSR and Purg1{lox}yEGFP-spo5DSR remained very similar (compare the long exposure for spo5DSR with the short exposure for NO DSR). These data demonstrate that DSR/Mmi1 RNA degradation pathway allows only a small percentage of DSR-containing transcripts to be translated prior to removal. Following the addition of uracil and the induction of Purg1{lox} transcription rate, the increased DSR-containing mRNA levels will result in higher translation efficiency and the kinetics of induction are maintained.

It has been shown that the disruption of mmi1 severely impairs cell growth [8] and we were concerned that over-expression of mRNAs containing DSR sequences may affect cell growth/viability by titrating the available Mmi1 activity. We therefore performed a spot-test, where cells containing DSRs were spotted onto media containing uracil to induce Purg1{lox} (Figure 2E). For a positive control, the strain AW507 was used [7]. In this strain (described in more detail in the next section) the expression of HO-endonuclease is under the control of the urg1 promoter (Purg1{lox}HO) and the cells contain the HO cut site (HOcs) within the S. pombe his3' selectable marker (HOcs-HIS). HO-dependent cleavage of HOcs-HIS thus prevents cell growth when the media does not contain histidine. As expected, growth in presence of uracil and absence of histidine lead to Purg1{lox}HO, HOcs-HIS cell inviability linked to the concentration of uracil in the growth media (Figure 2E). However, all the DSR containing strains tested grew equally as well on plates either with or without uracil.

Taken together these results show core-repeat and the spo5 DS elements retain selective removal activity when inserted into the 3’ UTR of mRNAs expressed at the urg1 locus and that the DSR/ Mmi1 RNA degradation system successfully reduced yEGFP levels without affecting the speed of induction or cell viability/growth.

The Mmi1/DSR mRNA degradation system attenuates HO expression levels

Because the detection limit of western blot analysis, we were unable to estimate the decrease in “OFF” levels of protein in the various repressed DSR cells (see Figure 2B). We therefore attempted to demonstrate lower Purg1{lox} OFF protein levels using biological assays. We used the previously described S. pombe single-strand annealing (SSA) assay [7]. The SSA strain contains the MATa minimal HO recognition sequence (HOcs) in-frame and within the coding sequence of the S. pombe his3' selectable marker. This construct, flanked by two homologous sequences, is integrated into chromosome 1 (HOcs-HIS; [7]). Regulation of the expression level of the HO endonuclease using Purg1{lox} induces double strand breaks (DSBs) at HOcs. DSB ends then undergo resection that results in single strand DNA (ssDNA) tails. If the chromosome is cut in G1 or both sister chromosomes are cut following replication of the region, homologous recombination (HR) repair is not an option and resection continues until both regions of homology become single stranded. Once this occurs, the homologous ssDNAs anneal, resulting in the repair of the chromosome at the expense of loss of the intervening sequences. These sequences include the HOcs and the his3' selectable marker. SSA rates can thus be measured by calculating the percentage of histidine auxotrophic cells by plating cells prior to and after induction of Purg1{lox}-HO onto histidine containing media and then replica plated onto media lacking histidine. SSA is an efficient repair mechanism, and thus the rate of marker loss reflects the HO expression level.

In S. pombe, for >80% of the cell cycle a sister chromatid is present, allowing DSB repair by homologous recombination. When HO is expressed at high levels in HOcs-HIS cells, both sister
Figure 3. DSR activity reduces both induced and repressed P \textit{urg1lox} protein levels. (A) Schematic illustration outlining the repair of a single HO-induced DSB in a G2 phase \textit{S. pombe} cell. Repair in a normal (WT) cell can occur by either by homologous recombination (HR) or single strand annealing (SSA) (left panel) whereas repair in an HR deficient \textit{rhp51}-delete cell can only occur via SSA (right panel). Grey box = region of homology, HOcs = HO endonuclease cut site. (B), Steady-state rate of \textit{his3}+ marker loss in WT HR proficient cells compared to HR deficient \textit{rhp51}-delete cells. The HO endonuclease ORF tagged at the C-terminus with yEGFP was inserted by Cre-mediated cassette exchange into the \textit{urg1} locus in WT cells containing the HOcs-HIS construct to create AW741 (NO DSR), AW743 (3XDSR), AW745 (4XDSR), AW747 (6XDSR), AW749 (8XDSR) and AW751 (spo5DSR) and in \textit{rhp51}-delete cells to create AW734 (NO DSR), AW816 (3XDSR), AW818 (4XDSR), AW820 (6XDSR), AW822 (8XDSR) and AW739 (spo5DSR). Logarithmically growing cells cultured in EMM+L were plated onto EMM+LH plates and grown at 30°C. Colonies were replica plated onto EMM+L plates and the percentage of histidine auxotrophic (his\textsuperscript{2}) cells calculated. The assay was repeated at least three times and the average numbers are presented as the mean +/- SD. (C) The kinetics of HO-cyEGFP protein accumulation is unaffected by DSR activity. Time-course showing accumulation of HO-cyEGFP protein levels following induction of P \textit{urg1lox}. Logarithmically growing AW671 (NO DSR) and AW673 (spo5DSR) cells (see Table 1) were induced by the addition of uracil at 0.25mg/ml. HO-cyEGFP protein levels were examined by western blot analysis as described in Figure 2B. Samples taken at time points shown (mins). (D) DSR activity slows \textit{his3}+ marker loss in the P \textit{urg1lox}-HO/HOcs-HIS SSA assay. Strains AW741 (NO DSR), AW743 (3XDSR), AW745 (4XDSR), AW747 (6XDSR), AW749 (8XDSR) and AW751 (spo5DSR) were grown in EMM+L to mid-log phase and uracil added at 0.25 mg/ml to induce P \textit{urg1lox}. Cells were plated onto EMM+LH plates and grown at 30°C. Colonies were replica plated onto EMM+L plates and the percentage of histidine auxotrophic cells calculated. Samples taken at time-points shown (mins). The assay was repeated twice and numbers shown are the mean. X axis = percentage of histidine auxotrophic cells, Y-axis = time in minutes. 

doi:10.1371/journal.pone.0083800.g003
chromatids are likely to be cut in a single cell. However, when HO is expressed at low levels (due to leakiness of the \( P_{\text{ugl}} \) promoter for example) the DSBs that occur are likely to be formed in only one of the two sister chromatids. This gives the cell the opportunity to use HR to repair the DSB. HR repair from the sister chromosome is silent: it restores both the HOcs site and retains the \( \text{his}^3 \) marker (Figure 3A). When the HR pathway is not available for repair, as in the \( rhp51 \)-deletant strain, a DSB on a single sister chromatid can only be repaired using SSA. Following sister segregation at mitosis, one daughter cell will be \( \text{his}^+ \) and the other \( \text{his}^- \). Therefore, the rate of marker loss at low levels of HO expression is expected to be higher in \( rhp51 \)-deletant cells when compared to WT cells (Figure 3A) and that rate should be a direct reflection of the number of DSBs introduced.

To test the effectiveness of the DSR/Mmi1 pathway, we subcloned the HO endonuclease ORF to create Cre-expression plasmids \( p\text{AW8ENdeI-HO-cyEGFP-NO DSR} \), \( 3\text{XDSR} \), \( 4\text{XDSR} \), \( 6\text{XDSR} \), \( 8\text{XDSR} \), and \( -\text{spo5DSR} \). Using an \( \text{urg1} \) base strain containing the HOcs-HIS construct (AW667 – Table 1), the HO-cyEGFP fusion ORF with and without DSR sequences was inserted at the \( \text{urg1} \) locus by cassette exchange. From the resulting strains, the steady-state level of histidine auxotrophic (\( \text{his}^+ \)) cells in otherwise wild type cells growing logarithmically in the absence of uracil \( ( P_{\text{ugl}} \text{ OFF}) \) was determined (Figure 3B). For \( P_{\text{ugl}} \)-HO-cyEGFP-NO DSR cells we observed 11.5 (+/− 2.4%) of cells had lost the \( \text{his}^3 \) marker with all strains carrying DSR elements showing significantly lower levels of marker loss. In cells containing 3 copies of the core DSR motif \( ( P_{\text{ugl}} \text{HO-cyEGFP-3XDSR}) \), the rate was reduced to 5.0 (+/− 2.0%) and increasing number of core motifs further decreases the percentage of histidine auxotrophic cells. The \( \text{HO-cyEGFP-spo5DSR} \) cells showed a steady state level of marker loss of 0.6 (+/− 0.5%), similar to that for \( \text{HO-cyEGFP-8XDSR} \) cells.

As discussed above, it is predicted that in HR deficient \( rhp51 \)-deletant cells the steady state level of \( \text{his}^3 \) marker loss will increase compared to wild type cells because the alternative DSB repair pathway (HR) has been removed. The HO-cyEGFP Cre-expression plasmids were transformed into an \( \text{urg1} \) base strain containing the HOcs-HIS construct where the \( \text{rhp51} \) gene is also deleted (AW686 – Table 1). Following cassette exchange, the strains were again analysed in the \( P_{\text{ugl}} \)-OFF condition to determine the state-of-state level of histidine auxotrophic cells. For all the \( rhp51 \)-deletant strains studied, the rate of marker loss increased relative to the HR proficient WT strains (Figure 3B). The level increased in \( P_{\text{ugl}} \)-HO-cyEGFP-NO DSR, \( rhp51 \)-d cells to ~ 43% compared to ~ 11% in WT \( rhp51 \) cells. As observed for HR-proficient \( rhp51 \) cells, marker loss decreased in cells where HO expression was attenuated by DSR regulatory elements with the steady state levels in 8XDSR and spo5DSR cells around 2%.

We next preformed a time course to monitor HO-cyEGFP proteins levels following induction of \( P_{\text{ugl}} \) by uracil addition. The samples were western blotted and probed with anti-GFP antibody (Figure 3C). As was observed for yEGFP protein levels (Figure 2D), following \( P_{\text{ugl}} \) induction the kinetics of HO-cyEGFP protein increase was similar for HO-cyEGFP-8xDSR cells and HO-cyEGFP-NO DSR cells, but a significant overall reduction in expression mediated proteins was evident when the spo5DSR was present (Figure 3C). To investigate the kinetics \( \text{his}^3 \) marker loss following induction of \( P_{\text{ugl}} \) cells containing either NO DSR, the spo5DSR, 3xDSR, 4xDSR, 6xDSR or 8xDSR were analysed for marker loss following induction by uracil (Figure 3D). The kinetics of marker loss was clearly influenced by DSR activity and correlated well with yEGFP levels observed in Figure 2. For example, in induced \( P_{\text{ugl}} \)-yEGFP-6xDSR and \( P_{\text{ugl}} \)-yEGFP-spo5DSR cells the protein levels were comparable (Figure 2A and 2B) and the levels and profile of \( \text{his}^3 \) marker loss in HO-cyEGFP-6XDSR and HO-cyEGFP-spo5DSR, HOcs-\\text{His} cells are also similar.

Overall, these data demonstrate that, despite being unable to detect the protein by western blot due to the low levels, the biological activity of the HO endonuclease in the \( P_{\text{ugl}} \text{ OFF} \) state is decreased by the presence of DSR motifs. This is consistent with protein levels in \( P_{\text{ugl}} \text{ repression cells being decreased when the transcript contains DSR elements. The implied “OFF” state protein levels mirror the protein levels observed by western blot analysis when the \( P_{\text{ugl}} \) promoter was induced by uracil addition. Increasing the tandem core DSR repeat number showed increasing DSR activity, presumably reflecting RNA processing. Despite containing 6 DSR repeats, the 15bp spo5 DSR element exhibits RNA processing activity in the “ON” state similar to that seen for 6 tandem core repeats (Figure 3B) but in the “OFF” state appears equivalent to 8XDSR repeats (see below). This suggests that other factors such as motif spacing may be important for efficient Mmi1 binding and RNA processing.

**Efficient regulation of replication fork barrier activity**

In \( S.\text{pombe} \), site-specific replication fork arrest and recombinant-dependent fork restart have been studied extensively \[15,16,17,18\]. The systems used involve the directional fork barrier sequence, RTS1, which is dependent for activity on the Myb-domain DNA binding protein Rtf1. To date, replication arrest at RTS1 has been regulated by transcriptional control of the \( rtf1 \) gene via the thiamine repressible promoter, \( \text{mmt1} \). However, the \( \text{mmt1} \) promoter is slow to induce (12–16 hrs) compared to the cell cycle time of \( S.\text{pombe} \) (2–3 hours). The \( \text{urg1} \) inducible system is quick to induce, with mRNA levels peaking 30 minutes after the addition of uracil [6]. However, previous attempts to regulate Rtf1 protein levels using \( P_{\text{ugl}} \) were unsuccessful because the repressed level of \( P_{\text{ugl}} \) transcription was too high for the system to be biologically off [7]. We therefore tested if the addition of the spo5DSR regulatory element was sufficient reduce Rtf1 “OFF” levels in \( P_{\text{ugl}} \) repressed cells.

The study of template exchange following fork restart has involved a system in which two inverted copies of \( ura4^{+} \) gene are flanked by RTS1 sequences \[17,18\]. This is referred to as the \( \text{RuiuR} \) construct (Figure 4A). We chose this system for testing the effectiveness of the spo5 DSR element. The \( \text{RuiuR} \) construct was crossed into \( \text{urg1} \) base strain (AW469) to create YSM077 (Table 1). Using plasmid \( p\text{AW8ENdeI-HO-cyEGFP-3XDSR} \) (see materials and methods) we created YSM098 (\( \text{urg1} \):\( P_{\text{ugl}} \)-\( rtf1 \)-spo5DSR, \( \text{RuiuR} \)) by Cre-mediated cassette exchange. Rtf1 activity in these cells can be monitored by detection of replication intermediates (RIs) arising from stalled replication forks using native two-dimensional gel electrophoresis (2DGE). Passive replication of the \( \text{RuiuR} \) locus (Rtf1 absent) is predicted to result in a Y-arc being detected (Figure 4B, left panel cartoon). However, upon site-specific fork arrest (Figure 4B, right panel cartoon), the intensity of the Y-arc is predicted to be reduced and an intense spot is predicted on the Y-arc, corresponding to the position of arrested forks.

In the complete absence of Rtf1 (\( rtf1 \) deleted: \( rtf1 \)D), replication this region is replicated passively (Figure 4C). When Rtf1 is under control of \( P_{\text{ugl}} \) in association with the spo5DSR and repressed (Figure 4C; \( t = 0 \)), the Y-arc is clearly visible with a faint spot corresponding to a low level of replication fork stalling. This is presumably because, as seen for the regulation HO using DSR sequences, the DSR/Mmi1 pathway of mRNA degradation is not 100% efficient. 180 minutes after the addition of uracil (Rtf1 induced), the Y-arc is no longer visible and an intense spot arising from fork arrest is seen (Figure 1C; \( t = 180 \)). These data show that
Table 1. S. pombe strains used in this study.

<table>
<thead>
<tr>
<th>urg1/ base strain employed</th>
<th>RCME plasmid used</th>
<th>Genotype of strain created</th>
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</table>
| h-, urg1::RMCE<sub>ramappa</sub>, leu1-32 (AW459)  
(Watson et al 2011) | pAW8Ndel-cyEGFP | h-, urg1::Purg1lox-cyEGFP, leu1-32 (AW640) |
|                            | pAW8Ndel-cyEGFP+1xDSR | h-, urg1::Purg1lox-cyEGFP+1xDSR, leu1-32 (AW726) |
|                            | pAW8Ndel-cyEGFP+2xDSR | h-, urg1::Purg1lox-cyEGFP+2xDSR, leu1-32 (AW728) |
|                            | pAW8Ndel-cyEGFP+3xDSR | h-, urg1::Purg1lox-cyEGFP+3xDSR, leu1-32 (AW730) |
|                            | pAW8Ndel-cyEGFP+4xDSR | h-, urg1::Purg1lox-cyEGFP+4xDSR, leu1-32 (AW732) |
|                            | pAW8Ndel-cyEGFP+5xDSR | h-, urg1::Purg1lox-cyEGFP+5xDSR, leu1-32 (AW694) |
|                            | pAW8Ndel-cyEGFP+6xDSR | h-, urg1::Purg1lox-cyEGFP+6xDSR, leu1-32 (AW696) |
|                            | pAW8Ndel-cyEGFP+7xDSR | h-, urg1::Purg1lox-cyEGFP+7xDSR, leu1-32 (AW698) |
|                            | pAW8Ndel-cyEGFP+8xDSR | h-, urg1::Purg1lox-cyEGFP+8xDSR, leu1-32 (AW700) |
|                            | pAW8Ndel-cyEGFP+BmxDSR | h-, urg1::Purg1lox-cyEGFP+BmxDSR, leu1-32 (AW702) |
|                            | pAW8Ndel-cyEGFP-spo5DSR | h-, urg1::Purg1lox-cyEGFP-spo5DSR, leu1-32 (AW638) |
|                            | pAW8Ndel-HO-cyEGFP | h-, urg1::Purg1lox-HO-cyEGFP, leu1-32 (AW671) |
|                            | pAW8Ndel-HO-cyEGFP+spo5DSR | h-, urg1::Purg1lox-HO-cyEGFP+spo5DSR, leu1-32 (AW673) |
| h-, urg1::RMCE<sub>ramappa</sub>, LEU-HOcs-his3<i>+</i>-i-EU2, leu1-32, his3<i>D1</i> (AW467 Watson et al 2011) | pAW8Ndel-HO-cyEGFP | h-, urg1::Purg1lox-HO-cyEGFP, LEU-HOcs-his3<i>+</i>-i-EU2, leu1-32, his3<i>D1</i> (AW741) |
|                            | pAW8Ndel-HO-cyEGFP+3DSR | h-, urg1::Purg1lox-HO-cyEGFP+3DSR, LEU-HOcs-his3<i>+</i>-i-EU2, leu1-32, his3<i>D1</i> (AW743) |
|                            | pAW8Ndel-HO-cyEGFP+4DSR | h-, urg1::Purg1lox-HO-cyEGFP+4DSR, LEU-HOcs-his3<i>+</i>-i-EU2, leu1-32, his3<i>D1</i> (AW745) |
|                            | pAW8Ndel-HO-cyEGFP+5DSR | h-, urg1::Purg1lox-HO-cyEGFP+5DSR, LEU-HOcs-his3<i>+</i>-i-EU2, leu1-32, his3<i>D1</i> (AW747) |
|                            | pAW8Ndel-HO-cyEGFP+8DSR | h-, urg1::Purg1lox-HO-cyEGFP+8DSR, LEU-HOcs-his3<i>+</i>-i-EU2, leu1-32, his3<i>D1</i> (AW749) |
| h-, smt0, urg1::RMCE<sub>ramappa</sub>, LEU-HOcs-his3<i>+</i>-i-EU2, rhp51::kanMX6 leu1-32, his3<i>D1</i> (AW866) | pAW8Ndel-HO-cyEGFP | h-, smt0, urg1::Purg1lox-HOcs-his3<i>+</i>-i-EU2, rhp51::kanMX6 leu1-32, his3<i>D1</i> (AW873) |
|                            | pAW8Ndel-HO-cyEGFP+3DSR | h-, smt0, urg1::Purg1lox-HOcs-his3<i>+</i>-i-EU2, rhp51::kanMX6, leu1-32, his3<i>D1</i> (AW834) |
|                            | pAW8Ndel-HO-cyEGFP+4DSR | h-, smt0, urg1::Purg1lox-HOcs-his3<i>+</i>-i-EU2, rhp51::kanMX6, leu1-32, his3<i>D1</i> (AW836) |
|                            | pAW8Ndel-HO-cyEGFP+5DSR | h-, smt0, urg1::Purg1lox-HOcs-his3<i>+</i>-i-EU2, rhp51::kanMX6, leu1-32, his3<i>D1</i> (AW838) |
|                            | pAW8Ndel-HO-cyEGFP+8DSR | h-, smt0, urg1::Purg1lox-HOcs-his3<i>+</i>-i-EU2, rhp51::kanMX6, leu1-32, his3<i>D1</i> (AW841) |
| h-, smt0, urg1::RMCE<sub>ramappa</sub>, Ruli1R, rtf1::natMX6, leu1-32, nda3-KM311 (YSM077) | pAW8Ndel-rtf1-spo5DSR | h-, smt0, urg1::Purg1lox-rtf1-spo5DSR, Ruli1R, rtf1::natMX6, leu1-32, nda3-KM311 (YSM077) |
| h+, urg1::RMCE<sub>ramappa</sub>, leu1-32, ade6::ade6<i>+</i>P<sub>atTIR1</sub>-skp1-AtTIR1-2NLS-9myc (AW617) | pAW8Ndel-rhp18-HAA17 | h+, urg1::Purg1lox-rhp18-HAA17, leu1-32 ade6::ade6<i>+</i>P<sub>atTIR1</sub>-skp1-AtTIR1-2NLS-9myc, rhp18::kanMX6<i>+</i> (YDP210) |
|                            | pAW8Ndel-rhp18-HAA17-spo5DSR | h+, urg1::Purg1lox-rhp18-HAA17-spo5DSR, leu1-32 ade6::ade6<i>+</i>P<sub>atTIR1</sub>-skp1-AtTIR1-2NLS-9myc, rhp18::kanMX6<i>+</i> (YDP231) |

Other S. pombe strains used in the study

<table>
<thead>
<tr>
<th>Strain number and source</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>AW501 (Watson et al 2011)</td>
<td>h-, leu1-32</td>
</tr>
<tr>
<td>JMM1015 (lab stock)</td>
<td>h-, rhp51::kanMX6, ade6-704, leu1-32, ura4-D18</td>
</tr>
<tr>
<td>AW507 (Watson et al 2011)</td>
<td>h-, urg1::Purg1lox-HOcs-his3&lt;i&gt;+&lt;/i&gt;-i-EU2, his3&lt;i&gt;D1&lt;/i&gt;, leu1-32</td>
</tr>
<tr>
<td>AW598 (lab stock)</td>
<td>h-, urg1::RMCE&lt;sub&gt;ramappa&lt;/sub&gt;, ade6-704, his3&lt;i&gt;D1&lt;/i&gt;, leu1-32</td>
</tr>
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</table>
the degradation of the rtf1-spo5DSR mRNA reduces the cellular concentrations of Rtf1 protein sufficiently to allow use of the rapidly inducible P_{urg1lox} system to study blocked replication forks by 2DGE. Importantly, this will allow the study of synchronised cells cultures to further elucidate the mechanisms of recombination-dependent fork restart in *S. pombe*.

Production of a null rhp18 phenotype by the addition of an auxin-inducible protein depletion system

The above experiments demonstrate that the addition of DSR sequences to destabilise the transcripts produced by the basal level of uninduced P_{urg1lox} provides a level of attenuation of the “OFF” level of protein function that is sufficient to allow the manipulation of a cellular function that is sensitive to low levels of protein. However, additional control of the protein stability would offer two additional advantages: first, it would allow even greater control of “OFF” level function and second, it would allow more rapid removal of residual protein upon "shut off" of P_{urg1} transcription, which would add to the versatility of the system. To establish a test system to validate the utility of combining the auxin degron (Figure 5A) with our P_{urg1lox} DSR system, we turned to a well characterised DNA repair function; Rhp18-dependent post replication repair (PRR). Rhp18 is the homolog of *S. cerevisiae* Rad18. The *S. pombe* Rhp18_Rad18 ubiquitin ligase is essential for PRR, allowing cells to progress through and survive S-phase in the presence of replication blocking lesions [19]. *rbp18*. *rad18* delete mutants are hypersensitive to DNA-damaging agents [20], allowing us to test for a null allele phenotype. Together with Rhp6 (*S. cerevisiae* Rad6 homologue), Rhp18_Rad18 mono-ubiquitylates the sliding clamp protein proliferating cell nuclear antigen (PCNA; Pen1 in *S. pombe*) in S phase and response to DNA lesions [19,21].

Using RMCE, we created strains where the rhp18*. *att1* ORF, tagged at the C-terminus with the IAA17 degron, was inserted at the *urg1* locus either with or without the spo5DSR element. Plasmids pAW8E_NdeI-rhp18IAA17 and pAW8E_NdeI-rhp18IAA17_neuDSR (see materials and methods) and the *urg1* base strain YDP273 were used to create strains YDP210 (P_{urg1lox}-rhp18_cIAA17) and YDP231 (P_{urg1lox}-rhp18_cIAA17_neuDSR) respectively (Table 1). Base strain YDP273 also contains the P_{urg1lox}-skp1-AIIRR fusion necessary for the efficient poly-ubiquitination of the IAA17 degron tag [Kanke et al 2011]. Strains YDP210 (rhp18*. *att1* delete, P_{urg1lox}-rhp18-cIAA17-spo5DSR), AW617 (rhp18*. *att1* delete) and YDP237 (rhp18*. *att1* delete) were serially diluted and spotted onto YEA media (control) and YEA media containing the UV memetic 4-Nitro-Quinoline-1-Oxide (4NQO) (Figure 5B). To regulate Rhp18_Rad18 induction, uracil was added or omitted from the growth media. To regulate Rhp18*. *att1* stability, the synthetic plant auxin NAA was either added or omitted. Following growth at 30°C, a null rhp18*. *att1* phenotype was only observed in rhp18-cIAA17-spo5DSR cells where P_{urg1lox} expression is repressed (uracil absent) and auxin-dependent Rhp18_IAA7 degradiation induced (NAA present) (Figure 5B bottom middle panel). The rhp18-cIAA17-spo5DSR cells were only partially sensitive to 4NQO in the absence of NAA (Figure 5B bottom left panel) demonstrating that transcription repression and RNA processing alone are insufficient to obtain the desired phenotype. These results demonstrate that...
protein destabilisation can add a further level of control when proteins are regulated via transcription from $P_{urg1lo}$.

Rhp18Rad18 is required for the ubiquitination of Pcn1 (S. pombe PCNA homolog), which occurs during S-phase and accumulates in cells treated with hydroxyurea [19]. Thus, the levels of Ub-Pcn1 in growing and hydroxyurea-treated cells provides a biochemical readout of Rhp18Rad111 activity. To compare the utility of the auxin degron, the regulation by DSR motifs and the combination of the two together we thus explored the levels of Ub-Pcn1 in a variety of strains and conditions. Cells in which Rhp18Rad111 IAA17 is regulated by $P_{urg1lo}$ either with or without an associated spo5DSR element were grown to mid-log phase and either treated,

**Figure 5. Use of an auxin-inducible degron allows for the generation of a conditional rhp18 mutant strain.** (A) Cartoon of the IAA17 degron system: addition of auxin allows binding of the TIR adaptor (fused to Skp1) to the IAA17 tag (IAA), which is fused to the target protein. This induces ubiquitination and proteasome degradation. (B) Strains AW617 (rhp18+), YDP273 (rhp18Δ) and YDP231 (rhp18Δ, $P_{urg1lo}$-rhp18-HAIAA17-spo5DSR) were serially diluted 10-fold in water and spotted on EMM+L plates supplemented as shown with uracil at 0.25 mg/ml, NAA at 0.5 mM and/or 4NQO at 400 nM. Time of incubation at 30°C. Top panels 3 days, bottom panels 5 days. (C) Schematic of experimental procedure used in D. HU = hydroxyurea, NAA = 1-naphthaleneacetic acid. (D) Ubiquitination of PCNA is abolished in $P_{urg1lo}$-rhp18-HAIAA17-spo5DSR cells in the presence of NAA. Logarithmically growing AW617 (rhp18+), YDP273 (rhp18Δ), YDP210 (rhp18- Δ) and YDP231 (rhp18Δ, $P_{urg1lo}$-rhp18-HAIAA17-spo5DSR) cells cultured in EMM+L at 30°C untreated (−) or treated with 10 mM HU (+) and grown for a 120 minutes or grown for 120 minutes in the presence of NAA at 0.5 mM or uracil at 0.25 mg/ml. Total protein extracts were separated by SDS PAGE prior to Western blotting using anti-PCNA antibody. (E) The auxin degron promotes protein degradation upon “shut-off”. $P_{urg1lo}$, YDP210 (rhp18-Δ, $P_{urg1lo}$-rhp18-HAIAA17) cells were grown in EMM+L and $P_{urg1lo}$ induced by the addition of uracil at 0.25 mg/ml. After 3 h induction, cells were pelleted by centrifugation, washed twice in EMM+L and re-suspended in EMM+L. Samples taken at time-points shown (mins). Total protein extracts were separated by SDS PAGE prior to Western blotting revealed protein levels using anti-HA to detect Rhp18-HAIA17 (upper panels) and anti-tubulin to detect tubulin as a loading control (lower panel). WT represents control strain AW501 (h*, leu1-32).

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Rhp18Rad111 is required for the ubiquitination of Pcn1 (S. pombe PCNA homolog), which occurs during S-phase and accumulates in cells treated with hydroxyurea [19]. Thus, the levels of Ub-Pcn1 in growing and hydroxyurea-treated cells provides a biochemical readout of Rhp18Rad111 activity. To compare the utility of the auxin degron, the regulation by DSR motifs and the combination of the two together we thus explored the levels of Ub-Pcn1 in a variety of strains and conditions. Cells in which Rhp18Rad111 IAA17 is regulated by $P_{urg1lo}$, either with or without an associated spo5DSR element were grown to mid-log phase and either treated,
or not, with 10 mM hydroxyurea. Where appropriate, 0.25 mg/ml of uracil was added to induce Rhp10\textsuperscript{16\textminus d}IAA17 and 0.5 mM NAA was added to induce Rhp10\textsuperscript{16\textminus d}IAA17 instability (for a schematic of experimental design, see Figure 5C). After 120 minutes incubation at 30°C, cell extracts were prepared and analysed by western blot using an S-PCNA antibody (Figure 4D).

As expected, in the control \textit{rhp18}\textsuperscript{+d} cells (\textit{rhp18}+), higher molecular weight Ub-Pcn1 species were observed in both logarithmically growing cells and, at higher levels, hydroxyurea arrested cells (Figure 5D, lanes 1 and 2; 11 and 12). These modifications were absent in the \textit{rhp18}\textsuperscript{16\textminus d}-deleted control (\textit{rhp18}Δ) strain (Figure 5D, lanes 3 and 13 and 14).

In both untreated and hydroxyurea-treated \textit{rhp18}16\textsuperscript{d}IAA17 (YPD210) and \textit{rhp18}16\textsuperscript{d}IAA17-spo5DSR (YPD231) cells, the levels of Ub-Pcn1 decreased in the repressed conditions (uracil absent) when compared to \textit{rhp18}16\textsuperscript{d}b (Figure 5D, lanes 5 and 6; 15 and 16). However, significant residual signal remained, even in the DSR-containing construct. Thus, while repressed P\textsubscript{nmt1} transcript levels appear lower that of the endogenous \textit{rhp18}16\textsuperscript{d} locus and this is further reduced by the presence of the spo5DSR, biological function is not completely ablated. When cells were concomitantly treated with the synthetic auxin, NAA, modification levels were further decreased in both strains. Importantly, \textit{rhp18}+ cells was undetectable in both untreated and HU-treated \textit{rhp18}16\textsuperscript{d}IAA17-spo5DSR cells (Figure 5D, lanes 17 and 18), while residual levels of modifications remained in \textit{rhp18}16\textsuperscript{d}IAA17 cells (Figure 5D, lanes 7 and 8). Over-expression of P\textsubscript{urg1lox}\textit{rhp18}16\textsuperscript{d}IAA17 (presence of uracil, absence of auxin) results in higher levels of PCNA ubiquitilation when compared to control \textit{rhp18}16\textsuperscript{d}b cells (Figure 5D, lanes 9 and 10; 19 and 20).

To establish if, upon shut-off of P\textsubscript{urg1lox}-dependent transcription, auxin addition resulted in more rapid removal of CIAA17-tagged protein, we grew cells in the presence of uracil for 3 hours before transferring them to fresh media without uracil, either supplemented, or not, with auxin (Figure 5E). Loss of the GFP signal was more rapid in the presence of auxin. Taken together, these results show that control over transcription rate, RNA turnover and protein depletion may all be required to obtain a null allele phenotype.

**Influence of arginine and urea**

During the course of our experiments, we have noticed that the level of P\textsubscript{urg1lox}-dependent transcription was significantly reduced in cells grown in EMM media containing arginine. Subsequent experiments demonstrated that, uniquely amongst the commonly used amino acid supplements, arginine significantly suppress the “ON” level (uracil present) of P\textsubscript{urg1lox}-dependent GFP expression (Figure 6A, lane 14). Importantly, the “OFF” level (uracil absent) was also reduced when compared to cells pre-cultured in the absence of arginine (Figure 6A, lane 6 versus lane 2) (see also figure 6C, lane 11 versus lane 2). A similar reduction was seen with the presence of adenine in this experiment, but unlike that seen with arginine, this was not always reproducible. To further improve the P\textsubscript{urg1lox} system, we thus investigated the potential use of arginine for reducing “OFF” level transcription. We first tested if, biologically, the presence of arginine could increase the sensitivity observed when \textit{rhp18} is under the control of P\textsubscript{urg1lox} and is suppressed by the absence of uracil (Figure 6B). Indeed, when grown in the absence of uracil (no induction) and the presence of arginine (inhibition), the phenotype of \textit{rhp18}d, P\textsubscript{urg1lox}\textit{rhp18}10-spo5DSR cells was closer to that seen for the \textit{rhp18} null mutant. We next investigated the kinetics of induction for P\textsubscript{urg1lox} in cells pre-cultured in arginine-containing medium and transferred into fresh arginine-free medium and induced immediately by addition of uracil (Figure 6D). To our surprise, the kinetics of induction was improved, with higher levels of yEGFP present at the earlier time points when compared to cells pre-cultured without arginine. Levels of yEGFP were comparable 3 hours post induction (Figure 6C). The use of arginine in the pre-culture can therefore markedly increase the dynamic range of the P\textsubscript{urg1lox} promoter system and increase the speed of induction.

A novel uracil catabolic pathway has recently been described in the budding yeast \textit{Saccharomyces kluveri}. This pathway is dependent on a pair of genes, \textit{URC1} and \textit{URC4}, that are highly conserved in many bacteria and fungi [22]. The \textit{S. kluveri} \textit{URC1} and \textit{URC4} genes are the orthologs of the \textit{S. pombe} \textit{urg1} and \textit{urg3}, respectively. In \textit{S. kluveri}, \textit{Urc1}, together with \textit{Urc4} and a set of other enzymes, breaks down uracil into urea and 3-hydroxypropionic acid [22]. When considering that the early commitment step of a metabolic pathway is usually subject to feedback inhibition by the final product of that pathway [23] and the fact that arginine can be broken down into urea by arginase when nitrogen is limiting [24], it is conceivable that \textit{Urc1}/\textit{Urg1} expression or activity might be subject to negative regulation by urea. As \textit{S. pombe} can use urea as a sole nitrogen source, we explored if replacing the ammonium in the EMM growth media with urea would have a similar effect on expression levels as was observed for arginine. Compared to cells grown in ammonium-containing media, there was no significant decrease of the “ON” level of P\textsubscript{urg1lox} when urea was used as the sole nitrogen source (Figure 6A, lane 15), indeed the level was higher (lane 15 versus lane 2). The equivalent “OFF” level was also higher (Figure 6A, lane 7). Furthermore, no reduction in the “ON” level of P\textsubscript{urg1lox}-dependent GFP expression was seen in ammonium-EMM media supplemented with lower concentrations of urea (0.5 mM and 25 uM) (Figure 6A lanes 16 and 17). As seen in Figure 6B, the addition of 25 uM urea also had no effect on the sensitivity of P\textsubscript{urg1lox}\textit{rhp18} cells to 4NQO. Despite these observations, an initial inhibition of yEGFP induction was evident when cells were grown in the presence of 10 mM urea before being transferred into fresh urea-free medium (with ammonium as a nitrogen source) and induced by addition of uracil (Figure 6D). Thus, while urea does directly or indirectly have an effect on \textit{urg1} promoter activity, a simple model of substrate inhibition does not explain the complexity of P\textsubscript{urg1} regulation.

**Discussion**

For \textit{S. pombe}, the control of gene expression has remained a problem for many years because a rapidly and easily inducible transcriptional regulation system, i.e. one equivalent to the P\textsubscript{GAL} system of \textit{S. cerevisiae}, has not been available. A number of regulatable expression systems have been established characterised, and each has advantages and disadvantages. The \textit{nmt1} promoter has most commonly been used to manipulate protein levels, and thus gene function, because it presents several distinct advantages: first, it is functional when integrated at different sites in the genome; second, it has a good dynamic range (~75 fold in our hands); third, through the use of TATA-box mutations several different strengths of promoter are available. Importantly, these maintain the dynamic range between “ON” and “OFF” states. However, the \textit{nmt1} promoter has one major disadvantage: it takes between 12 and 16 hours to induce and induction is not particularly synchronous. This has limited its utility for the many experiments that require rapid and synchronous induction to study, for example, the cell cycle specificity of a proteins function.

The recently described \textit{urg1} promoter [6], which is induced by the addition of uracil to the media, offers the most plausible alternative to P\textsubscript{urg1} since it has a similar dynamic range (~1:75).
and is induced within 30 minutes by a simple media manipulation: the addition of uracil, which does not otherwise significantly alter cell physiology. However, \( P_{urg1} \) does suffer from a number of disadvantages: first it does not work well outside of its normal locus; second, its basal level of transcription is relatively high; and third, it is induced during meiosis. In previous work [7] we

Figure 6. The effect of arginine and urea on \( P_{urg1\text{lox}} \) expression levels. (A) Arginine reduces both \( P_{urg1\text{lox}} \) induced and repressed protein levels. AW640 (\( P_{urg1\text{lox}-cyEGFP} \)) cells were grown in EMM+L or EMM+L supplemented with adenine (A), histidine (H), arginine (R), EMM media where \( \text{NH}_4\text{Cl} \) was substituted for 22 mM glutamic acid (E), EMM media where \( \text{NH}_4\text{Cl} \) was substituted for urea at 10 mM (U) or EMM media supplemented with urea at 0.5 mM (U 0.5 mM) or 25 \( \mu \text{M} \) (U 25 \( \mu \text{M} \)). Cells were induced by the addition of uracil at 0.25 mg/ml and cells grown for 2h. A long exposure of the \( P_{urg1\text{lox}} \) OFF and a short exposure of the \( P_{urg1\text{lox}} \) ON are shown. Arrows mark band of interest. (B). Strains AW617 (\( rhp18^{+} \)), YDP273 (\( rhp18^{-} \)), YDP210 (\( rhp18^{-} \), \( P_{urg1\text{lox}}-rhp18 \)) and YDP231 (\( rhp18^{-} \), \( P_{urg1\text{lox}}-rhp18\text{-spo5DSR} \)) were serially diluted 10-fold in water and spotted on EMM+L plates or supplemented as shown with urea at 25 \( \mu \text{M} \) or arginine at 100 \( \mu \text{g/ml} \), with or without 4NQO at 400 nM. Time of incubation at 30°C was 3 days. (C). The induction kinetics of \( P_{urg1\text{lox}} \) is improved when cells are pre-cultured in EMM supplemented with arginine. AW640 (\( P_{urg1\text{lox}-cyEGFP} \)) cells were pre-cultured in EMM+L or EMM+L supplemented with arginine at 100 \( \mu \text{g/ml} \) (\( P_{urg1\text{lox}} \) OFF). Cells were pelleted by centrifugation, washed twice in EMM+L and re-suspended in EMM+L containing uracil at 0.25 mg/ml (\( P_{urg1\text{lox}} \) ON). Samples taken at time-points shown (mins). (D). The induction kinetics of \( P_{urg1\text{lox}} \) is significantly delayed when cells are pre-cultured in the presence of urea. AW640 (\( P_{urg1\text{lox}-cyEGFP} \)) cells were pre-cultured in EMM+L or EMM+L where the nitrogen source is 10 mM urea (\( P_{urg1\text{lox}} \) OFF). Cells were pelleted by centrifugation, washed twice in EMM+L and re-suspended in EMM+L containing uracil at 0.25 mg/ml (\( P_{urg1\text{lox}} \) ON). Samples taken at time points shown (mins). For (A), (C) and (D), total protein extracts were separated by SDS PAGE prior to Western blotting using anti-GFP to detect yEGFP (upper panels) and anti-tubulin to detect tubulin as a loading control (lower panel). WT represents control strain AW501 (\( h^{-} \), \( leu1-32 \)).

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described a system that overcame the first of these disadvantages. We established a Recombination Mediated Cassette Exchange system that allowed the rapid and simple replacement of the urg1 ORF with any sequence of interest. In this report, we have overcome the second of these disadvantages by providing two additional levels of regulation: one at the level of RNA stability and the second at the level of protein stability.

While we do not provide any analysis of how the P_{urg1} promoter functions, we note two things that may be informative: First, the locus is part of a widely conserved operon that has been shown to carry out a novel uracil catabolic cascade in response to nitrogen availability. In addition to the urg1 and urg9 genes, this operon also includes genes that are predicted to encode for a uracil transporter, uracil phosphoribosyltransferases, and perhaps most importantly, a putative transcription factor, which belongs to the Zinc finger family of transcriptional factors [22]. The conserved genomic organization of this bouquet of genes in a wide diversity of fungi and bacteria suggests that the transcriptional regulation of urg1 expression is likely to be complex. This may be one reason why it has not been possible to transfer the dynamic range of the P_{urg1} promoter available at the native locus to a plasmid-based system. Second, we observed that arginine, when supplemented into the growth media, represses uracil-dependent induction by P_{urg1lox}. In particular, arginine suppresses the “OFF” state transcription – i.e. reducing the “leakiness” of the promoter and improves the induction kinetics. Our analysis shows that, serendipitously, this provide an additional opportunity, when combined with our DSR sequences and/or cIAA17 degron tag, to tightly regulate processes that are particularly sensitive to very low protein levels.

While the use of the modified P_{urg1} system we describe here will not solve all the problems associated with gene regulation in S. pombe, we have demonstrated, both here and in our own unpublished data, that the system is both versatile, robust, easy to use and applicable to a range of biological questions. Most importantly, we have succeeded in regulating protein functions which are sensitive to low levels of protein in cells and have exploited the system to study induced conditions in a cell cycle-dependent manner. For our own purposes we chose to regulate HO-dependent site-specific DSR formation, Rtf1-dependent replication fork arrest and Rlp10-dependent post replication repair. However, other functions can also be regulated by application of this modified P_{urg1lox} system. The availability of the generic “base strain” required for RMCE and the convenience of a range of plasmids compatible with the Cre-mediated site specific recombination on which RMCE is based, mean that any sequence can be simply and easily cloned into an appropriate RMCE plasmid and targeted directly to the urg1 locus via a simple transformation and selection procedure at an efficiency that is routinely greater than 30% of cells. Once integrated, the sequence (whether an ORF) will be under transcriptional control such that it can be regulated simply by the addition of uracil. Based on which RMCE vector the sequence of interest is cloned into, both the “ON” and “OFF” state transcript levels can be attenuated by the desired amount due to the inclusion of one or more DSR elements in the non-translated region of the resulting transcript. Similarly, protein stability can be regulated by inclusion of a protein tag derived from the Arabidopsis IAA17 degron, which is regulated by the addition of auxin. Here we have shown that both these systems function and that they can be combined to generate genuine conditional null allele phenotypes. Finally, the versatility of the P_{urg1lox} system can be further enhanced by the simple addition of arginine in pre-induction cultures.

Materials and Methods

Strains and growth conditions

Strains used in this work are listed in Table 1 and all strains grown at 30°C. The media composition was as described [25]. The nitrogen source used in Edinburgh Minimal Media was either 3 g/litre NH4Cl (94 mM), 3.75 g/litre L-glutamic acid (22 mM) or 0.6 g/litre urea (10 mM). In the text, EMM refers to nitrogen source used as NH4Cl unless stated otherwise. For selection of G418, hygromycin (HPH) and nourseothrin (NAT) resistant cells, G418 disulphate (Melford), hygromycin B (Melford) and nourseothrin-dihydrogen sulphate (Melford) were added to YEA plates at a final concentration of 200 μg/ml, 200 μg/ml and 100 μg/ml respectively. Synthetic plant auxin 1-Naphthaleneacetic acid (NAA) Sigma powder was dissolved in a small volume of 0.1N NaOH and then diluted with double distilled water to the required concentration (0.5 M). EMM media was supplemented with leucine (L), adenine (A), arginine (R) and histidine (H) at 100 μg/ml as required. Yeast transformations were performed using a lithium acetate method [26]. Cell pre-cultures for P_{urg1lox} induction assays were not grown to stationary phase before subculturing. E. coli strain DH5α was used for all cloning procedures.

Construction of DSR plasmids for cassette exchange at the urg1 locus

Complementary oligonucleotides containing 1 to 8 repeats of the DSR core element (TTAAAC) (1xDSR to 8xDSR) and 8 repeats of the mutated core element (GTAAAC) (8mxDSR) were synthesised (P1 to P18 - Table 2). The core motifs were separated by 6 nucleotides of randomly selected bacteriophage lambda DNA sequence. After annealing complementary oligonucleotides, the resulting DNA duplex was blanked by overhangs compatible with BglII and XmaI restriction enzymes. The annealed oligonucleotides were ligated into BglII/XmaI restricted pAW8E_{NdeI-CTAP} [7], replacing the CTAP tag to create pAW8E_{NdeI-L-1xDSR} through to pAW8E_{NdeI-L-8xDSR} and pAW8E_{NdeI-L-8mxDSR}. The yeast codon optimised yEGFP ORF from pAW8E_{NdeI-cyEGFP} (Watson et al 2011) was sub-cloned as a BglII fragment into the DSR plasmids to create pAW8E_{NdeI-cyEGFP-1xDSR} through to pAW8E_{NdeI-cyEGFP-8xDSR} and pAW8E_{NdeI-cyEGFP-8mxDSR}. The 3HA sequence (encoding 3 copies of the hemagglutinin epitope tag) from pAW8E_{NdeI-c3HA} (Watson et al., 2011) was sub-cloned as a BglII fragment into pAW8E_{NdeI-L-3xDSR}, pAW8E_{NdeI-L-4xDSR}, pAW8E_{NdeI-L-6xDSR} and pAW8E_{NdeI-L-8xDSR} to create pAW8E_{NdeI-c3HA-3xDSR}, pAW8E_{NdeI-c3HA-4xDSR}, pAW8E_{NdeI-c3HA-6xDSR} and pAW8E_{NdeI-c3HA-8xDSR} respectively.

The 157bp DSR element of the S. pombe spo5 gene as identified by Harigaya et al. (2006) was amplified using the KOD HotStart DNA polymerase system (Novagen - used for all subsequent PCR reactions) from total genomic DNA using primers P19 and P20 (Table 2). The product was cloned into XmaI restricted pAW8E_{NdeI-cyEGFP} to create pAW8E_{NdeI-cyEGFP-spo5DSR}. The 3HA sequence from pAW8E_{NdeI-c3HA} was sub-cloned into pAW8E_{NdeI-cyEGFP-spo5DSR} as a BglII fragment to create pAW8E_{NdeI-c3HA-spo5DSR}.

The IAA17 degron tag sequence was amplified from the plasmid template pMK43 [13] using primers P21 and P22 and the resulting fragment was cloned into the BglII site of pAW8E_{NdeI-cyEGFP} and pAW8E_{NdeI-cyEGFP-spo5DSR}, replacing the yEGFP sequence, to create pAW8E_{NdeI-cIAA17} and pAW8E_{NdeI-cIAA17-spo5DSR} respectively. A single copy of the HA hemagglutinin epitope tag was inserted between the MCS and the poly-TGS linker by annealing complimentary oligonucleotides P23 and P24.
and cloning the resulting DNA duplex into SpeI restricted pAW8E-NdeI-cIAA17 and pAW8E-NdeI-cIAA17-spo5DSR to create pAW8E-NdeI-cHAIA17 and pAW8E-NdeI-cHAIA17-spo5DSR respectively.

To create non-tagging Cre-expression DSR plasmids, the sequence located between the loxP and loxM3 sites of pAW8E (Figure 1) was replaced with a construct containing the 37bp urg1 promoter fragment, an MCS of NdeI-SphI-SacI-SalI-SpeI and the required DSR sequence. Constructs were synthesised (Genscript) and sub-cloned into NheI/XmaI restricted pAW8E-NdeI to create pAW8E-NdeI-3xDSR, pAW8E-NdeI-4xDSR, pAW8E-NdeI-6xDSR, pAW8E-NdeI-8xDSR and pAW8E-NdeI-spo5DSR.

Plasmids created are listed in Figure 1 including Genbank accession numbers for each. Plasmids are available from Addgene.

**DSR plasmid inserts**

The S. pombe rtf1 ORF was amplified from total genomic DNA using primers P25 and P26 and cloned into pAW8E-NdeI-cyEGFP as an NdeI/SpeI fragment to generate pAW8E-NdeI-rtf1-cyEGFP. The BglII restriction enzyme site was removed from the rtf1 ORF of pAW8E-NdeI-rtf1-cyEGFP using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and the primers P27 and P28. The mutated rtf1 ORF was amplified using P29 and P30 and cloned into pAW8E-NdeI-cyEGFP spo5DSR as an NdeI/BglII fragment, removing cyEGFP tag, to generate pAW8E-NdeI-rtf1-spo5DSR.

The mutated plasmid insert was confirmed by sequencing.

The HO endonuclease ORF was sub-cloned from pAWE-NdeI-HO-cyEGFP [7] as an NdeI/SpeI fragment into pAW8E-NdeI-cyEGFP-DSR plasmids to create pAWE-NdeI-HO-cyEGFP, pAWE-NdeI-HO-cyEGFP-3xDSR, pAWE-NdeI-HO-cyEGFP-4xDSR, pAWE-NdeI-HO-cyEGFP-6xDSR, pAWE-NdeI-HO-cyEGFP-8xDSR, and pAWE-NdeI-HO-cyEGFP-spo5DSR.

**S. pombe strain construction**

All **P**<sup>ug**</sup>**lox** strains were generated using Cre-mediated cassette exchange. See Table 1 for a list of strains created, plus the base

**Table 2. Primers used in this study.**

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<th>NAME</th>
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<tbody>
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<td>CCGGGGTTTAAA</td>
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<tr>
<td>P3</td>
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<td>P4</td>
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</table>

**Fission Yeast Tools for Transcriptional Regulation**

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strain and pAW3E_Ndh Cre-expression plasmid used for each. Other strains used in this study are also listed in Table 1. To create ugt1 base strain AW666, strains AW469 and JMM1015 were crossed (Table 1). The ugt1 base strain AW617 was generated by crossing HM2468 with AW590 (Table 1). The rhp10/urf1 gene locus in AW617 was deleted using the natMX6 selectable marker to create strain YDP273 (Table 1).

Cassette exchange
Cassette exchange was performed essentially as described [7]. The procedure was adapted for the introduction of HO-endonuclease gene sequences into HR-deficient rhp51/rhp18-rad18 single-strand annealing (SSA) system. After transformation of the Cre-expression plasmids containing the HO gene into the rhp51/rhp18-deleted ugt1 base strain AW686 (Table 1), cells were plated directly onto EMM plates supplemented with 15 μM thiamine (EMM+T - Psnal OFF). Following incubation at 30°C for 4–5 days, transformants were re-streaked onto fresh EMM+T plates. Transformants were grown in 50 ml liquid EMM media supplemented with leucine but with thiamine omitted (EMM+L) overnight to approximately 1×10^6 cells/ml and 500 cells plated onto EMM+L plates and grown at 30°C until colonies appear. Colonies were replica plated onto YE plates supplemented with hygromycin at 200 μg/ml. Following incubation overnight at 30°C, colonies sensitive to hygromycin were re-streaked onto EMM+L plate and replica plated onto EMM plates to confirm loss of the plasmid. The leucine auxotrophic colonies were used for subsequent experiments.

SSA assay growth conditions and genetic colony assay
Logarithmically growing cells grown at 30°C in EMM+L were pelleted and re-suspended in pre-warmed EMM+LH (P_ugt1lox OFF) or EMM+LH supplemented with uracil at 0.25 mg/ml (P_ugt1lox ON) and incubation continued at 30°C. At the indicated time points 500 cells were plated on EMM+LH agar and grown at 30°C until colonies appeared. The resulting colonies were replica plated onto EMM+L agar, grown at 30°C and the percentage of histidine auxotrophic colonies calculated.

Preparation of total cell extract and Western blot analysis
Preparation of cell extracts for SDS-PAGE and Western blotting was performed as previously described [7]. Mouse monoclonal anti-GFP antibody (Roche) was diluted 1:5,000, rabbit anti-PCNA antibody (Gift: A. Lehmann) was diluted 1:2,000 and mouse monoclonal anti-HA (Santa Cruz Biotechnology) diluted 1:2,500. As a loading control, mouse monoclonal anti-tubulin antibody (diluted 1:10,000; Sigma) was used.

2D gel electrophoresis
Cells were grown in EMM media supplemented with adenine and leucine (EMM+AL) at 30°C to a density of approximately 1×10^7 cells/ml and 1.25×10^7 cells harvested by centrifugation. P_ugt1lox/rhf1 expression was induced by the addition of uracil at 0.25 mg/ml (P_ugt1lox ON). Chromosomal DNA was extracted using standard procedures, embedded in agarose plugs and digested using 30 units of Ael. Digested chromosomal DNA was analysed by 2D gels [27], using 0.35% and 0.9% agarose for the first and second dimensions, respectively. Replication intermediates were visualized using pCen (centromere proximal to the snf1 gene) as a probe. Probe pCen template DNA was amplified from total genomic S. pombe DNA using primers P31 and P32. Autoradiography was performed using a storage phosphor screen/Storm PhosphorImager system.

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Author Contributions
Conceived and designed the experiments: AMC ATW JMM. Wrote the paper: ATW AMC.

References

