Role of the RPA-Sgs1 interaction in stabilizing stalled replication forks

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch – Naturwissenschaftlichen Fakultät
der Universität Basel

von

Anna Maria Hegnauer
aus Deutschland

Basel, 2011

creative commons

Namensnennung-Keine kommerzielle Nutzung-Keine Bearbeitung 2.5 Schweiz

---

Sie dürfen:

das Werk vervielfältigen, verbreiten und öffentlich zugänglich machen

Zu den folgenden Bedingungen:

Namensnennung. Sie müssen den Namen des Autors/Rechteinhabers in der von ihm festgelegten Weise nennen (wodurch aber nicht der Eindruck entstehen darf, Sie oder die Nutzung des Werkes durch Sie würden entlohnt).

Keine kommerzielle Nutzung. Dieses Werk darf nicht für kommerzielle Zwecke verwendet werden.

Keine Bearbeitung. Dieses Werk darf nicht bearbeitet oder in anderer Weise verändert werden.

- Im Falle einer Verbreitung müssen Sie anderen die Lizenzbedingungen, unter welche dieses Werk fällt, mitteilen. Am Einfachsten ist es, einen Link auf diese Seite einzubinden.
- Jede der vorgenannten Bedingungen kann aufgehoben werden, sofern Sie die Einwilligung des Rechteinhabers dazu erhalten.
- Diese Lizenz lässt die Urheberpersönlichkeitsrechte unberührt.

Quelle: http://creativecommons.org/licenses/by-nc-nd/2.5/ch/ Datum: 3.4.2009

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät
auf Antrag von Frau Prof. Dr. Susan Gasser und Herr Prof. Dr. Primo Schär.

Prof. Dr. Susan Gasser
(Referent)

Prof. Dr. Primo Schär
(Ko-Referent)

Basel, den 27. April 2010

Prof. Dr. E. Parlow
(Dekan)

Summary

S phase is the period of the cell cycle when all genomic DNA is copied precisely twice. During this complex process, replication forks frequently encounter obstacles such as tightly bound protein-barriers or are challenged by genotoxic insults creating DNA damage. As a consequence replication forks stall and form fragile DNA structures that need to be stabilized and restarted in order to prevent DNA double strand break (DSB) formation and aberrant homologous recombination (HR). Therefore, the intra-S phase checkpoint, a sophisticated surveillance mechanism, is activated to restrain potential fork collapse and to regulate cell cycle progression, DNA repair and late origin firing. Two important proteins in stabilizing arrested replication forks are the checkpoint kinase Mec1 and the RecQ helicase Sgs1 in *S. cerevisiae*. It has been proposed that both pathways in maintaining fork integrity converge on replication protein A (RPA). In fact, RPA had been shown to recruit Mec1-Ddc2 to stalled replication forks and to bind Sgs1. Therefore, this PhD work aimed to study which impact the RPA-Sgs1 interaction has in stabilizing stalled replication forks in response to the replication fork inhibitor hydroxyurea (HU).

During the first part of this PhD project, I have determined the interaction site between Sgs1 and the single strand binding heterotrimer RPA. On Sgs1, I have identified an unstructured, acidic region N-terminal to the helicase domain, which binds Rpa70 and had not been characterized before. I have created a new mutant, *sgs1*-r1, which completely disrupts Rpa70 interaction by two hybrid analysis. Indeed, we found that *sgs1*-r1 partially displaces DNA pol α from HU-stalled replication forks. However, in contrast to *sgs1*Δ, *sgs1*-r1 behaves epistatic to the S-phase specific *mec1*-100 mutant in response to HU, indicating that both factors act on the same pathway for replisome stability. Our data suggests that RPA-binding and helicase function of Sgs1 are necessary for full DNA pol α association at HU-arrested replication forks. Furthermore, we demonstrate that

the same Sgs1 region that interacts with RPA is also a Mec1 target *in vitro* and is important for Rad53 activation after exposure to HU.

The main binding site on RPA was mapped to the N-terminal oligonucleotide binding (OB) fold of the largest RPA subunit, Rpa70. To gain structural insights, we have solved the structure of the N-OB fold of *S. cerevisiae* Rpa70 (this was performed by M. Vogel and P. Amsler in collaboration with N. Thomae's laboratory). Despite low sequence conservation, the crystal structure of yeast Rpa70(3-133) displays high 3D conservation with the N-OB fold of human RPA70. It also consists of a five-stranded ß-barrel, capped by short α-helices and a basic cleft in the center. This cleft has been reported to mediate different protein-interactions in human cells. Therefore, we made use of the *rfa1*-t11 mutant, which carries a charge reversal mutation pointing towards this basic cleft. Indeed, *rfa1*-t11 partially disrupts Sgs1 binding as monitored by two-hybrid analysis. In addition, *rfa1*-t11 affects DNA pol α association at HU-stalled replication forks and displays a genome-wide replication defect in response to replication stress. These phenotypes for *rfa1*-t11 are stronger than for *sgs1*Δ, which indicates that only a fraction can be assigned to the loss of Sgs1 binding. However, we observe an epistatic relationship between *rfa1*-t11 and proteins involved in homologous recombination (HR) such as *mre11* and *rad51*. We therefore suspect that impaired HR in *rfa1*-t11 cells might be the reason for the failure to restart DNA synthesis at stalled or collapsed replication forks.

Table of contents

1. Introduction

1.1. The mitotic cell cycle

The mitotic cell cycle is a highly regulated process by which a eukaryotic cell grows and divides into two daughter cells. As well as growing in size, before division, a cell has to duplicate all its essential components. Of particular importance is the faithful replication of genetic material, the DNA, into exactly two identical copies, which must then be precisely segregated into the new daughter cells. These operations occur in separate stages of the cell cycle: DNA synthesis takes place during the synthesis (S) phase and chromosome segregation during mitosis (M) phase (see Figure 1). S and M phase are normally separated by two gap phases, also known as the G1 and G2 phases.

Groundbreaking work towards understanding cell cycle progression has been done by L. Hartwell, P. Nurse and T. Hunt. Hartwell *et al.* identified temperature sensitive cell-division cycle (cdc) mutants in *S. cerevisiae* that blocked specific stages of cell cycle progression (Hartwell, Culotti et al. 1970). This led to the model whereby entry into a new cell cycle depends on transition through a point in G1 phase, called START. Once a cell has passed the START transition it irreversibly commits to a new round of DNA synthesis and mitosis, until it reaches the next G1 phase (Hartwell, Culotti et al. 1974).. Whether a yeast cell commits to a new round of division depends on different factors. It has to have reached a critical size, the DNA should be intact and mating pheromone (e.g., α-factor in the case of a-type cells) must be absent (Morgan 2007). Progression through the cell cycle is unidirectional and requires the successive activation and inactivation of different cyclin-dependent kinase (CDK)-cyclin complexes. In contrast to a large number present in mammalian cells, the serine-threonine kinase Cdc28 is the only CDK in budding yeast. It interacts with nine different cyclins (Cln1-3, Clb1-6) to regulate specific tasks throughout the cell cycle, e.g., initiation of replication or G2/M transition (Morgan 2007). CDK activity is

controlled by various mechanisms, such as those regulating cyclin expression levels (e.g., through phosphorylation and activation of the transcription factors SBF and MBF), ubiquitin-mediated degradation of cyclins (e.g., through the anaphase promoting complex, Cdc20/APC), or CDK inhibitors (e.g., degradation of the Sic1 inhibitor during the G1/S transition), or phosphorylation and dephosphorylation of CDK by Swe1 and Mih1 (Morgan 2007).

For all organisms, it is crucial that the duplication of the DNA within the chromosomes and division of the cellular components are performed with extreme precision and reliability over generations. The fidelity of cell reproduction depends not only on accurate DNA replication and chromosome segregation, but also on the correct order the events occur during the cell cycle. For example, it is necessary that DNA duplication is completed before an attempt is made to segregate the chromosomes into the new daughter cells. To achieve faithful transmission of genetic material from one cell to the other, eukaryotic cells possess sophisticated surveillance mechanisms called cell cycle checkpoints. An overview of cell cycle checkpoints will be presented in Section 1.1.1 below.

1.1.1. Cell cycle checkpoints

Cell cycle checkpoints form a highly conserved regulatory network that monitors the completion of important cell-cycle events and halts cell cycle progression if something goes wrong (Figure 1). A central target of cell cycle checkpoints is CDK and its interaction with the different cyclins, whose levels oscillate throughout the different stages of the cell cycle (Morgan 2007).

The G1/S checkpoint controls entry into a new cell cycle in mid to late G1 (also called START transition or restriction point in animal cells). Activation of the G1/S checkpoint temporarily stops the cell cycle if conditions are not ideal. As mentioned above, START is prevented if a cell has not reached a critical size, if

DNA damage is sensed, or depending on the presence of signals from outside the cell (for example pheromone or mitogens). The G2/M checkpoint monitors the completion of DNA replication and prevents the entry into mitosis, or M phase, until the DNA is accurately and completely replicated, by controlling the activity of M-phase cyclin-CDK complexes. A checkpoint at the metaphase-to-anaphase transition controls for correct spindle assembly, which is required for mitosis. It inhibits the initiation of sister-chromatid separation until the spindle is ready.

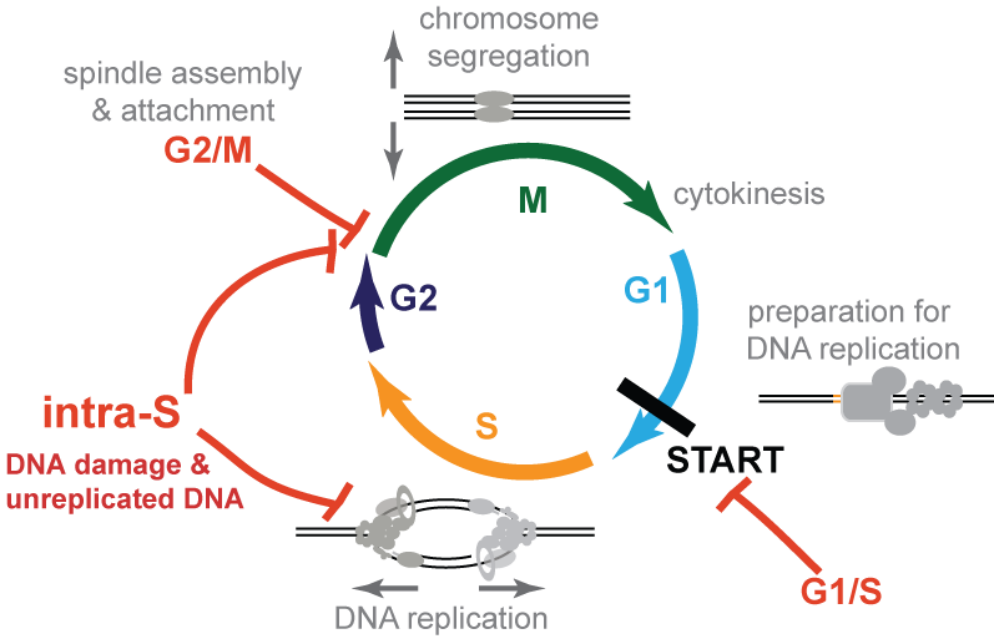


Figure 1 - Schematic representation of the eukaryotic cell cycle. During late M and G1 phase origins are licensed and the preRC complex assembled. Once a cell has passed START, it has committed itself to a new cell cycle until it reaches the next G1 phase. During S phase the whole genome is replicated exactly into two copies. The duplicated chromosomes segregate into new daughter cells during M phase. G2 phase separates S and M phase. Cell cycle checkpoints and their time of action are indicated in red. G1, S, G2 and M represent the four phases of the cell cycle.

Importantly, the cell cycle can be blocked during all these transitions if chromosomal DNA is extensively damaged. DNA damage can be caused by either extrinsic or intrinsic genetic insults, such as irradiation, chemical compounds, reactive metabolic products or DNA replication stress. If not repaired by continuous active DNA repair mechanisms, this can result in severe DNA

lesions that form a major threat to genomic stability. Therefore, signaling pathways exist which recognize DNA damage to regulate DNA repair, apoptosis and cell cycle progression by sending inhibitory signals to the cell cycle checkpoints. This response to DNA damage is also referred to as DNA damage checkpoint response.

During S-phase, additional signaling pathways exist, which monitor not only DNA damage but also replication stress. Upon replication stress, fragile DNA structures are formed that if not stabilized result in replication fork collapse and the formation of DNA double strand breaks (DSB), which are one of the most deleterious lesions for the cell. To avoid this, the intra-S phase checkpoint has a major role in stabilizing the replisome at stalled replication forks. In addition, it regulates replication fork progression, late origin firing, DNA repair and aberrant homologous recombination. The molecular mechanisms of intra-S phase checkpoint activation and function at stalled replication forks will be discussed in more detail in chapter 1.3

1.2. DNA replication in eukaryotes

1.2.1. Origins of replication

Chromosome replication begins at specific sites in the genome, which are called replication origins. In contrast to most eukaryotes, *S. cerevisiae* origins contain specific DNA elements that are important for origin determination and to ensure sufficient origin activity (Marahrens and Stillman 1992), (Gilbert 2004). Budding yeast origins contain an autonomously replicating sequence (ARS), which when transferred into any piece of DNA enables its replication in S phase (Bell and Dutta 2002). Each ARS is about 100-200 base pair (bp) long and consists of a highly conserved A element and two or three poorly conserved B elements. The A element possesses an 11 bp ARS consensus sequence (ACS), is AT-rich and is flanked by the B1 element (Marahrens and Stillman 1992). The A and B1 elements together form the central binding site for the origin recognition complex

(ORC), while the other B regions are thought to act as enhancers for origin efficiency (Sclafani and Holzen 2007). Those origins are distributed throughout the genome at an average interval of about 30 kb (Morgan 2007). In fission yeast or metazoans, no discrete sequence elements for initiation have been found. Fission yeast, *S. pombe,* ARS elements are 500-1000 bp long, AT-rich and are nearly randomly distributed along the chromosomes (Segurado, de Luis et al. 2003). In metazoans, replication origins tend to be organized in replicon clusters that are activated at the same time during S phase. A replicon is defined as the region of DNA that is replicated from a single origin, and can vary in length from as little as 10 kb to 1000 kb (Morgan 2007).

1.2.2. Initiation of DNA replication

Origin activation must be carefully regulated to ensure that DNA replication occurs once and only once per cell cycle. To achieve this, the initiation process is divided into two temporally different steps, as shown in Figure 2 and described in more detail in the following sections. In the first step, during late mitosis and early G1, the pre-replicative complex (pre-RC) assembles at origins and prepares them for firing (Blow and Dutta 2005). This process involves the loading of inactive Mcm2-7 helicase (MCM), which is believed to be the replicative helicase. This event is also known as licensing. In the second step, during S phase, the pre-RC is transformed into an active pre-initiation complex (pre-IC), which unwinds the origin and loads on the replication machinery. When DNA synthesis is initiated and the replisome moves away from the origin, the pre-RC at that origin is dismantled and the pre-RC components are destroyed or inhibited. This is mainly facilitated by the activation of S-CDKs during early S-phase. S- and M-CDKs prevent re-assembly of the pre-RC until late mitosis, when all CDK activities are reduced (Morgan 2007). Thus, pre-RC complex formation is restricted to late mitosis and early G1 by a simple and elegant mechanism, which ensures that DNA is only replicated once per cell cycle.

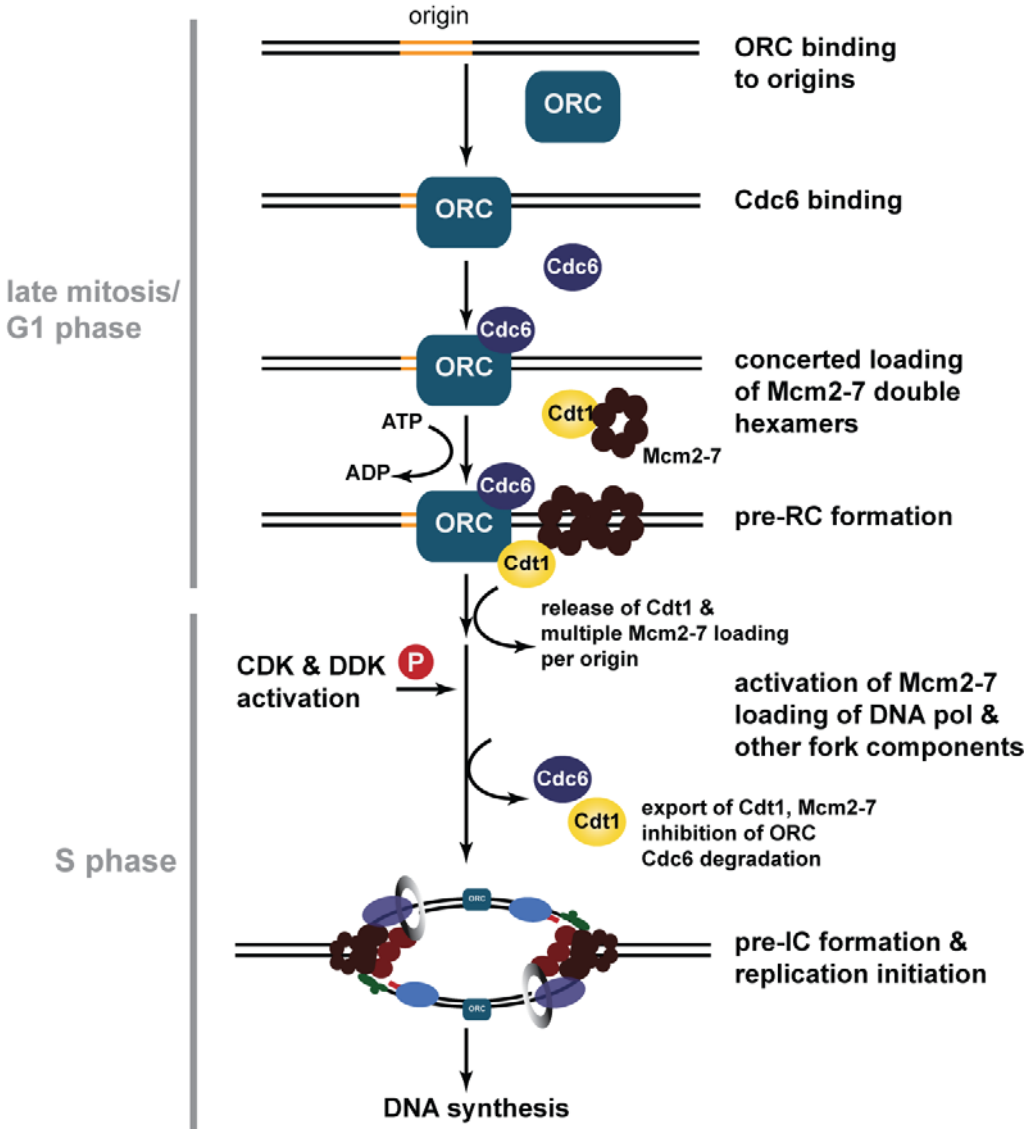


Figure 2 - Model for origin licensing and replication initiation. During late mitosis and early G1 the pre-replicative complex (pre-RC) is assembled at origins., ORC binds to origins and recruits Cdc6 and Cdt1-Mcm2-7 heptamers thereby forming the pre-RC. Cdt1 is released upon Mcm2-7 binding. Several inactive Mcm2-7 hexamers are loaded per origin. This process is also referred to as licensing. In late G1, the pre-RC is converted into an active helicase complex, which can initiate DNA synthesis. This requires the activation of CDK and DDK kinases and the recruitment of additional factors to the origin. In parallel, further licensing is prevented by inhibition or degradation of the pre-RC components. This ensures that the genome is only replicated once cell cycle.

1.2.2.1. Pre-RC formation and licensing

Central to pre-RC formation is the conserved origin recognition complex (ORC), which consists of 6 subunits (Orc1-6), see also Table 1. ORC binds to origins of replication and recruits other initiation proteins. As cells exit mitosis, Cdc6 binds to ORC and in an ATP-dependent manner they recruit heptamers of Cdt1-Mcm2-7 (Randell, Bowers et al. 2006), (Chen, de Vries et al. 2007). Recently, it was shown that single Cdt1-Mcm2-7 heptamers are loaded cooperatively, such that double Mcm2-7 hexamers are formed, which are connected head-to-head via their N-terminal rings (Remus, Beuron et al. 2009). Surprisingly, not just one MCM double hexamer is loaded per origin, but 10 – 40 molecules, which are distributed at significant distances away from where ORC is bound (Blow and Dutta 2005). The function of the excess MCM hexamers is still under debate. However, in human and *Xenopus* cells, it was proposed that excess MCM loading licenses dormant origins that are not activated during normal DNA replication, but could provide a backup mechanism in response to replication stress (Woodward, Gohler et al. 2006), (Zhu, Ukomadu et al. 2007). Consistently, when MCM levels were lowered in human cells by small interfering RNA (siRNA), replication rate was not influenced under unchallenged conditions (Zhu, Ukomadu et al. 2007). However, in response to replicative stress, lowered chromatin-bound MCM levels inhibited the firing of dormant origins and reduced viability compared to normal cells with excess MCM levels.

1.2.2.2. Pre-IC formation and replication initiation

The inactive pre-RC is converted into an active helicase complex by further recruitment of several factors including Cdc45 and GINS (Tercero, Labib et al. 2000), (Kanemaki, Sanchez-Diaz et al. 2003), (Gambus, Jones et al. 2006). Together, Cdc45 and GINS form a stable complex with the MCM helicase (the

Table 1 - Proteins involved in replication initiation in S. cerevisiae and metazoan cells.

	S. cerevisiae	Metazoan	Function
Pre-RC formation	Orc1-6 (ORC)	ORC1-6 (ORC)	binds origins, crucial factor for pre-RC formation
	Cdc6	CDC6	required for Mcm2-7 loading to origins
	Cdt1	CDT1	required for Mcm2-7 loading to origins
	Mcm2-7 (MCM)	MCM2-7 (MCM)	replicative helicase (inactive)
Pre-IC formation and replication initiation	Cdc28-Clb5/6 (CDK)	CDK2-CYCLIN A/E (CDK)	important kinase for replication initiation and modulating MCM helicase activity
	Cdc7-Dbf4 (DDK)	CDC7-DBF4/DRF4 (DDK)	important kinase for activation of the Mcm2-7 helicase and replication initiation
	GINS (Sld5-Psf1-Psf2-Psf3)	GINS (SLD5-PSF1-PSF2-PSF3)	form with Mcm2-7 the active helicase (Cdc45-Mcm2-7-GINS or CMG complex)
	Cdc45	CDC45	forms with Mcm2-7 the active helicase (Cdc45-Mcm2-7-GINS or CMG complex)
	Sld2	RECQ4	important for initiation and elongation
	Sld3	no known homologue	interacts with Cdc45
	Dpb11	CUT5/TOPBP1	initiation with Sld2 to recruit GINS
	Mcm10	MCM10	important for DNA pol alpha loading and replication elongation
	Ctf4	AND-1	important for DNA pol alpha loading and replication elongation

CMG complex or ‘unwindosome’), which is necessary for the establishment and progression of replication forks. Assembly of the CMG complex is promoted by activation of both S-CDKs and another kinase, called Cdc7-Dbf4 (DDK) in yeast. Similar to CDK, Cdc7 is stably expressed throughout the cell cycle, but its activity is highly regulated and depends on its association with Dbf4, whose levels oscillate during the cell cycle. During G1, DDK is inactive, because Dbf4 is targeted for proteosomal degradation by the anaphase-promoting complex (APC). However, at the G1/S boundary Dbf4 levels rise and DDK gets activated as a result of CDK-dependent inactivation of the APC. Dbf4 levels remain high until

the cell exits from mitosis. Different biochemical and genetic data suggest that DDK specifically targets the MCM complex. Indeed, DDK phosphorylates several Mcm2-7 subunits in *S. cerevisiae*, and a mutation in Mcm5, *mcm5-bob1*, bypasses the role of DDK in replication. Recently, Sheu *et al.* reported that the essential DDK function in promoting normal S-phase progression is to relieve an inhibitory activity of the Mcm4 N-terminal domain (Sheu and Stillman). Deletion of this serine/threonine rich (NSD)-domain in $mcm4^{\Delta 74\text{-}174}$ bypasses the requirement of DDK for the formation of a stable Cdc45-MCM complex at each origin. However, DDK is still required for the timely assembly of the Cdc45-MCM complex in the $mcm4^{\Delta 74\text{-}174}$ mutant and for proper intra-S phase checkpoint activation in response to the replication fork inhibitor hydroxyurea (HU) (Sheu and Stillman).

In addition to CMG, other factors, such as *S. cerevisiae* Sld2, Sld3, Dpb11 and Mcm10, are necessary for the assembly of the pre-IC at origins, which then loads the replisome and initiates DNA synthesis (Sclafani and Holzen 2007). Recently, it was shown that S-CDK targets both Sld2 and Sld3 (***s****ynthetic* ***l****ethal with* ***d****pb11-1*), which in their phosphorylated forms can bind to the C- or N-terminal BRCT domains of Dpb11 (Tanaka, Umemori et al. 2007). In addition, it was reported that Sld3 forms a stable complex with Cdc45 and that Dpb11-Sld2 and GINS are loaded onto origins in a mutually dependent manner, in association with DNA pol ε (Masumoto, Sugino et al. 2000), (Takayama, Kamimura et al. 2003), (Kanemaki and Labib 2006). Muramatsu *et al* 2010 suggested that CDK promotes the formation of a soluble pre-loading (pre-LC) complex consisting of Sld2, Dbb11, GINS and DNA pol ε, which associates with origins and thereby loads DNA pol ε (Muramatsu 2010 G&D). Interestingly, it was shown that the minimal set of S-CDK targets required for DNA replication are Sld2 and Sld3 in yeast (Zegerman and Diffley 2007), (Tanaka, Umemori et al. 2007). This suggests that the S-CDK phosphorylation-dependent interaction between Dpb11, Sld2 and Sld3 is sufficient for replication initiation in G1, when Dbf4 is overexpressed and therefore DDK is active (Zegerman and Diffley 2007), (Tanaka, Umemori et al.

2007). Bypassing both DDK (with the mcm5-bob1 mutation) and CDK (with sld2-T84D and a fusion of Sld3 to Dpb11) leads to synthetic lethality (Zegerman and Diffley 2007). This suggests that cell cycle regulation of DNA replication is completely abrogated and that both kinases are necessary to regulate helicase activation and replisome loading.

In contrast to *S. cerevisiae*, recruitment of Cdc45 and GINS to the pre-RC is less well understood in human cells. So far no mammalian homologue for Sld3 has been identified. Recent data suggest that human RecQ4 is required for replication initiation and might be a poorly conserved homologue of Sld2: the N-terminus of RecQ4 possesses limited homology to Sld2. Depletion of RecQ4 in HeLa cells, significantly inhibited CMG assembly at chromatin (Im, Ki et al. 2009). However, the proposed Dpb11/Cut5 interaction domain in *Xenopus* RecQ4 is absent in human RecQ4. Consistently, no direct interaction between human RecQ4 and TopBP1, the human Dpb11/Cut5 homologue, could be detected (Xu, Rochette et al. 2009). In line with this finding, Im *et al.* recently reported that depletion of TopBP1 did not significantly affect CMG complex formation in HeLa cells (Im, Ki et al. 2009). This suggests that the molecular mechanisms leading to pre-IC formation in human cells might differ somewhat from *S. cerevisiae*.

Furthermore, replication initiation requires loading of the DNA polymerases and other factors to origins, to form the replisome progression complex (RPC). However, how the RPC is formed is currently not well understood. Different studies have implicated Mcm10 and Ctf4/And-1 in DNA pol α recruitment in *S. cerevisiae*, *Xenopus* extracts and mammalian cells (Ricke and Bielinsky 2004), (Zhu, Ukomadu et al. 2007). Mcm10 binds to origins after pre-RC assembly and promotes Cdc45 loading (Wohlschlegel, Dhar et al. 2002), (Sawyer, Cheng et al. 2004), (Ricke and Bielinsky 2004). After activation of the CMG complex by CDK and DDK, the DNA at the origin is unwound and replication protein A (RPA) is recruited to and binds the resulting single stranded DNA (ssDNA) (Tanaka and Nasmyth 1998). Mcm10 is required for loading Ctf4/And-1, which subsequently

facilitates loading of DNA pol α (Ricke and Bielinsky 2004), (Zhu, Ukomadu et al. 2007). Consistently, disruption of the Mcm10-Ctf4/And-1 interaction by an antibody in *Xenopus* also interferes with the loading of Ctf4/And-1 and DNA pol α to chromatin and inhibits DNA synthesis (Zhu, Ukomadu et al. 2007). Both Mcm10 and Ctf4 physically interact with DNA pol α and stabilize the catalytic subunit of DNA pol α (Ricke and Bielinsky 2004), (Zhou and Wang 2004). Additionally, Mcm10 was shown to stimulate polymerase activity of DNA pol α *in vitro* (Fien, Cho et al. 2004). After synthesis of a 7-12 nucleotide (nt) RNA primer followed by a short stretch of DNA, DNA pol α is displaced by replication factor C (RFC) and proliferating cell nuclear antigen (PCNA) is loaded onto dsDNA. Subsequently DNA pol ε or DNA pol δ are loaded to the PCNA-primer complex and the replisome is assembled (Kunkel and Burgers 2008).

1.2.3. Components of the replication fork and replication elongation

After replication initiation, the assembled replisomes move away from the origin in both directions as the DNA is synthesized. Replication is directed and can only occur from the 5' to the 3' end of a polynucleotide. Therefore, only one DNA strand can be synthesized continuously (leading strand), while the other strand (lagging strand) has to be synthesized discontinuously in 200 bp short Okazaki fragments (Morgan 2007). The Okazaki fragments are quickly ligated to form a continuous DNA strand, such that new nucleosome assembly can occur already very close to the fork junction (Burgers 2009). Central to eukaryotic replication elongation are three major DNA polymerases: DNA pol α/primase, DNA pol ε and DNA pol δ (for review see (Burgers 2009)). They are associated with the moving fork and use a single-stranded template for DNA synthesis (see Figure 3, Table 2). However, only DNA pol α/primase is able to initiate a new DNA strand. Therefore, it plays a crucial role in origin activation and also in the initiation of Okazaki fragments during lagging strand synthesis. Based on recent genetic studies in *S. cerevisiae*, it was possible to place the other two replicative DNA

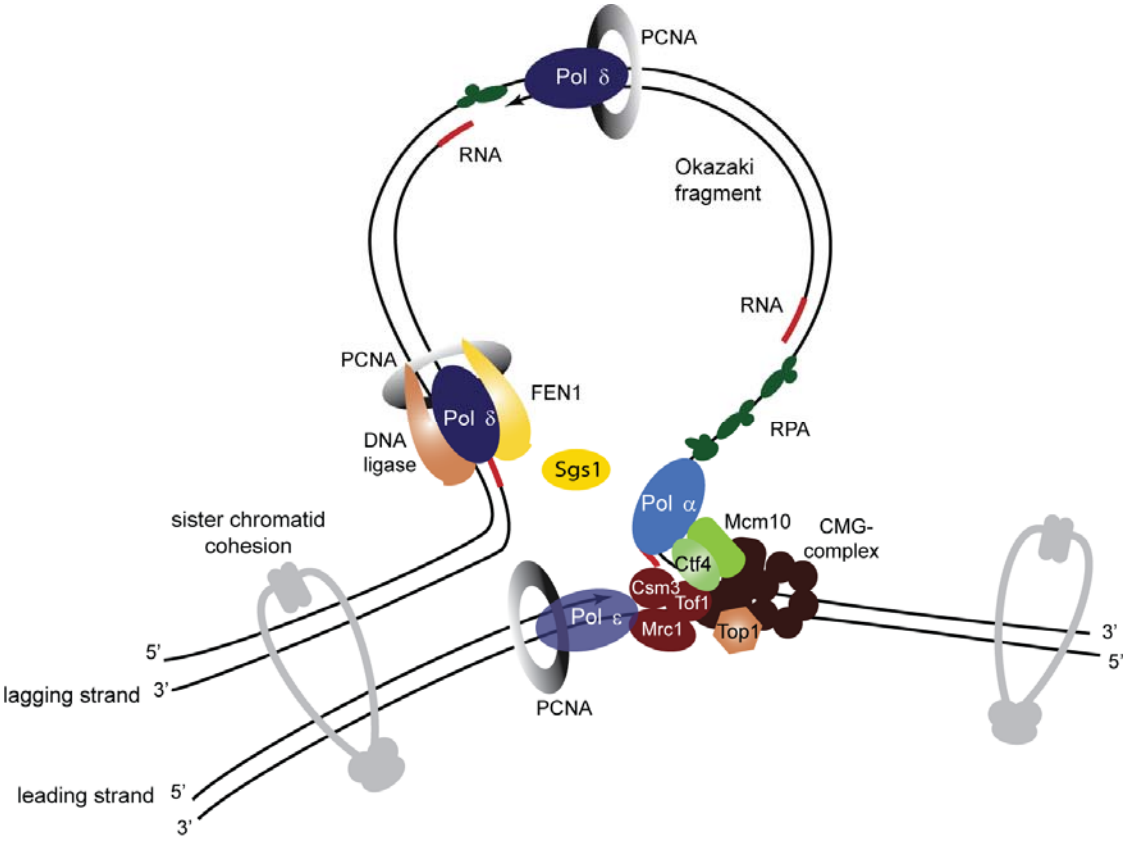


Figure 3 - Simplified schematic representation of a moving fork in *S.cerevisiae*. During replication elongation dsDNA is unwound by the replisome progression complex: the Cdc45-Mcm2-7-GINS (CMG) complex forms the active helicase. The CMG-complex is coupled to the replicative DNA polymerases DNA pol α and DNA pol ε by Mcm10, Ctf4 and Mrc1-Tof3-Csm4. DNA pol ε facilitates leading strand DNA synthesis and DNA pol α and DNA pol δ duplicate the lagging strand. RPA binds ssDNA during replication and protects it from nuclease digestion and prevents inter- and intra-strand reannealing. Okazaki fragments are processed by the combined action of DNA pol δ, FEN1, Dna2 and DNA ligase. The RecQ helicase Sgs1 travels with the fork, stabilizes the DNA polymerases, prevents aberrant HR and functions in Okazaki fragment maturation.

polymerases; suggesting that DNA pol ε is the leading strand polymerase and DNA pol δ the lagging strand polymerase (Pursell, Isoz et al. 2007), (Nick McElhinny, Gordenin et al. 2008). In one study, the active site of DNA pol ε was altered in such a way that its polymerase mutation rate during replication was increased, leaving a specific molecular signature (Pursell, Isoz et al. 2007). Using

a *URA3* reporter gene that was placed in different orientations on opposite sites of two replication origins, Pursell and coworkers analyzed the resulting mutations introduced during DNA replication. Their data suggested that DNA pol ε functions in leading strand synthesis. Similar experiments were carried out with DNA pol δ, placing it consistently on the lagging strand (Nick McElhinny, Gordenin et al. 2008). However, there is evidence that DNA pol δ can also function under certain circumstances in the leading strand synthesis, which explains why deletion of the catalytic subunit of DNA pol ε is dispensable for cell growth in *S. cerevisiae* (Dua, Levy et al. 1999), (Kesti, Flick et al. 1999).

As for replication initiation, DNA pol α/primase starts an Okazaki fragment by synthesis of a short RNA-DNA primer (Burgers 2009). Loading of the sliding clamp PCNA effects a switch to DNA pol δ, which continues lagging strand synthesis and corrects errors made by DNA pol α/primase, whose fidelity is lower due to the lack of proof-reading activity (Pavlov, Frahm et al. 2006). The heterotrimer PCNA acts as a processivity factor for both DNA pol δ and DNA pol ε and its posttranslational modification by ubiquitylation or sumoylation plays an important role in coordinating replication-associated repair events (Chilkova, Stenlund et al. 2007), (Hoege, Pfander et al. 2002). During Okazaki fragment maturation, PCNA complexes with DNA pol δ, FEN1 and DNA ligase I (Dionne, Nookala et al. 2003). The dominant pathway for removal of the initiator RNA primer in wild-type cells during unchallenged conditions is probably the short flap pathway, where polymerase activity and 3'-exonuclease activity of DNA pol δ are precisely coordinated with the 5'-flap endonuclease activity of FEN1 (Garg, Stith et al. 2004). Every time DNA pol δ reaches the 5'-end of the downstream Okazaki fragment, it adds 1-2 nt to the new strand in a strand displacement mode. The resulting 1-2 nt flap is recognized by FEN1 and cleaved off. This process is repeated over multiple cycles until all initiator RNA is removed and a regular DNA-DNA nick is generated, which is subsequently connected by DNA ligase I. Deletion of FEN1 (*rad27*Δ) is not lethal, because other flap endonucleases such as Exo1 can compensate for its loss (Tran, Erdeniz et al.

2002). The absence of both flap endonucleases FEN1 and Exo1 in a *rad27*Δ *exo1*Δ double mutant, leads to a severe growth defect or synthetic lethality (Tishkoff, Boerger et al. 1997).

Table 2- Proteins involved in replication elongation in S. cerevisiae and metazoan cells

	S. cerevisiae	**Metazoan**	**Function**
Replication fork progression complex (RPC)	CMG	CMG	active replicative helicase
	Top1	TOP1	releases torsional tension, coordinates replication with transcription
	Mrc1	CLASPIN	connects DNA pol epsilon to the CMG complex
	Tof1-Csm3	TIM-TIPIN	control replication fork progression at replication fork barriers
	Mcm10	MCM10	connects DNA pol alpha to the CMG complex
	Ctf4	AND-1	connects DNA pol alpha to the CMG complex
	FACT	FACT	chromatin remodeller
DNA polymerases	DNA pol α/ primase	DNA pol α /primase	initiates new DNA strands during replication initiation & elongation
	DNA pol δ	DNA pol δ	lagging strand polymerase, elongation and maturation of Okazaki fragments, 3'-exonuclease function for proof-reading
	DNA pol ε	DNA pol ε	leading strand polymerase, 3'-exonuclease function for proof-reading
Other proteins associated with the moving fork	PCNA	PCNA	sliding clamp, processivity factor fo DNA pol epsilon and delta
	RFC	RFC	clamp loader, loads PCNA onto primer junctions
	RPA	RPA	crucial for replication initiation and elongation, binds ssDNA
	FEN1	FEN1	5'-flap endonuclease important for Okazaki fragment maturation
	DNA ligase I	DNA ligase I	ligates DNA-DNA nicks during Okazaki fragment maturation
	Dna2	DNA2	essential endonuclease/helicase processes long Okazaki fragments
	Exo1	EXO1	5'-flap endonuclease, Okazaki fragment maturation
	Pif1	PIF1	5'-3' helicase, Okazaki fragment maturation, contributes to formation of long flaps
	Sgs1	BLM/WRN	RecQ helicase implicated in faulty Okazaki fragment processing

An alternative pathway of Okazaki fragment maturation is required when long flaps are formed, which fold back or bind other proteins such that FEN1 action is inhibited. A reason for this could be FEN1 dysfunction at unusual DNA structures or sequences, or due to unusual extensive strand displacement synthesis by DNA pol δ (Burgers 2009). Consistently, *rad27*Δ mutants accumulate duplications of up to 100 nt. *In vitro* studies have indicated that Dna2 is activated by long flaps bound by RPA (Kao, Veeraraghavan et al. 2004). The essential endonuclease/helicase Dna2 is thought to degrade those flaps to 2-6 nt, which are then further processed by FEN1/DNA pol δ. This model is supported by genetic data, where overexpression of Dna2 rescues the synthetic lethal phenotype of a *rad27*Δ *pol3-exo*$^-$ double mutant (Jin, Ayyagari et al. 2003). Other proteins have been implicated in Okazaki fragment maturation, such as the 5'-3' helicase Pif1, RNase H and the RecQ helicase Sgs1 (Burgers 2009), (Kang, Lee et al.).

Unwinding of the parental double stranded DNA is facilitated by the RPC, with the Cdc45-Mcm2-7 -GINS complex as the core (Gambus, Jones et al. 2006). The *S. cerevisiae* RPC additionally contains Mcm10, Ctf4, Mrc1, Tof1-Csm3, FACT and Top1 (Gambus, Jones et al. 2006). It was suggested that one crucial function of the RPC is to couple MCM helicase to other components of the replisome such as DNA polymerases. Indeed, Lou *et al.* reported that Mrc1 associates with Cdc45, MCM helicase and DNA pol ε (Lou, Komata et al. 2008). Mrc1 regulates replication fork progression and is likely to couple MCM helicase to DNA pol ε, although this needs to be shown directly. Furthermore, recent data suggest that during replication elongation a complex of GINS and Ctf4 connects MCM helicase to DNA pol α (Gambus, van Deursen et al. 2009). Mcm10 has also been suggested to link DNA pol α to the CMG complex (Ricke and Bielinsky 2004), (Lee, Liachko et al.).

The torsional tension, which stems from unwinding of the long DNA duplexes, is released by Top1, a type I topoisomerase which travels with the replication fork

(Gambus, Jones et al. 2006). In budding yeast, Top1 seems to be one of the major swivels for replication fork movement. Consistently, deletion of Top1 reduces fork progression rate by 50% (Tuduri, Crabbe et al. 2009). However, *top1*Δ mutants are viable, because another topoisomerase, Top2, can substitute for its loss. Recently, it was suggested that mammalian Top1 also plays a critical role in coordinating replication and transcription thereby preventing genomic instability (Tuduri, Crabbe et al. 2009).

S. cerevisiae Tof1 (topoisomerase I interacting factor) and Csm3 (chromosome segregation in meiosis) are associated with Mrc1 at the moving fork (Bando, Katou et al. 2009). Tof1-Csm3 (Tim-Tipin in human cells) plays a role in controlling replication fork progression and stabilization at sites where non-nucleosomal proteins bind tightly to DNA. In the rDNA locus, Tof1-Csm3 is required for Fob1-dependent replication barrier activity (Calzada, Hodgson et al. 2005). Furthermore, different studies link yeast Tof1-Csm3 or human Tim-Tipin to sister chromatid cohesion (Leman, Noguchi et al.), (Mayer, Pot et al. 2004). Consistently, deletion of Tof1-Csm3 in budding yeast or depletion of their homologues in *C. elegans*, *Xenopus* or human tissue causes cohesion defects of different extents (McFarlane, Mian et al.). Sister chromatid cohesion ensures that the newly synthesized sister chromatids are properly aligned during mitosis and meiosis. Additionally, sister chromatid cohesion can provide a partner for repair by homologous recombination in case of chromosome breakage or replication fork restart. Hence, it is not surprising that sister chromatid cohesion and DNA replication are intimately linked and that several cohesion establishment factors have been linked to the replication machinery (Uhlmann 2009).

1.3. The Intra-S phase checkpoint

Here, we will talk about the intra-S phase checkpoint as containing both the DNA damage response in S phase, which is similar to the checkpoint response in

other cell cycle phases, and additional measures that are activated upon replication stress to maintain functional replication forks (replication checkpoint). The Intra-S phase checkpoint response is illustrated in Figure 4 and discussed in the sections below.

1.3.1. DNA damage checkpoint

To maintain genome stability, it is essential that all cells can cope with different kinds of genotoxic insults that create DNA damage. The DNA damage checkpoint is activated in response to DNA lesions throughout the different cell cycle stages and directs an appropriate cellular response, including regulation of cell cycle progression, DNA repair or apoptosis. The DNA damage checkpoint response is a signaling transduction cascade and can be classified into different steps: where the signal is sensed, transmitted and amplified. In the first step, the DNA lesion is recognized by sensor proteins, leading to the activation of the sensor kinase Mec1/ATR (Tourriere and Pasero 2007). The signal is further transmitted by phosphorylation of adaptor proteins, which results in the recruitment of the downstream effector checkpoint kinases Rad53/CHK2 and Chk1/CHK1. Mec1/ATR-dependent phosphorylation of the effector kinases leads to their activation, which is crucial for checkpoint response. During S phase, budding yeast Rad53 and metazoan CHK1 function as the principal effector kinases in modulating the downstream events of checkpoint control (Tourriere and Pasero 2007). Although there is recent evidence that *S.cerevisiae* Chk1 can also act in replication fork stabilization, compared to Rad53, it plays a minor role during the intra-S-phase checkpoint (Segurado and Diffley 2008).

1.3.1.1. Sensor kinases

Central components of the conserved DNA damage checkpoint are the *S. cerevisiae* PI3K-like kinases Mec1 and Tel1, which are homologues of metazoan ATR and ATM, respectively (Gottifredi and Prives 2005). These checkpoint kinases Mec1/ATR and Tel1/ATM share many biochemical similarities, they both

target SQ/TQ sites of overlapping substrates and have partially redundant functions in checkpoint activation (Cimprich and Cortez 2008). However, they sense different DNA lesions: ATM is primarily activated in response to DSBs, while Mec1/ATR responds to a wide range of DNA lesions including base adducts, DNA crosslinks, DSBs and replication stress (Cimprich and Cortez 2008). During S phase, Mec1/ATR plays an important role in stabilizing replisome components at stalled forks and is crucial for a global DNA damage and replication checkpoint response, which is also referred to as intra-S phase checkpoint response (Tourriere and Pasero 2007). Recent findings on Mec1/ATR activation and its function during the intra-S phase checkpoint will be discussed in more detail in chapter 1.5.

1.3.1.2. Sensing damage

It is currently thought that different forms of DNA damage can be converted into one common intermediate, which is sensed by Mec1/ATR. This intermediate probably contains several long stretches of ssDNA coated by RPA (Zou and Elledge 2003), (Shimada, Pasero et al. 2002). Indeed, both budding yeast Mec1 and human ATR form a stable complex with Ddc2 or ATRIP, respectively (Rouse and Jackson 2002). Ddc2/ATRIP binds RPA and was shown to recruit the complex to sites of damaged chromatin (Zou and Elledge 2003). Furthermore, DNA damage is sensed independently by the RFC-like protein Rad24, which interacts with Rfc2-5 and loads the DNA damage checkpoint clamp 9-1-1 (Rad17-Ddc1-Mec3 in budding yeast and RAD9-RAD1-HUS1 in human cells) onto primer-template junctions adjacent to ssDNA coated by RPA (Majka, Niedziela-Majka et al. 2006). Additionally, yeast Dpb11 or human TOPBP1 are recruited to the lesion and implicated in the activation of Mec1/ATR (Navadgi-Patil and Burgers 2008), (Delacroix, Wagner et al. 2007).

1.3.1.3. Transduction

Following the sensing of DNA damage, the signal is amplified by adaptor proteins. In budding yeast, the best characterized adaptor protein between Mec1 and

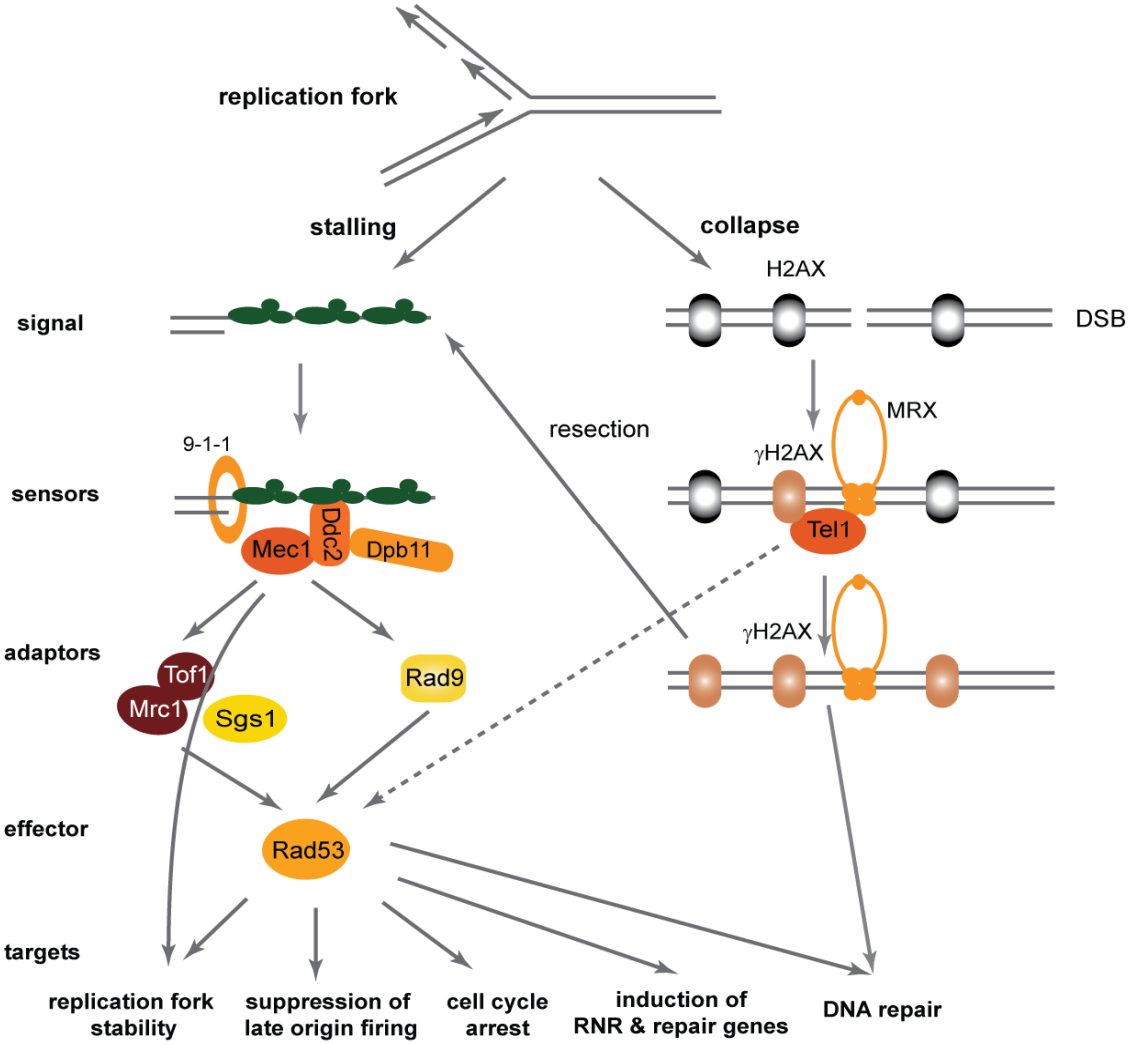


Figure 4 - The intra-S phase checkpoint response in *S. cerevisiae*. Replication stress or DNA damage can lead to fork stalling or collapse, which activates the intra- S phase checkpoint. Important roles during signaling cascade play the PI3-like kinases Mec1 and Tel1. Tel1 is mainly activated in response to DSB, while Mec1 responds to a wide range of DNA lesions including replication stress. After activation, Mec1 and Tel1 phosphorylate various substrates including adaptor proteins, which amplify the signal and facilitate the activation of effector kinases such as Rad53 and Chk1 (not shown in the figure). Rad53 is the main effector kinase and modulates crucial down-stream events of the checkpoint response such as replisome stability, late origin firing, cell cycle progression and DNA repair. In addition to Rad53 activation, Mec1 also acts directly at the fork to maintain replication fork integrity.

Rad53 is Rad9 (Sweeney, Yang et al. 2005). Rad9 is recruited to the site of DNA damage where it becomes extensively phosphorylated by Mec1 (Emili 1998), (Schwartz, Duong et al. 2002). Furthermore, phosphorylated Rad9 oligomerizes and recruits the effector kinase Rad53, which leads to Mec1-dependent phosphorylation of Rad53 (Gilbert, Green et al. 2001), (Schwartz, Duong et al. 2002). In addition, it is thought that Rad9 acts as a scaffold for Rad53 autophosphorylation, which contributes to a full activation of Rad53 (Gilbert, Green et al. 2001). Recent data suggests that Rad9 oligomerization is not required for initial Rad53 activation, but it is necessary for the maintenance of checkpoint signaling (Usui, Foster et al. 2009). Finally, activated Rad53 is released from Rad9 in order to phosphorylate downstream targets that regulate different events, including late origin firing, entry into mitosis or the induction of repair and ribonucleotide reductase genes (Branzei and Foiani 2009).

1.3.1.4. Effectors

Regulation of dNTP levels via Rad53 and its upstream kinase Mec1 is crucial for cell survival. Deletion of either *RAD53* or *MEC1* results in cell death (Zhao, Muller et al. 1998). This lethality is explained by deregulation of the downstream kinase Dun1, which controls genes encoding for ribonucleotide reductase (RNR) and several DNA damage inducible genes (Zhao and Rothstein 2002). The lack of Rad53-dependent phosphorylation of Dun1 leads to activation of Sml1, which is an inhibitor of ribonucleotide reductase *RNR1*, and therefore decreased dNTP pools (Zhao and Rothstein 2002). The lethality of *rad53* and *mec1* can be rescued by additional inactivation of Sml1 or overexpression of *RNR1* which however does not restore the checkpoint function of *rad53* or *mec1* (Zhao, Muller et al. 1998).

1.3.1.5. Detection of DNA double strand breaks (DSB)

During S-phase, DSBs are sensed in a pathway involving the checkpoint kinase Tel1/ATM (Harrison and Haber 2006). The heterotrimeric complex Mre11-Rad50-Xrs2 (MRX) in *S.cerevisiae* or MRE11-RAD50-NBS1 (MRN) in human cells

recognizes the DNA lesion and plays a critical role in holding both DSB ends together (Dupre, Boyer-Chatenet et al. 2006), (see Figure 5). Furthermore, MRX/MRN is crucial for the recruitment and activation of Tel1/ATM (Nakada, Matsumoto et al. 2003), (Dupre, Boyer-Chatenet et al. 2006). Tel1/ATM can phosphorylate a number of substrates, including the histone variant H2AX at nucleosomes surrounding the lesion and other adaptor proteins such as BRCA1, 53BP1, MDC1 and NBS1 in human cells (Czornak, Chughtai et al. 2008).

In *S. cerevisiae*, Tel1 together with MRX facilitates processing of DSB to the single stranded 3' end, which is necessary for the repair by homologous recombination (HR) (Mantiero EMBO R, 2007). Resection requires the combined action of different enzymes: in the first step MRX and Sae2 create short 3' overhangs, which are then further cleaved by Sgs1-Dna2 or Exo1 (Zhu, Chung et al. 2008), (Mimitou and Symington 2008), (Gravel, Chapman et al. 2008). The resulting 3' overhang is coated by RPA, which recruits the checkpoint kinase Mec1/ATR. Thus both kinases Tel1 and Mec1 contribute checkpoint activation, although Mec1 was shown to have the principal role in Rad53 activation (Naiki, Wakayama et al. 2004).

1.3.1.6. DSB repair

There are two main pathways for DSB repair: either by error-prone non-homologous end joining (NHEJ) or by precise homologous recombination (HR). NHEJ connects DNA ends with little or no sequence homology and occurs mainly in G1 or M phase (Misteli and Soutoglou 2009). HR takes place during S and G2 phase and requires a homologous template such as the homologous sister chromatid. During S phase, HR is also believed to facilitate the restart of stalled or collapsed replication forks in addition to DSB repair (see also chapter 1.3.3). Therefore, a simplified model for HR is introduced below or in Figure 6: After resection of the DSB, the resulting single stranded 3' end is coated by RPA. This recruits not only Mec1/ATR but also the recombination proteins Rad51 and Rad52, leading to nucleofilament formation. During HR, the nucleofilament

invades the homologous dsDNA and anneals with the complementary strand thereby creating a D-loop. The second broken DNA strand is also captured by Rad51 and Rad52 and anneals with the displaced DNA strand. Both invading ends act as primers for DNA synthesis and the gaps are filled in by DNA polymerases. In the last step of HR repair, the resulting Holliday junction is resolved and the repaired duplexes are released (For review see (West 2003), and section 1.3.3).

1.3.2. Replication checkpoint

As discussed in chapters 1.2 and 1.4, DNA replication is tightly regulated and MCM helicases are loaded only once per cell cycle. . Therefore, to ensure the completion of chromosome replication, it is crucial that replication fork components are maintained once they have been loaded. To achieve this, an additional checkpoint pathway exists during S phase, called the replication checkpoint, which stabilizes stalled replication forks in response to replication stress. The replication checkpoint prevents irreversible replication fork collapse and preserves the integrity of replication forks to allow fork restart once the source that halts the fork is eliminated.

The replication checkpoint response shares many similarities with the DNA damage checkpoint during S phase, including the activation of Mec1/ATR and Rad53/CHK1 and overlapping downstream events (for further reading see (Tourriere and Pasero 2007), (Paulsen and Cimprich 2007), (Segurado and Tercero 2009)). That is why both checkpoints are often referred to as the intra-S phase checkpoint response. However, the reason for replication checkpoint activation is not DNA damage, but replication fork stalling due to replicative stress. Replication forks can arrest due to physical obstructions like protein-DNA complexes, unusual DNA structures formed at DNA repeats or common fragile sites or due to depletion of dNTP levels, which can be triggered by hydroxyurea

(HU), (Tourriere and Pasero 2007), (Branzei and Foiani). It is currently believed that replication stress causes the functional uncoupling of the MCM helicases from the replicative DNA polymerases thereby creating long stretches of ssDNA (Byun, Pacek et al. 2005), (Paulsen and Cimprich 2007). This ssDNA is subsequently coated by RPA and leads to the recruitment of Mec1-Ddc2 in yeast or ATR-ATRIP in human cells (Zou and Elledge 2003), (Rouse and Jackson 2002). After its activation Mec1/ATR posphorylates several components of the replisome including RPA and Mrc1/Claspin resulting in amplification of the checkpoint signal (Brush, Morrow et al. 1996), (Brush and Kelly 2000), (Kumagai and Dunphy 2000), (Alcasabas, Osborn et al. 2001), (Osborn and Elledge 2003). Different studies suggest that in response to replication stress, Mrc1 in yeast and Claspin in human cells act as the adaptor proteins between Mec1/ATR and Rad53/CHK1 (Alcasabas, Osborn et al. 2001), (Kumagai and Dunphy 2000). Thus mutation of the Mec1-dependent phosphorylation sites in the mrc^{AQ} mutant suppresses Rad53 hyperphosphorylation in *S. cerevisiae (Osborn and Elledge 2003)*. However, the molecular mechanism details as to how Mrc1 activates Rad53 are not understood, since no direct interaction between Rad53 and Mrc1 has yet been shown. In contrast to yeast, the *Xenopus* homologue of Mrc1, CLASPIN acts as a *bona fide* checkpoint adaptor and was shown to directly interact with CHK1 (Kumagai and Dunphy 2000), (Kumagai and Dunphy 2003). In budding yeast, Mrc1 function as a checkpoint mediator can be partially substituted by Rad9 (Alcasabas, Osborn et al. 2001). However, it is likely that Mrc1 and Rad9 promote distinct Rad53 phosphoisoforms with different biological functions.

Another mediator of the replication checkpoint in budding yeast is the fork-associated Tof1-Csm3 complex (Foss 2001). In contrast to Mrc1, Tof1-Csm3 probably plays a minor role in activating Rad53 but unlike Mrc1 it is crucial for replication fork maintenance at natural pause sites such as the replication fork barrier (RFB) of ribosomal DNA (Tourriere, Versini et al. 2005), (Hodgson, Calzada et al. 2007). It was suggested that Tof1-Csm3 might act as a molecular

break, inhibiting fork progression when the fork encounters non-nucleosomal proteins that are tightly bound to DNA (Mohanty, Bairwa et al. 2006). Consequently, deletion of Tof1 and Csm3 (but not Mrc1) completely abolishes replication fork stalling at RFBs. Fork progression at such protein-DNA barriers requires the activity of the DNA helicase Rrm3 (Azvolinsky, Dunaway et al. 2006). Rrm3 is also replication fork-associated and is probably needed to remove protein complexes or DNA-RNA hybrids ahead of the fork. It has been suggested that Tof1-Csm3 inhibits Rrm3 function at RFBs thereby facilitating replication fork arrest (Mohanty, Bairwa et al. 2006).

It is interesting to note that forks arrested at natural pause sites do not trigger the replication checkpoint response (Tourriere and Pasero 2007). The block at protein-DNA barriers inhibits progression of the MCM helicase and therefore prevents formation of ssDNA, which would be necessary to recruit and activate Mec1/ATR. Similarly, the replication checkpoint is blind to drugs that inhibit Top1 (CPT) or cause inter-strand crosslinks like mitomycin C or nitrogen mustard.

In addition to its function in activating a global checkpoint response, Mec1/ATR was shown to act locally at stalled replication forks to prevent irreversible fork collapse (see also chapter 1.5, (Friedel, Pike et al. 2009)). Using a separation of function mutant, *mec1*-100, it was demonstrated that fork stabilization and suppression of late origin firing are genetically distinct functions (Paciotti, Clerici et al. 2001), (Tercero, Longhese et al. 2003). *mec1-100* has delayed and reduced levels of Rad53 activation in S-phase in response to HU or MMS, but is proficient for the G2/M checkpoint. Although *mec1-100* mutant cells de-repressed late origin firing similarly to *mec1*Δ mutants, in contrast to *mec1Δ*, they were not hypersensitive to HU or MMS and could largely maintain stalled replication forks (Tercero, Longhese et al. 2003). Therefore it was proposed that the most critical function of the intra-S-phase checkpoint is to stabilize replication forks. Furthermore, Cobb *et al* reported a direct role for Mec1 in stabilizing polymerases at HU-arrested replication forks (Cobb, Schleker et al. 2005). Remarkably, this

role was largely separable from its function in Rad53 activation. Cells carrying the *mec1-100* mutation provoked a partial loss of DNA pol α and DNA pol ε in response to HU which was not observed in wild type cells or the *rad53*Δ mutant (Cobb, Schleker et al. 2005), (Cobb, Bjergbaek et al. 2003).

1.3.3. RecQ helicases have multiple roles in the maintenance of replication fork integrity

RecQ helicases are crucial for the maintenance of genome integrity during mitosis and meiosis. They are implicated in different processes such as DSB repair by HR, Okazaki fragment maturation, in replication fork stabilization and replication fork restart, intra-S-phase checkpoint activation, telomere maintenance and meiotic recombination (for review see (Bachrati and Hickson 2008), (Wu 2007), (Rossi, Ghosh et al.). RecQ helicases are conserved from *E. coli* to man and possess ATP-dependent 3'-5' helicase activity. Bacteria or lower eukaryotes such as *S. cerevisiae* only contain one representative of the RecQ

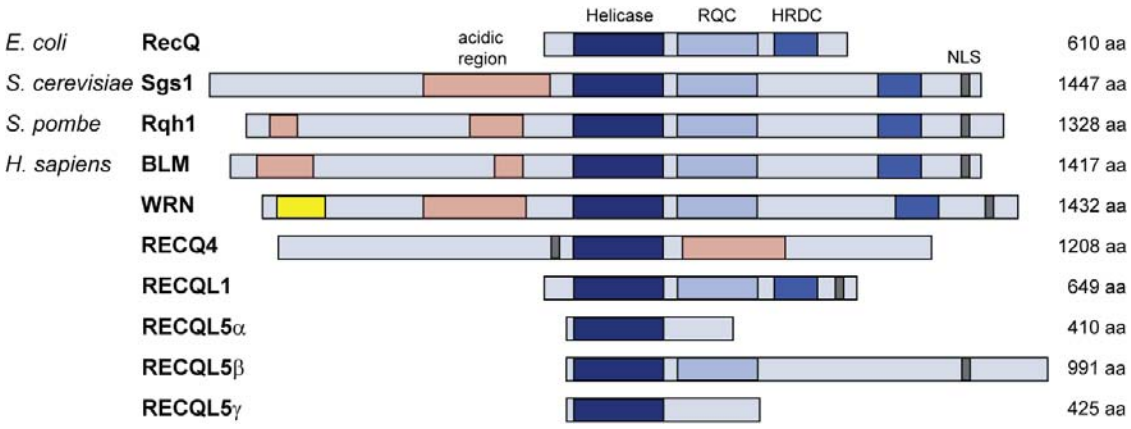


Figure 5 - Schematic diagram of selective members of the RecQ helicase family. *E.coli*, *S.cerevisiae* and *S.pombe* only possess one RecQ homologue. In contrast, there are at least five members known in human cells. The helicase domain is shown in dark blue, the RQC domain in light blue, the HRDC domain in blue, the NLS in black, the acidic region in red and the exonuclease domain of WRN in yellow.

family (RecQ and Sgs1 respectively), whereas there are at least five known homologues in human cells (BLM, WRN, RecQ4, RecQ1 and RecQ5). Mutations in three of the human RecQ genes give rise to diseases associated with cancer predisposition and premature ageing (BLM is mutated in Bloom's syndrome, WRN in Werner's syndrome and RECQ4 is mutated in Rothmund-Thomson, Baller-Gerold or Rapadilino syndrome). In addition to a conserved helicase domain, most members of the RecQ family helicases comprise other discernable motifs like a RecQ C-terminal (RQC) and a Helicase and RNaseD C-terminal (HRDC) domain. The RQC domain is unique to RecQ helicases and contains a Zn-binding region and a winged helix turn helix motif, which is probably the primary dsDNA binding site (Killoran and Keck 2006). A recent study on WRN revealed that the winged helix motif of the RQC domain is necessary for structure specific DNA binding and unwinding of DNA at branched points (Kitano, Kim et al.). RecQ helicases prefer structures that resemble replication or recombination intermediates and show weak activity towards linear dsDNA with blunt ends due to steric hindrance of the ß-wing of the winged helix motif with paired bases (Kitano, Kim et al.). The HRDC domain was also shown to bind DNA and mutations in the HRDC domain affected structure specific binding and unwinding activities (Bernstein and Keck 2005). Furthermore, some members of the RecQ family comprise domains, important for nuclear localization (NLS), protein interaction, oligomerization or additional enzymatic activity like the exonuclease domain of WRN (Hoadley and Keck).

1.3.3.1. Sgs1 contributes to the maintenance of stalled replication forks

There is strong evidence that RecQ helicases function directly at the fork to preserve replication fork stability. In *S. cerevisiae*, the sole RecQ helicase Sgs1 can be detected at replication forks in the presence or absence of HU (Cobb, Bjergbaek et al. 2003). Deletion of Sgs1 increases the rate of spontaneous gross chromosomal rearrangements (GCR), which can be exacerbated by nucleotide depletion using HU or exposure to the alkylating agent MMS (Myung and

Kolodner 2002), (Cobb, Schleker et al. 2005). Using chromatin immunoprecipiation (ChIP) it was reported that in wild-type cells DNA pol α and DNA pol ε remain bound to forks stalled by hydroxyurea for up to one hour (Cobb, Bjergbaek et al. 2003), (Cobb, Schleker et al. 2005). In contrast, both DNA polymerases were partially displaced from HU-arrested forks in cells where Sgs1 was deleted, suggesting that Sgs1 is necessary for the stable association of DNA polymerases in response to replication stress (Cobb, Bjergbaek et al. 2003). However, the molecular mechanism of how Sgs1 contributes to polymerase stability is not clear. Curiously, no direct interaction between Sgs1 and DNA pol α or DNA pol ε has been reported so far (Cobb, Bjergbaek et al. 2003). However, Sgs1 was shown to bind RPA, which in turn promotes the initiation of primer synthesis by DNA pol α. Therefore, it might be possible that Sgs1 acts indirectly on DNA pol α by inducing a conformational change in RPA, which affects its association with DNA pol α. Interestingly, combination of the *sgs1*Δ mutant with *mec1*-100 results in a synergistic increase in GCR rates, indicating that Sgs1 and Mec1 contribute to replication fork stability in an additive fashion (Cobb, Schleker et al. 2005). These cells also display a rapid loss of DNA polymerases and RPA from HU-arrested replication forks, resulting in fork collapse.

1.3.3.2. Sgs1 functions in intra-S phase checkpoint activation

In addition to its function in replication fork stabilization, Sgs1 also participates in intra-S checkpoint activation. Although deletion of Sgs1 does not affect HU-induced Rad53 activation, Sgs1 becomes essential, when Rad24 is deleted (Frei and Gasser 2000), (Bjergbaek, Cobb et al. 2005). It was proposed that Sgs1, which is epistatic with Mrc1 for checkpoint activation, activates the checkpoint at stalled forks, while Rad24 and the 9-1-1 complex primarily activate the checkpoint response at broken forks, which might arise after replication fork collapse (Bjergbaek, Cobb et al. 2005). Interestingly, Rad53 activation by Sgs1 in response to HU does not require the helicase activity of Sgs1 nor depend on Rad51 or Top3. Therefore, it seems likely that checkpoint activation is in part

mediated by an interaction between Sgs1 and Rad53. Indeed, Sgs1 interacts with Rad53 and both proteins colocalise in S phase specific nuclear foci (Frei and Gasser 2000), (Bjergbaek, Cobb et al. 2005). The Rad53 binding site of Sgs1 was mapped to the large region comprising the helicase, the RQC domain and an acid region N-terminal of the helicase domain. Within Rad53, it is the FHA1 domain that binds Sgs1 independently of Rad53's phosphorylation status (Bjergbaek, Cobb et al. 2005). However, the precise role of the interaction of those domains in checkpoint activation has not been elucidated yet.

1.3.3.3. Roles of Sgs1 during HR

Replication fork collapse and subsequent replisome dissociation leads to aberrant DNA structures that are targeted by exonucleases or undergo homologous recombination (Cotta-Ramusino, Fachinetti et al. 2005). Recent data suggest that HR plays both a positive and a negative role during replication. On the one hand, aberrant or unscheduled HR at stalled replication forks is a major source of genomic instability in yeast. On the other hand, there is growing evidence that proteins involved in HR function in the restart of stalled or collapsed replication forks and their activity is controlled by the intra-S phase checkpoint (Alabert, Bianco et al. 2009), (Petermann, Orta et al.).

The *S. cerevisiae* RecQ helicase, Sgs1, interacts tightly with the type IA topoisomerase Top3 and Rmi1 (RTR complex), reviewed in (Ashton and Hickson). The RTR complex is involved in the HR repair pathway and deletion of any RTR member causes a hyper-recombination phenotype. This hyper-recombination phenotype can be partially suppressed by deleting genes involved in early stages of HR (Ashton and Hickson). As discussed in chapter 1.3.1 recent data suggest that Sgs1 already functions in early steps of HR during DNA end resection. However, a more established role for Sgs1 and the RTR complex is in double Holliday junction (DHJ) dissolution. In budding yeast, DHJs can be resolved by three pathways. The first pathway involves the cleavage by Holliday

junction (HJ) resolvases, which cleave the junction symmetrically on opposing strands creating products that can be later ligated together (Ashton and Hickson). The first identified HJ resolvase was RuvC in *E.coli* (Dunderdale, Benson et al. 1991). In budding yeast this function is performed by Yen1 and Slx1-Slx4 (Fricke and Brill 2003), (Ip, Rass et al. 2008). The second pathway is asymmetrical cleavage catalyzed by the 5'-flap endonuclease Mus81-Mms4 (Boddy, Gaillard et al. 2001). DHJs resolved by Mus81-Mms4 produce a mixture of flapped and gapped linear duplexes that need to be further processed by flap-endonucleases and gap-filling polymerases before they can be ligated. The third pathway is by DHJ dissolution, which is mediated by the RTR complex (Ira, Malkova et al. 2003), (Wu and Hickson 2003). In contrast to the other two pathways, which produce a mixture of cross-over and non-cross-over products, DHJ dissolution exclusively creates non-cross-over products (Ashton and Hickson). In general, cross-over formation is more dangerous for the cell, because it involves the strand exchange of large DNA sections between the two homologous chromosomes. This can lead to translocations, inversions, deletions and loss of heterozygosity. Cross-over formation is also suppressed by the Srs2 helicase, which channels recombination intermediates into the synthesis-dependent strand annealing (SDSA) pathway (Ashton and Hickson). Srs2 was shown to act as an antirecombinase by disruption of Rad51-ssDNA filaments (Krejci, Van Komen et al. 2003), (Veaute, Jeusset et al. 2003). Overexpression of Sgs1 rescues the hyper-recombination phenotype of *srs2*Δ, suggesting that the functions of Sgs1 and Srs2 overlap (Mankouri, Craig et al. 2002), (Ira, Malkova et al. 2003). Interestingly, deletion of both *SGS1* and *SRS2* helicase causes a synthetic lethal phenotype, which can be rescued by deletion of genes involved in HR, such as *RAD51*, *RAD55* or *RAD57* (Gangloff, Soustelle et al. 2000). This suggests that Srs2 and Sgs1 act on parallel pathways to suppress aberrant homologous recombination. In their combined absence, aberrant HR intermediates accumulate, which are either not processed or are resolved through deleterious reciprocal exchange.

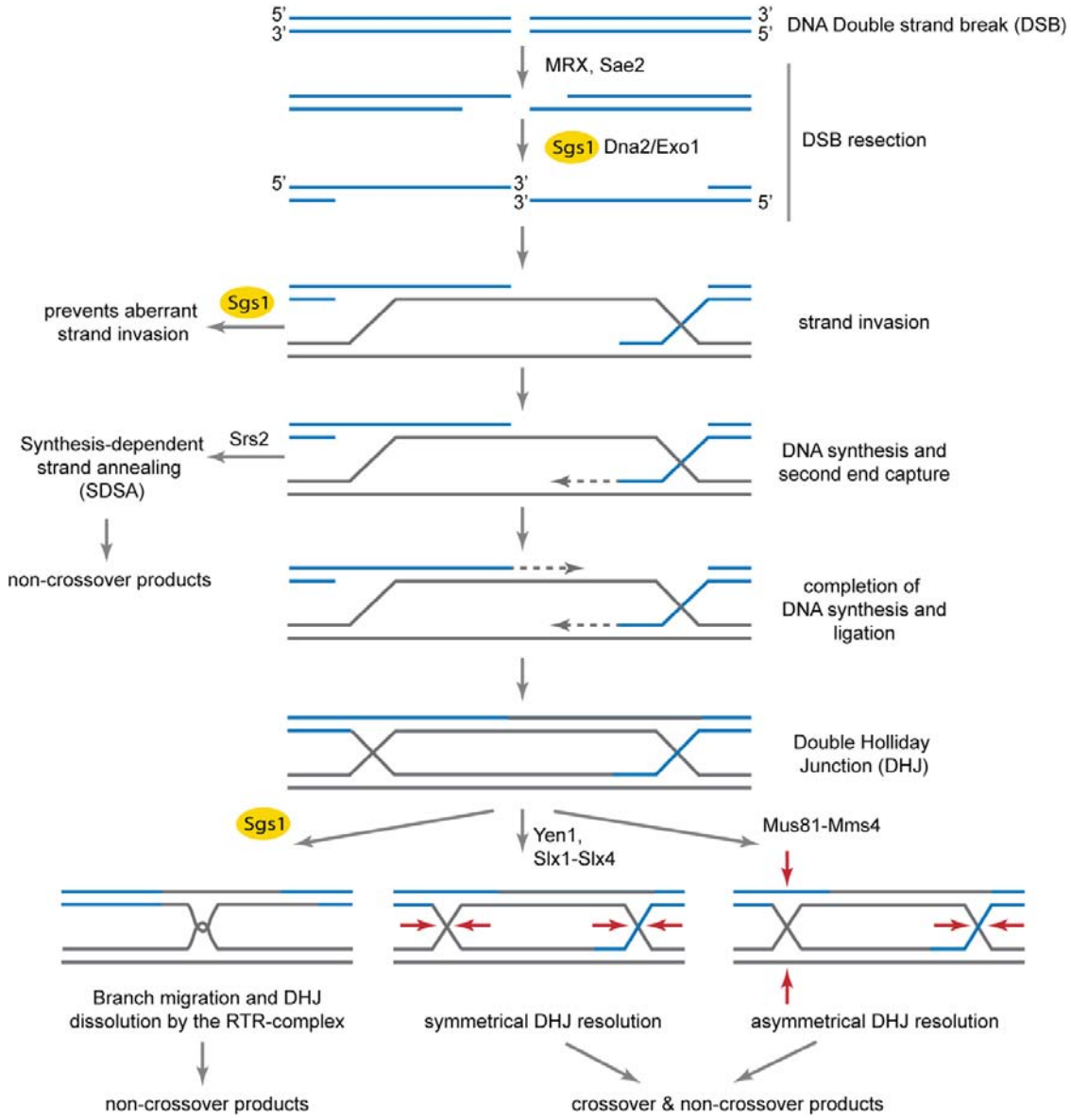


Figure 6 - Different Sgs1 functions during HR (adapted from (Ashton and Hickson). Replication fork stalling or collapse can create DSB or replication intermediates that can only be resolved by HR. Sgs1 functions already early in DSB repair mediating DNA end resection. Sgs1 also functions as an antirecombinase thereby preventing aberrant HR. After formation of the double Holliday junction (DHJ) Sgs1 can mediate branch migration and dissolution of the DHJ producing exclusively non-crossover products.

1.3.3.4. The human RecQ helicases BLM and WRN also maintain replication fork stability

Like *sgs1*Δ mutants, human cells lacking WRN and BLM display aberrant replication intermediates, HU and MMS-sensitivity and hyper-recombination (Bachrati and Hickson 2008). Ectopic expression of either WRN or BLM in *S. cerevisiae* partially rescues the increased rates of spontaneous recombination in *sgs1*Δ cells, indicating that the functions are conserved among both species (Yamagata, Kato et al. 1998), (Heo, Tatebayashi et al. 1999). BLM seems to be the closer homologue since, in contrast to WRN, it can complement the HU sensitivity and reduced life span of *sgs1*Δ mutants (Heo, Tatebayashi et al. 1999). Indeed, similarly to Sgs1, BLM was also shown to interact with the human homologues of Top3 and Rmi1 (TOPIIIα and RMI1) to facilitate DHJ resolution of recombination intermediates and regression of stalled replication forks (reviewed in (Liu and West 2008)). Recently, a new member of the RTR complex has been identified in human cells, RMI2, which binds an oligonucleotide binding (OB) fold in the C-terminal region of RMI1 not present in the yeast homologue (Xu, Guo et al. 2008), (Singh, Ali et al. 2008). The human RecQ helicases BLM and WRN are also implicated in the intra-S phase checkpoint. Both RecQ helicases are phosohorylated by the checkpoint kinase ATR in response to the replication inhibitor HU or aphidicolin (Davies, North et al. 2004), (Pichierri, Rosselli et al. 2003). Cells lacking BLM fail to maintain replication fork integrity and affect the suppression of new origin firing after replication stress (Davies, North et al. 2007). BLM's ability to stabilize stalled replication forks depends on its helicase activity and ATR-dependent phosphorylation (Davies, North et al. 2007). It was suggested that BLM promotes replication fork regression at stalled forks to facilitate restart of DNA synthesis by template switching and recombination once the stress is alleviated (Davies, North et al. 2007), (Ralf, Hickson et al. 2006). A similar function has been reported for WRN, which is especially important for the stability of fragile sites (Pirzio, Pichierri et al. 2008). Furthermore, BLM and WRN

are both implicated in Okazaki fragment maturation and directly interact with the replication proteins FEN1 and DNA polδ (Bachrati and Hickson 2008), (see also chapter 1.2.3). Interestingly, the interaction between RecQ helicases and RPA is also conserved from yeast to human cells (Cobb, Bjergbaek et al. 2003), (Doherty, Sommers et al. 2005). The implications of the RecQ helicase-RPA interaction on replication fork stability and intra-S phase checkpoint activation are the subject of this study and are also discussed in more detail in chapters 1.3.4, 2, and 3.

1.3.4. RPA structure and function

Replication protein A (RPA) is a central player for almost all processes during DNA metabolism (Wold 1997), (Binz, Sheehan et al. 2004). RPA is an evolutionarily conserved ssDNA binding protein. It binds ssDNA with high affinity and low specificity thereby protecting it from nuclease digestion and preventing unwanted inter- and intra-strand reannealing. Furthermore, RPA mediates multiple protein interactions and is crucial for replication initiation, replication elongation, intra-S phase checkpoint activation and DNA repair (see chapters 1.2.2-1.3.3).

1.3.4.1. Structure of RPA

RPA is a heterotrimeric protein composed of structurally related subunits of 70 kDa, 32 kDa and 14 kDa, named RPA70, RPA32 and RPA14 according to their molecular weight (Wold 1997). All three subunits interact and are essential for stable complex formation. In yeast RPA is encoded by the essential genes *RFA1*, *RFA2* and *RFA3*. The best characterized RPA homologue is from human cells. RPA consists of six oligonucleotide binding (OB) folds, which can all interact with ssDNA (see Figure 7), (Fanning, Klimovich et al. 2006), (Gao, Cervantes et al. 2007). The largest subunit of human RPA, RPA70 (Rpa70 in *S. cerevisiae*) consists of four oligonucleotide binding domains: DNA binding domain (DBD) A,

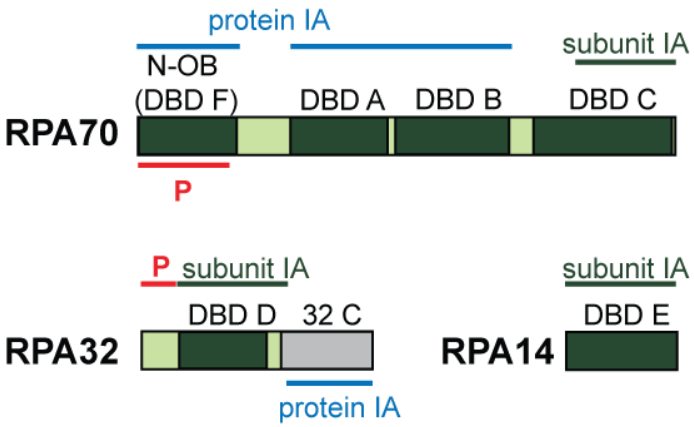


Figure 7 - Domain structure of RPA. The DNA binding domains are indicated (DBD). Regions important for protein interactions (IA) are indicated by blue horizontal lines, regions for subunit interactions by green horizontal lines and regions that are phosphorylated during the cell cycle or in response to DNA damage are in labeled with a red horizontal line (P).

B, C and F. These bind ssDNA with differing affinity, with DBD A and B being the tightest binders. The second subunit RPA32 comprises one OB fold (DBD D) in the center that is flanked by an unstructured N-terminal region which is phosphorylated in a cell cycle dependent manner or in response to DNA damage and replication stress (Binz, Sheehan et al. 2004). The C-terminus of RPA32 consists of a winged-helix turn helix motif (RPA32C) implicated in protein interaction (Bochkarev and Bochkareva 2004). The smallest subunit RPA14 contains a single OB fold (DBD E) and is necessary for RPA complex formation. In yeast, RPA14 was also reported to bind weakly to telomeric DNA (Gao, Cervantes et al. 2007). Although canonical RPA consists of RPA70, RPA32, RPA14 (or RPA1, RPA2 and RPA3 respectively), some organisms such as some plants (*Arabidopsis thaliana* or *Oryza satvia*) contain multiple RPA subunit genes forming alternative RPA complexes that seem to have different functions (Ishibashi, Kimura et al. 2006). Keshav *et al* 1995 discoverd a new subunit of human RPA, RPA4 (Keshav, Chen et al. 1995). RPA4 is a mammalian specific homologue of RPA32 and stably interacts with RPA70 and RPA14. Recently it was reported that human RPA4, unlike RPA32, does not support chromosomal

replication and cell cycle progression, but might be instead involved in maintaining cell quiescence (Haring, Humphreys et al.).

1.3.4.2. Binding mode of RPA

Although the structure of many RPA fragments has been solved by NMR or crystallization, the structure of intact heterotrimeric RPA is not known yet. It has been proposed that RPA binds ssDNA sequentially (for review see and references therein (Bochkarev and Bochkareva 2004), (Fanning, Klimovich et al. 2006)). In the first step, DBD A and DBD B of RPA70 bind to ssDNA with a defined 5'-3' polarity (DBD A at the 5' end) to occlude approximately 8-10 nt of DNA (Figure 8). In the second step, DBD C joins to form the so-called 12-23 nt binding mode. Finally, in the last step, which is also referred to as the 30 nt binding mode, DBD D (RPA32) contacts the ssDNA. The cooperative binding of all four RPA DBDs increases the affinity of RPA for DNA by several orders of magnitude. Thus RPA changes its conformation upon ssDNA binding from a globular binding mode with low affinity to an elongated high-affinity binding mode.

The exact molecular mechanism as to how RPA orchestrates the different specific protein interactions that it needs to make during DNA metabolism are not known. One model suggests that proteins trade places with ssDNA via RPA

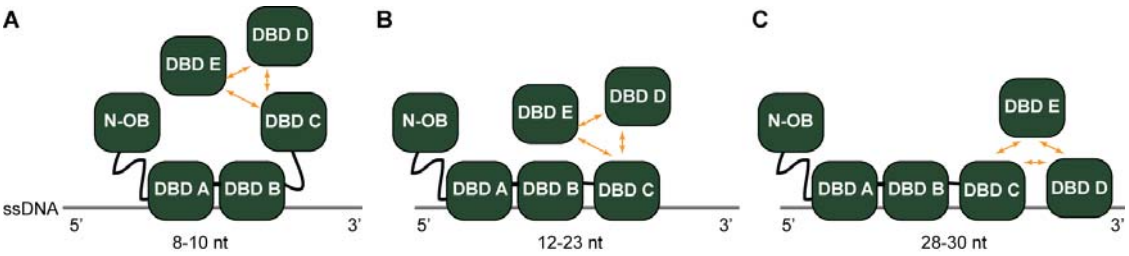


Figure 8 - Binding mode of RPA. A) ssDNA binding is initiated by DBD A and DBD B of RPA70 (8-10 nt binding mode). In this binding mode RPA has probably a globular confirmation with low affinity for DNA. **B)** In the 12-23 nt binding mode also DBD C of RPA70 contacts the DNA. **C)** During the last step, DBD C of RPA32 joins the DBD A-C in DNA binding (30 nt binding mode) resulting in an elongated RPA complex with high affinity for ssDNA.

specific binding sites (“hand-off” mechanism) (Fanning, Klimovich et al. 2006). The incoming protein replaces the preceding one, because it has a greater affinity for RPA and competes with it for ssDNA. Alternatively, it was proposed that other proteins induce conformational changes in RPA to a weaker ssDNA binding mode to allow access. Most RPA-protein interactions are mediated by DBD F (also referred to as N-OB), DBD A, DBD B and the winged helix-turn-helix motif of RPA32C. A study in SV40 virus replication revealed a model for protein-mediated RPA dissociation, where T-antigen helicase promotes primer synthesis of DNA polymerase α/primase on ssDNA coated by RPA (Arunkumar, Klimovich et al. 2005). It was proposed that T-antigen hexamer interacts with RPA (RPA32C, DBD A and DBD B) thereby changing RPA to a globular, low-affinity conformation. This releases a short stretch of ssDNA, which is immediately bound by DNA pol α/primase associated with T-antigen. DNA pol α/primase then initiates primer synthesis, displacing the weakly bound RPA and T-antigen during primer elongation.

The N-terminal OB-fold of human and yeast RPA (DBD F, N-OB) is also involved in mediating protein interaction and phosporylated in response to DNA damage or replication stress (Brush and Kelly 2000), (Nuss, Patrick et al. 2005). Bochkareva *et al* 2005 reported the crystal structure of the RPA70 N-OB fold bound to the N-terminal region of the p53 transactivation domain (Bochkareva, Kaustov et al. 2005). The p53 transactivation region that binds the N-OB fold is largely disordered in solution, but induces two short amphiphatic helices upon RPA binding. One of the amphiphatic helices mimics ssDNA binding to the basic cleft of the N-OB fold. In addition, a peptide that imitates RPA32 hyperphosphorylation that occurs after DNA damage or replication stress is able to compete with p53 and can dissociate bound p53 peptide from the N-OB fold of RPA70 (Bochkareva, Kaustov et al. 2005). This suggests that hyperphosphorylation of the RPA32 N-terminus by checkpoint kinases might regulate the interaction between RPA and other proteins such as p53. Recent studies in human cells have revealed that other checkpoint proteins such as

ATRIP, MRE11 and RAD9 also interact with the RPA N-OB fold employing a similar mechanism to p53 (Ball, Ehrhardt et al. 2007), (Xu, Vaithiyalingam et al. 2008). As in human cells, the *S. cerevisiae* Rpa70 N-OB fold was reported to mediate interaction with Ddc2 and is necessary for retention of the checkpoint kinase Mec1 at sites of DNA damage (Ball, Ehrhardt et al. 2007). Consistently, Ddc2 recruitment to DSB is abolished in the *rfa1*-t11 mutant, which carries a charge reversal mutation (K45E) in the N-OB fold of yeast Rpa70 (Umezu, Sugawara et al. 1998), (Zou and Elledge 2003), (Dubrana, van Attikum et al. 2007). Interestingly, *rfa1*-t11 seems to be a separation of function mutant and is quite well characterized, because deletion of the yeast N-OB fold is lethal (Philipova, Mullen et al. 1996). Although *rfa1*-t11 cells were shown to be proficient for DNA replication under normal conditions, they display sensitivity to MMS, UV and HU and defects in recombination and DNA damage checkpoint activation (Umezu, Sugawara et al. 1998), (Kim and Brill 2001), (Kanoh, Tamai et al. 2006), (Soustelle, Vedel et al. 2002), (Kantake, Sugiyama et al. 2003).Thus, whereas deletion of the yeast N-OB fold is lethal (Philipova, Mullen et al. 1996), *rfa1*-t11 seems to be a separation of function mutant.

1.3.4.3. RPA interacts with RecQ helicases

Several studies have reported that RPA interacts with various RecQ helicases, including BLM and WRN in human cells or Sgs1 in budding yeast (Cobb, Bjergbaek et al. 2003), (Doherty, Sommers et al. 2005). In human cells, RPA70 DBD A and DBD B have been shown to bind an N-terminal region of WRN or BLM. There is evidence that this interaction is functionally relevant since RPA stimulates the unwinding activity of both BLM and WRN and enhances the branch migration activity of WRN (Doherty, Sommers et al. 2005), (Sowd, Wang et al. 2009). A recent study suggested that RPA can only efficiently stimulate processive DNA unwinding by BLM when multiple BLM molecules are present (Yodh, Stevens et al. 2009). Interestingly, it seems that a single BLM molecule can only unwind short stretches of DNA even in the presence of RPA. The same

study reported that monomeric BLM repetitively unwinds DNA. After unwinding a certain length of DNA, BLM switches strands and reverses the unwinding reaction (Yodh, Stevens et al. 2009). During these cycles, RPA stimulated unwinding re-initiation and BLM was shown to both load and displace RPA from ssDNA (Yodh, Stevens et al. 2009). This repetitive unwinding and strand switching by BLM could be important for its antirecombinase function to prevent aberrant HR and for recovery of stalled replication forks.

1.4. Regulation of origin choice and replication initiation

1.4.1. Control of origin choice and replication timing

There is evidence that activation of eukaryotic origins occurs throughout S-phase according to a temporal program (Sclafani and Holzen 2007). In yeast, as in mammals, replication origins are not fired all at the same time, some origins are activated earlier in S-phase and others later (Raghuraman, Winzeler et al. 2001). In S. cerevisiae, origin timing is established in G1 (Raghuraman, Brewer et al. 1997), suggesting that late firing origins are already marked as such. It was proposed that timing of origin firing depends on neighbouring sequences and chromatin structure (Friedman, Diller et al. 1996). Indeed, in mammalian cells, origins located in active euchromatic regions tend to replicate early, while origins in heterochromatic regions are mainly activated late in S-phase. Thus, it was suggested that transcription in euchromatic regions “opens up” chromatin thereby enabling easy access to the replication machinery. Furthermore, there is evidence that coordinated activation of origins takes place at discrete replication foci. Those nuclear structures are believed to contain clusters of replicons, which can be detected as chromatin loops anchored to a nuclear skeleton (nuclear matrix), when most of the chromatin proteins are removed by high salt or lithium 3,5-diiodosalicylate (Cayrou, Coulombe et al.). Studies in *Xenopus* and mammalian cells indicate that an event occurring at mitosis is important for the selection of origins that can be used in the following S-phase (Buongiorno-

Nardelli, Micheli et al. 1982), (Courbet, Gay et al. 2008). They also link chromosome architecture with the regulation of origin localization and usage in higher eukaryotes (Cayrou, Coulombe et al.).

Additionally, there is a connection between replication timing and the intra-S-phase checkpoint response. If replication is inhibited due to replication stress or DNA damage, the checkpoint kinases Mec1 and Rad53 in yeast (ATR and CHK1 in human cells) get activated and suppress late origin firing (Santocanale and Diffley 1998), (Shirahige, Hori et al. 1998). In *S. cerevisiae*, it was shown that Dbf4 is phosphorylated by Rad53, which inhibits DDK activity and removes it from chromatin (Weinreich and Stillman 1999), (Pasero, Duncker et al. 1999). Interestingly, in *Xenopus* and mammalian cells, the ATR and CHK1 kinases have been also been implicated in the regulation of origin activation during normal replication (Shechter, Costanzo et al. 2004), (Marheineke and Hyrien 2004), (Syljuasen, Sorensen et al. 2005). This checkpoint function in normal S-phase suggests that the status of active replicons within replication foci is sensed via a CHK1-dependent pathway. The molecular mechanism is still unknown, but it is likely that the ATR/CHK1 pathway modulates origin usage to regulate the density of active origins during S-phase (Shechter and Gautier 2005). This might activate late or dormant origins, which are suppressed during normal DNA synthesis, but which could rescue replication if replication forks collapse (Maya-Mendoza, Tang et al. 2009).

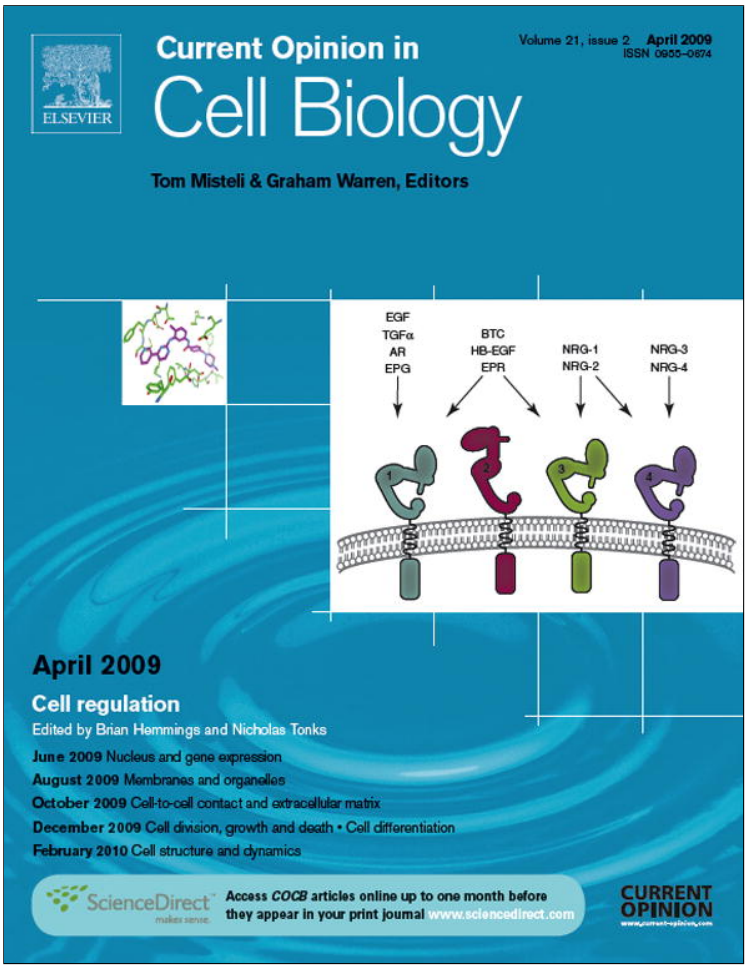
1.4.2. Prevention of re-replication

In budding yeast, new licensing of already replicated origins is inhibited in S and G2 phase mainly due to CDK-dependent phosphorylation of different pre-RC components. A major target of CDK phosphorylation at the G1-S transition is Cdc6 (Blow and Dutta 2005). Phosphorylated Cdc6 is rapidly degraded by the ubiquitin ligase SCF. This ensures that Cdc6 is only present in G1, when CDK is inactive. Furthermore, ORC activity is regulated by CDK-dependent

phosphorylation. CDKs are directly recruited to ORC, which is associated with origins throughout the cell cycle in *S. cerevisiae.* ORC phosphorylation maintains chromatin-bound ORC in an inactive state during S and G2 phase and is important to prevent re-replication. Another CDK-dependent control mechanism is the nuclear export of Mcm2-7 and Cdt1 (Blow and Dutta 2005), (Morgan 2007). This prevents the assembly of new MCM complexes at origins during S phase, but does not affect the MCM complexes that are already chromatin bound. Thus, multiple pathways exist to prevent re-licensing and over-replication. Indeed, studies in budding yeast showed that only inactivation of all CDK-dependent control mechanisms resulted in significant re-replication (Nguyen, Co et al. 2001).

In metazoan cells, CDKs also contribute to the regulation of the licensing system. However, their effects are less well understood and seem to vary in different organisms (Blow and Dutta 2005), (Morgan 2007). However, a major pathway that prevents pre-RC assembly in S and G2 phase is facilitated by downregulation of Cdt1 and the activation of geminin (Wohlschlegel, Dwyer et al. 2000), (Nishitani, Taraviras et al. 2001). At the end of G1 and early S-phase Cdt1 is degraded in a CDK-dependent by Skp2-containing SCF ubiquitin ligases (Pichierri, Rosselli et al. 2003), (Liu, Li et al. 2004). Aditionally, there is evidence that Cdt1 is also degraded in a CDK-independent manner (Blow and Dutta 2005). Later in G2 and M phase, Cdt1 binds to geminin, which protects it from degradation and allows Cdt1 levels to build up in an inactive form (Ballabeni, Melixetian et al. 2004). In late mitosis, geminin is down-regulated by proteolysis or CDK-dependent ubiquitination, which results in the release of Cdt1 and pre-RC formation. Experiments in human tissue and Xenopus revealed that down-regulation of Cdt1 levels is crucial to prevent re-replication, despite any CDK-dependent inhibitory phosphorylation of ORC, Cdt6 or MCM (Vaziri, Saxena et al. 2003), (Li and Blow 2005). Thus, overexpression of Cdt1 or inhibition of geminin synthesis during S phase resulted in extensive re-replication of DNA.

1.5. Review: ATR/Mec1 – coordinating fork stability and repair



Available online at www.sciencedirect.com
ScienceDirect
Current Opinion in Cell Biology

ATR/Mec1: coordinating fork stability and repair

Anna M Friedel, Brietta L Pike and Susan M Gasser

During S phase, eukaryotic cells unwind and duplicate a tremendous amount of DNA, generating structures that are very sensitive to both endogenous and exogenous insults. The collision of DNA polymerases with damaged DNA or other obstructions to fork progression generates replication stress, which can evolve into fork collapse if the replisome components are not stabilized. To ensure genome integrity, stalled replication forks are recognized by a checkpoint, whose central player is the human kinase ATR or Mec1 in *S. cerevisiae*. This review will discuss recent findings revealing roles of the ATR/Mec1 kinase: both in stabilizing the replisome directly and in activating the checkpoint response to regulate origin firing, DNA repair, fork restart, and cell cycle progression.

Address
Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, CH-4058 Basel, Switzerland

Corresponding author: Gasser, Susan M (susan.gasser@fmi.ch)

Current Opinion in Cell Biology 2009, **21**:237–244

This review comes from a themed issue on
Cell regulation
Edited by Brian Hemmings and Nikolas Tonks

Available online 21st February 2009

0955-0674/$ – see front matter

DOI 10.1016/j.ceb.2009.01.017

Introduction

Maintaining genome integrity is crucial for all organisms. Eukaryotes have developed sophisticated control mechanisms called checkpoints that recognize DNA damage and act to slow progression through the cell cycle and coordinate repair. During S phase, cells are particularly sensitive to DNA insult, which can arise from genotoxic lesions (e.g. provoked by MMS) or by endogenous replication stress (e.g. fork delay), which can be exacerbated by treatment with hydroxyurea (HU). Consequently, additional checkpoint response mechanisms exist in S phase to deal with stalled replication forks and/or DNA damage. Central to checkpoint signal transduction pathways are the phosphoinositide 3-kinase (PI3K)-related protein kinases ATR and ATM, or their budding yeast homologs Mec1 and Tel1. While ATM/Tel1 is activated principally by DNA double-strand breaks (DSBs), ATR/Mec1 responds to a wide range of DNA damage, including replication stress, base adducts, UV-induced nucleotide damage, and DSBs [1•]. This review will examine the roles of ATR/Mec1 as it interacts with fork components to stabilize the replisome, and as it activates the checkpoint signal transduction pathway that controls origin firing, DNA repair and prevents early initiation of mitosis.

Activation and recruitment of the checkpoint kinase ATR/Mec1

The current model suggests that ATR/Mec1 does not recognize the primary lesion itself, but long stretches of single-stranded DNA (ssDNA), which are generated either by the functional uncoupling of replicative helicases and polymerases during fork stalling or by nucleolytic processing of DSBs (Figure 1) [2,3]. The regions of ssDNA are not naked but are coated by replication protein A (RPA). RPA itself is a target of ATR/Mec1, but also plays a central role in recruiting ATR/Mec1 via its co-factor, the ATR-interacting protein (ATRIP; Ddc2 in budding yeast, see Table 1) [4]. Visible ATRIP/Ddc2 foci demonstrate the accumulation of the complex at sites of repair [5,6]. However, RPA-coated ssDNA alone is not sufficient for ATR/Mec1 activation. It has been reported that primers created by Polymerase α (Pol α)/primase are required for checkpoint activation in *Xenopus* egg extracts and that primed ssDNA with a free 5′ end, but not unprimed ssDNA, is needed to activate the downstream checkpoint kinase CHK1 [7–9].

Other factors have been implicated in activation of the ATR/Mec1 kinase. The 9-1-1 checkpoint clamp, a heterotrimer structurally related to PCNA but consisting of RAD9-RAD1-HUS1 in humans and Rad17-Mec3-Ddc1 in *S. cerevisiae*, is loaded onto primer-template junctions adjacent to RPA-coated ssDNA [1•,10]. The clamp loader RAD17-RFC2-5 (human) or Rad24-Rfc2-5 (yeast) loads 9-1-1 independently of ATR-ATRIP, although recent data suggest that phosphorylation of RAD17 Ser635 and Ser645 by ATR affects the turnover of RAD9 molecules in foci and therefore contributes to 9-1-1 retention close to DNA lesions [11]. In humans, 9-1-1 then recruits the BRCT domain-containing topoisomerase-binding protein-1 (TOPBP1) that binds ATRIP and thus contributes to ATR activation [12,13]. By contrast, it has been reported that *S. cerevisiae* 9-1-1 can activate Mec1-Ddc2 directly *in vitro* [10]. Intriguingly, the co-localization of these sensors, 9-1-1 and Mec1/Ddc2, appears to be sufficient to activate the checkpoint even without induced damage [14•].

Recently, two studies have shown that Dpb11 plays a role in checkpoint activation, acting like its human homolog TOPBP1 [15,16•]. In this case however, the C-terminus

Figure 1

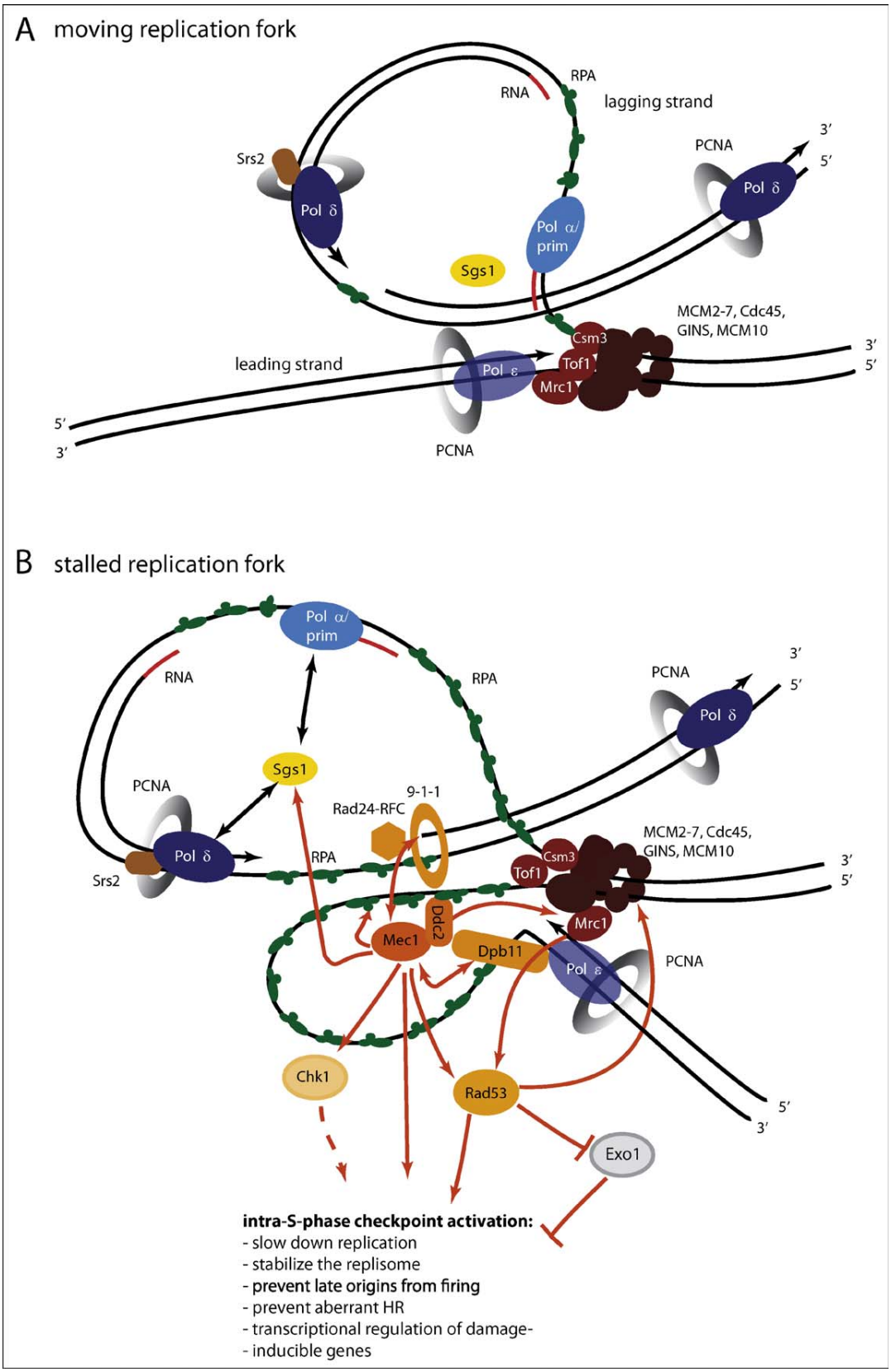


Table 1

Conserved *S. cerevisiae* and *H. sapiens* checkpoint proteins and their functions.

S. cerevisiae	*H. sapiens*	Function
RFA-ssDNA	RPA-ssDNA	Signal
Rad24-RFC	RAD17-RFC	Sensor (RFC-like complex, clamp loader)
Ddc1-Rad17-Mec3	RAD9-RAD1-HUS1	Sensor (9-1-1, DNA damage checkpoint clamp)
Dpb11	TOPBP1	Sensor (required for ATR activation)
Mre11-Rad50-Xrs2	MRE11-RAD50-NBS1	Sensor (MRX/MRN)
Mec1/Ddc2	ATR/ATRIP	Sensor (signaling kinase)
Tel1	ATM	Sensor (signaling kinase)
Pol ε	POL ε	Fork associated (leading strand polymerase)
Sgs1	BLM, WRN	Fork associated (RecQ helicase)
Tof1/Csm3	TIM/TIPIN	Fork associated
Ino80	INO80	Fork associated (chromatin remodeler)
Mrc1	Claspin	Mediator
Rad9	BRCA1/53BP1	Mediator
Rad53	CHK2	Effector (signaling kinase)
Chk1	CHK1	Effector (signaling kinase)

of Dpb11, and not its BRCT repeats, interacts with Ddc2, leading to Mec1 activation. Moreover, Mordes *et al.* 2008 observed that Mec1-dependent phosphorylation of Dpb11 (Thr731) further enhances the ability of Dpb11 to amplify Mec1-Ddc2 activity. Additional research is needed to confirm exactly how Dpb11 is recruited to damage since it appears to colocalize with Pol ε during initiation, but not during elongation [17]. In one model, 9-1-1 and Mec1-Ddc2 are recruited independently of RPA-ssDNA, and Mec1 subsequently phosphorylates the Ddc1 subunit of 9-1-1. In an alternative model, 9-1-1 and Dpb11 act in parallel to activate Mec1-Ddc2. In this case Dpb11 could be recruited to RPA-ssDNA via its interaction with other proteins, for example, Pol ε or Sld2 and Sld3 [17–19]. Data from the Burgers laboratory has also suggested that Dpb11 and 9-1-1 can independently activate Mec1-Ddc2 *in vitro*, although synergism is observed when both activators are present [15].

Direct interaction of ATR/Mec1 with components of the replication fork

By studying mutations in the budding yeast Mec1, or in targets of Mec1, it is clear that ATR/Mec1 functions at stalled replication forks to keep replication polymerases engaged, and that this is largely separable from its role in activating downstream checkpoint kinases such as Rad53 [2,20] (see Table 2). For instance, even in the absence of exogenous genotoxic stress, *mec1* mutants accumulate gross spontaneous chromosomal rearrangements (GCRs) at rates far higher than mutants lacking the downstream kinase Rad53, or in the adaptor proteins that function in the checkpoint response [21,22]. Spontaneous GCRs are thought to arise from inappropriate fork-associated recombination events. The direct role of Mec1 in maintaining DNA polymerases at forks has been demonstrated by Chromatin immuno-precipitation (ChIP) assays for both DNA Pol α and Pol ε. Both polymerases are stably associated at forks stalled on HU for roughly an hour in a Mec1/Ddc2-dependent manner, whereas *rad53*-11 or *rad53*Δ mutations had little or no effect on polymerase stability under identical conditions [20,23]. Moreover, ChIP has shown that Mec1/Ddc2 accumulates at stalled replication forks, which is not the case for the downstream kinase Rad53 [23]. A partial loss of function mutant, *mec1*-100, which compromises the S phase checkpoint but leaves the G2/M activation of Rad53 intact, provokes a partial loss of replicative polymerases at stalled forks, yet this mutation leads to a complete fork collapse when the RecQ helicase Sgs1 is deleted. The polymerase loss phenotype observed in *mec1*-100 *sgs1Δ* mutants correlates with compromised replication fork recovery and a dramatic increase in the rate of GCR. By contrast, cells lacking the Rad53 kinase show no loss of polymerases and no synergism with *sgs1Δ*, although there is significant displacement of the MCM helicase from stalled forks [23]. This latter may result from release of MCM from the replisome allowing extensive DNA unwinding and a subsequent collapse of forks in *rad53Δ* cells. Finally it

Model of a moving and stalled replication fork in *S. cerevisiae*. **(a)** At an unperturbed replication fork MCM helicases/Cdc45 unwind the parental DNA double strand. Polymerase ε (Pol ε) is responsible for leading strand synthesis, while polymerase α (Pol α/prim) initiates Okazaki-fragment-synthesis at the lagging strand that is completed by polymerase δ (Pol δ). **(b)** At a stalled replication fork single stranded DNA coated by RPA (RPA-ssDNA) accumulates owing to functional uncoupling of MCM/Cdc45 and the replicative polymerases. This triggers an S phase-specific checkpoint response, where 9-1-1 and the checkpoint kinase Mec1-Ddc2 are recruited independently to RPA-ssDNA. Subsequently, Mec1-Ddc2 is activated, leading to phosphorylation of various downstream targets including mediator proteins Mrc1 or Rad9, which contribute to the activation of the downstream effector kinases Rad53 and Chk1. Especially, Rad53 was shown to play a crucial role in stabilizing the replisome, preventing late origins from firing, preventing homologous recombination (HR), and mediating DNA repair. Furthermore, Mec1-Ddc2 was also shown to stabilize the replication fork by acting locally at the stalled fork.

Table 2

Contribution of Mec1/ATR versus Rad53/CHK1 to S phase checkpoint events.

Mec1/ATR target	S phase checkpoint events	Rad53/CHK1 target
Phosphorylation of Mrc1	Slowed rate of replication	Downregulation of dNTP levels
Maintenance of polymerases and RPA	Stabilization of the replisome at stalled replication forks	Maintenance of the MCM complex
Sgs1/BLM	Protection of stalled or damaged replication forks	Inhibition of Exo1 activity
(via Rad53)	Prevention/delay of late firing origins	Downregulation of Cdc7/Dbf4 activity
(via Rad53)	Transcriptional regulation of damage-inducible genes	Activation of Dun1 kinase
Locally: by MCM2 phosphorylation and Plx1 recruitment	Replication fork restart	Globally: by Rad53-dephosphorylation

has been shown that in a *rad53*Δ strain MMS-induced lethality, presumably due to fork collapse, can be suppressed by ablation of Exo1 that works together with Sgs1 to resect DSBs [24••,25,26], while *mec1*Δ defects cannot [27••]. Together, these observations provide compelling arguments that the ATR/Mec1 kinase has a crucial role in the maintenance of functional replication forks independent of its well characterized function in the activation of Rad53 (see Table 2).

In human cells, it has been shown not only that the RecQ Bloom's Syndrome helicase (BLM) is a target of ATR but also that ATR-dependent phosphorylation of residue Thr99 of BLM is required for efficient replication fork restart and suppression of new origin firing after aphidicolin treatment [28••]. Thus BLM helicase, like its yeast homolog, has both an anti-recombinase function to resolve deleterious strand invasion, in addition to a role as an ATR target in protecting stalled or damaged forks. It is unclear whether this also involves reversing fork regression, or possibly maintaining polymerases for fork restart. Finally, there is accumulating evidence that ATR and another human RecQ helicase, Werner's syndrome helicase (WRN), work together to suppress fragile site instability [29]. The molecular mechanism remains unclear, but may involve recovery of stalled replication forks at fragile sites, which are prone to replisome pausing and/or strand breaks. However unlike yeast, where deletion of the RecQ helicase is synergistic with *mec1*-100 [23], siRNA-mediated downregulation of human ATR and WRN suggests that these two activities are epistatic for fragile site suppression in the presence of low doses of aphidicolin [29].

The replication fork component Mrc1 is a key target of Mec1/Ddc2 and is important both for S phase checkpoint activation and for the stabilization of replication forks even in an unperturbed S phase. Mrc1 is not an essential protein, but several studies have shown that the rate of replication fork movement decreases significantly in *mrc1*Δ mutants in the absence of exogenous damage [30–32]. It was proposed that loss of Mrc1 results in uncoupling of the processive Cdc45/MCM helicase from the leading strand Pol ε [33••,34••], causing ssDNA to accumulate. Recently, a study from Petermann *et al.* found that depletion of Claspin, the human Mrc1 homolog, similarly decreases replication fork progression [35]. Furthermore, Claspin-depletion and CHK1-depletion slow fork progression in an additive manner, suggesting that Claspin does not only mediate CHK1 activation but also stabilizes forks. A mutant form of the yeast protein in which all possible Mec1-target sites (SQ/TQ) have been changed to non-phosphorylatable AQ sites (mrc1AQ) has been used to distinguish the function of Mrc1 in replication from its role in Rad53 activation [31]. *mrc1AQ* mutants display normal rates of replication fork progression but fail to activate Rad53. This suggests that Mrc1 prevents fork collapse primarily through its presence at the fork and not through Rad53 activation [31].

Not only in the presence of HU but also in unperturbed cells, Mrc1 has been shown to interact via its N-terminal and C-terminal domains with the N-terminal and C-terminal domains of the Pol ε catalytic subunit [34••]. Phosphorylation, presumably by Mec1/Ddc2, disrupts the N-terminal interaction of Mrc1 with Pol ε, and this may be responsible for slowing replication fork progression in response to damage by binding and inhibiting other proteins at the fork, for example, the MCM helicase. Finally, the interaction of Mrc1's C-terminus with the C-terminal domain of Pol ε is not affected during the S phase checkpoint, possibly providing a mechanism through which Mrc1 stabilizes the fork, that is, by anchoring Pol ε to DNA during HU-treatment. Indeed, the binding of yeast Pol ε to HU-stalled replication forks is decreased in *mrc1*Δ mutants and this again is highly synergistic with loss of Sgs1 [34••,36].

S phase checkpoint activation: the downstream effector kinases CHK1 and Rad53

Activated ATR/Mec1 gives rise to a global checkpoint response by phosphorylation and activation of numerous targets, including downstream effector kinases Chk1 and Rad53 in yeast, and CHK1 and CHK2 in humans. In particular, the kinases Rad53 in *S. cerevisiae* and human CHK1 are thought to be crucial for the S phase-specific checkpoint response. Mediator proteins like Rad9/53BP1 or Mrc1/Claspin contribute to the activation of downstream kinases, and the amplified signal leads to a

response that includes cell cycle arrest, activation of DNA repair, transcription of damage inducible genes, and S phase-specific mechanisms to prevent replication fork collapse on MMS and to prevent late origins from firing [37,38]. Importantly, replication fork stabilization enables the resumption of DNA synthesis once the stress is removed since *de novo* assembly of the pre-replication complex required for replication fork firing is not possible during S phase [1•,34••]. Phosphorylation of CHK1 by ATR leads to its activation and rapid release from chromatin, allowing amplification of the checkpoint signal through phosphorylation of multiple downstream targets. One is CDC25, which inhibits CDK activation to prevent entry into mitosis [1•]. Histone H3 Thr11 (H3-T11) is another target of chromatin-bound CHK1 as recently reported by Shimada *et al.*, and decreased phosphorylation after DNA damage correlates with transcriptional repression. They suggested the following mechanism for CHK1 function in damage-induced transcriptional repression: upon DNA damage CHK1 dissociates from chromatin and H3-T11 becomes hypophosphorylated, which impairs the recruitment of the histone acetyltransferase GCN5 and therefore leads to decreased histone H3K9 acetylation and thus reduced transcription [39].

Rad53 is the main effector of the *S. cerevisiae* S phase checkpoint response and its phosphorylation is sufficient and necessary for checkpoint activation [40]. It has been suggested that the primary role of Rad53 in response to MMS, UV, and IR is to prevent Exo1-dependent replication fork breakdown since it has been shown that deletion of *EXO1*, which encodes an exouclease with a 5′-flap endonuclease activity, rescues the MMS, UV, and IR but not HU sensitivity of *rad53* mutants [27••]. This is especially interesting in connection with studies from three independent laboratories that report that Exo1 collaborates with Sgs1 to resect DNA DSBs [24••,25,26]. A two-step model for DSB end resection was proposed, in which Mre11-Rad50-Xrs2 (MRX) and Sae2 initiate 5′-strand resection and Sgs1 and/or Exo1 play a role in long-range resection to create ssDNA that serves as a substrate for Rad51 leading to homologous recombination. However, it is presently unknown how Exo1 contributes to replication fork integrity. It is conceivable that in the absence of Rad53, MMS treatment leads to excessive DNA unwinding due to MCM release or loss of replisome components and increased DNA breaks or DSB-like structures that are then processed by Exo1. Alternatively, the Lydall laboratory proposed that Rad53 may directly downregulate Exo1 activity [41], since Exo1 is phosphorylated in a Mec1/Rad53-dependent manner upon telomere uncapping or treatment with the break-inducing agents bleomycin or camptothecin. This suggests a negative feedback loop of Exo1 phosphorylation that limits ssDNA accumulation and checkpoint activation [41].

In addition to preventing replication fork collapse, another function of the S phase-specific checkpoint is to prevent late origin firing [42]. Indeed, DNA Pol α was shown to bind early but not late firing origins in wild-type cells treated with HU, while in the *rad53* mutant Pol α could be recovered efficiently at both [2,20]. A new study from Alvino *et al.* challenges the long standing model that Rad53 specifically inhibits late origin firing during a replication block [43•]. They suggest that late origin firing is not specifically impeded, but instead the entire S phase is delayed according to the speed of replication fork progression. Their model proposes that Rad53 downregulates the activity of the Cdc7/Dbf4 kinase that is required for replication initiation and thereby regulates origin firing by the rate of replication elongation while the order in which origins fire is maintained. Further research should reveal if this model is accurate, and if so how cells maintain the precise order of origin firing once the S phase checkpoint has been activated.

Replication fork restart after DNA damage

Although S phase checkpoint activation has been studied intensively, not much is known about how cells inactivate the checkpoint once the replication stress is removed ('recovery') or how they downregulate the checkpoint when DNA repair fails ('adaptation'). Curiously, Trenz *et al.* recently demonstrated that ATR-dependent phosphorylation of MCM2 Ser92 in response to replication stress induces the binding of Polo-like kinase 1 (PLX1) to chromatin leading to origin firing in *Xenopus* egg extracts [44••]. This function was shown to be independent of PLX1-mediated phosphorylation of Claspin, which facilitates adaptation and the onset of mitosis after prolonged ATR activation. A model was suggested in which ATR-mediated MCM2 phosphorylation and subsequent PLX1 recruitment acts locally to activate dormant origins neighboring stalled replication forks This mechanism disengages the global ATR/CHK1-dependent suppression of origin firing to complete replication in areas with replication fork stalling. This is consistent with the observation that PLX1-depletion leads to DSB accumulation when forks stall after aphidicolin treatment.

Consistent with Rad53 as the main S phase checkpoint effector, recovery and adaptation correlate with the disappearance of phosphorylated Rad53, largely due to the function of Ser/Thr phosphatases [40]. It has been proposed that Rad53 regulates fork restart and late origin firing by independent mechanisms. It is likely that different phosphatases could recognize specific Rad53 phosphorylation patterns and therefore control distinct checkpoint responses [45•,46]. New data suggest a mechanistic link between Rad53 deactivation by the Psy2-Pph3 phosphatase and replication fork restart after MMS treatment [45•,47]. Szyjka *et al.* also reported that MMS-treated *pph3Δ* cells display reduced fork progression and decreased Rad53 phosphorylation levels.

In a *pph3Δ* background, deletion of Ptc2, another Rad53 phosphatase that was previously shown to be necessary for DSB adaptation [48], results in a block of replication fork progression and lethality in MMS, which can be rescued by overexpressing a dominant negative kinase-dead *rad53* allele. Interestingly, neither Pph3, Ptc2 nor Ptc3 deletion alone or in combination is required for Rad53 deactivation after HU-induced replication stress, suggesting that another not yet identified phosphatase might be responsible [45•,46]. Thus, different phosphatases seem to be necessary to recognize and dephosphorylate specific Rad53 phosphorylation patterns that correspond to distinct genotoxic insults as well as to differential mechanisms to lead to fork restart or late origin firing. Further research on how phosphatases regulate Rad53 inactivation after replication stress is needed to help understand the complex mechanism of S phase checkpoint recovery and adaptation.

Interestingly, recent data suggest that the evolutionary conserved Ino80 chromatin remodeling complex is not only important for DSB repair but may also play a crucial role to restart HU-stalled forks [49,50,51••]. Ino80 is recruited to DSBs by Mec1/Tel1-dependent phosphorylation of histone H2A (γ-H2A) [49]. Shimada *et al*. observed enhanced Ino80 binding at stalled forks and at late firing origins after HU-treatment, and consistently replication fork collapse and increased rates of DSBs when important Ino80 subunits are deleted [51••]. The model they proposed hypothesizes that Ino80 promotes recovery by mobilizing histones ahead of the fork to mediate polymerase resumption. Furthermore, the Ino80 subunit Ies4 is phosphorylated by Mec1/Tel1 after DNA damage and surprisingly genetic data suggest redundant functions for phosphorylated Ies4 and the S phase-specific checkpoint regulator Tof1 [52]. It is of great interest to understand how exactly this chromatin remodeler contributes to replication fork restart.

Conclusion

The conserved checkpoint kinase ATR or Mec1 serves multiple roles in the cellular response to endogenous replication stress and DNA damage. It functions directly at stalled forks (i.e. stabilizing replicative polymerases) and is also crucial for activation of a checkpoint response during S phase. The combination of strains mutated in both replication machinery components and checkpoint proteins demonstrate that the roles of ATR/Mec1 are complex. A major enigma is the mechanism by which ATR/Mec1 contributes to polymerase stabilization to allow resumption of replication and successful completion of DNA synthesis once the insult has been removed. Which target(s) of ATR/Mec1 stabilize replicative polymerases at the fork? Is this sufficient to suppress fork-associated recombination events? Although many potential targets of ATR/Mec1 at the fork have emerged, including RecQ helicases, Claspin/Mrc1, and RPA, further work is required to answer these questions.

Acknowledgements

The Gasser laboratory is supported by grants from the EU and the Swiss National Science Foundation, as well as the Novartis Research Foundation, and a HFSP fellowship to B Pike. We thank Kenji Shimada and Stephanie Küng for helpful comments.

References and recommended reading

Papers of particular interest published within the period of review have been highlighted as:

- • of special interest
- •• of outstanding interest

1. • Cimprich KA, Cortez D: **ATR: an essential regulator of genome integrity**. *Nat Rev Mol Cell Biol* 2008, **9**:616-627.
Gives a comprehensive overview on ATR in DNA-damage signaling in comparison to ATM.

2. Aparicio OM, Stout AM, Bell SP: **Differential assembly of Cdc45p and DNA polymerases at early and late origins of DNA replication**. *Proc Natl Acad Sci U S A* 1999, **96**:9130-9135.

3. Paulsen RD, Cimprich KA: **The ATR pathway: fine-tuning the fork**. *DNA Repair (Amst)* 2007, **6**:953-966.

4. Zou L, Elledge SJ: **Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes**. *Science* 2003, **300**:1542-1548.

5. Melo JA, Cohen J, Toczyski DP: **Two checkpoint complexes are independently recruited to sites of DNA damage *in vivo***. *Genes Dev* 2001, **15**:2809-2821.

6. Rouse J, Jackson SP: **Lcd1p recruits Mec1p to DNA lesions *in vitro* and *in vivo***. *Mol Cell* 2002, **9**:857-869.

7. Michael WM, Ott R, Fanning E, Newport J: **Activation of the DNA replication checkpoint through RNA synthesis by primase**. *Science* 2000, **289**:2133-2137.

8. Byun TS, Pacek M, Yee MC, Walter JC, Cimprich KA: **Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint**. *Genes Dev* 2005, **19**:1040-1052.

9. MacDougall CA, Byun TS, Van C, Yee MC, Cimprich KA: **The structural determinants of checkpoint activation**. *Genes Dev* 2007, **21**:898-903.

10. Majka J, Niedziela-Majka A, Burgers PM: **The checkpoint clamp activates Mec1 kinase during initiation of the DNA damage checkpoint**. *Mol Cell* 2006, **24**:891-901.

11. Medhurst AL, Warmerdam DO, Akerman I, Verwayen EH, Kanaar R, Smits VA, Lakin ND: **ATR and Rad17 collaborate in modulating Rad9 localisation at sites of DNA damage**. *J Cell Sci* 2008, **121**:3933-3940.

12. Delacroix S, Wagner JM, Kobayashi M, Yamamoto K, Karnitz LM: **The Rad9-Hus1-Rad1 (9-1-1) clamp activates checkpoint signaling via TopBP1**. *Genes Dev* 2007, **21**:1472-1477.

13. Lee J, Kumagai A, Dunphy WG: **The Rad9-Hus1-Rad1 checkpoint clamp regulates interaction of TopBP1 with ATR**. *J Biol Chem* 2007, **282**:28036-28044.

14. • Bonilla CY, Melo JA, Toczyski DP: **Colocalization of sensors is sufficient to activate the DNA damage checkpoint in the absence of damage**. *Mol Cell* 2008, **30**:267-276.
By using an artificial tethering system it was demonstrated that colocalization of Mec1-Ddc2 and 9-1-1 is sufficient to activate a checkpoint response in absence of DNA damage.

15. Navadgi-Patil VM, Burgers PM: **Yeast DNA replication protein Dpb11 activates the Mec1/ATR checkpoint kinase**. *J Biol Chem* 2008, **283**:35853-35859.

16. Mordes DA, Nam EA, Cortez D: **Dpb11 activates the**
• **Mec1-Ddc2 complex**. *Proc Natl Acad Sci U S A* 2008, **105**:18730-18734.
References [15] and [16•] report that *S. cerevisiae* Dbp11 activates Mec1-Ddc2 *in vitro* and therefore is a functional ortholog of human TopBP1.

17. Masumoto H, Sugino A, Araki H: **Dpb11 controls the association between DNA polymerases alpha and epsilon and the autonomously replicating sequence region of budding yeast**. *Mol Cell Biol* 2000, **20**:2809-2817.

18. Zegerman P, Diffley JF: **Phosphorylation of Sld2 and Sld3 by cyclin-dependent kinases promotes DNA replication in budding yeast**. *Nature* 2007, **445**:281-285.

19. Tanaka S, Umemori T, Hirai K, Muramatsu S, Kamimura Y, Araki H: **CDK-dependent phosphorylation of Sld2 and Sld3 initiates DNA replication in budding yeast**. *Nature* 2007, **445**:328-332.

20. Cobb JA, Bjergbaek L, Shimada K, Frei C, Gasser SM: **DNA polymerase stabilization at stalled replication forks requires Mec1 and the RecQ helicase Sgs1**. *EMBO J* 2003, **22**:4325-4336.

21. Myung K, Datta A, Chen C, Kolodner RD: **SGS1, the Saccharomyces cerevisiae homologue of BLM and WRN, suppresses genome instability and homeologous recombination**. *Nat Genet* 2001, **27**:113-116.

22. Myung K, Kolodner RD: **Suppression of genome instability by redundant S-phase checkpoint pathways in *Saccharomyces cerevisiae***. *Proc Natl Acad Sci U S A* 2002, **99**:4500-4507.

23. Cobb JA, Schleker T, Rojas V, Bjergbaek L, Tercero JA, Gasser SM: **Replisome instability, fork collapse, and gross chromosomal rearrangements arise synergistically from Mec1 kinase and RecQ helicase mutations**. *Genes Dev* 2005, **19**:3055-3069.

24. Zhu Z, Chung WH, Shim EY, Lee SE, Ira G: **Sgs1 helicase and two**
•• **nucleases Dna2 and Exo1 resect DNA double-strand break ends**. *Cell* 2008, **134**:981-994.
Report together with references [25] and [26] that the RecQ helicase Sgs1 collaborates with Exo1 in DSB end resection. A two-step model was proposed in which MRX together with Sae2 initiates 5′ degradation, which is further processed by Exo1/Sgs1 to generate long ssDNA tracts that serve Rad51 as a substrate for homologous recombination.

25. Mimitou EP, Symington LS: **Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing**. *Nature* 2008, **455**:770-774.

26. Gravel S, Chapman JR, Magill C, Jackson SP: **DNA helicases Sgs1 and BLM promote DNA double-strand break resection**. *Genes Dev* 2008, **22**:2767-2772.

27. Segurado M, Diffley JF: **Separate roles for the DNA damage**
•• **checkpoint protein kinases in stabilizing DNA replication forks**. *Genes Dev* 2008, **22**:1816-1827.
This work provides further evidence that Mec1 has separable functions in replication fork stabilization from Rad53. The *rad53Δ*, but not *mec1Δ*, lethality on MMS can be rescued by deletion of the 5′-3′exonuclease and flap endonuclease Exo1.

28. Davies SL, North PS, Hickson ID: **Role for BLM in replication-**
•• **fork restart and suppression of origin firing after replicative stress**. *Nat Struct Mol Biol* 2007, **14**:677-679.
Using a dual-labeling chromosome fiber spreading technique it was shown that ATR-catalyzed BLM phosphorylation on T99 is important for replication fork restart after HU or aphidicolin treatment and for suppression of new origin firing.

29. Pirzio LM, Pichierri P, Bignami M, Franchitto A: **Werner syndrome helicase activity is essential in maintaining fragile site stability**. *J Cell Biol* 2008, **180**:305-314.

30. Hodgson B, Calzada A, Labib K: **Mrc1 and Tof1 regulate DNA replication forks in different ways during normal S phase**. *Mol Biol Cell* 2007, **18**:3894-3902.

31. Szyjka SJ, Viggiani CJ, Aparicio OM: **Mrc1 is required for normal progression of replication forks throughout chromatin in *S. cerevisiae***. *Mol Cell* 2005, **19**:691-697.

32. Tourriere H, Versini G, Cordon-Preciado V, Alabert C, Pasero P: **Mrc1 and Tof1 promote replication fork progression and recovery independently of Rad53**. *Mol Cell* 2005, **19**:699-706.

33. Pursell ZF, Isoz I, Lundstrom EB, Johansson E, Kunkel TA: **Yeast**
•• **DNA polymerase epsilon participates in leading-strand DNA replication**. *Science* 2007, **317**:127-130.
It is shown for the first time that polymerase (in *S. cerevisiae* is involved in leading strand DNA synthesis.

34. Lou H, Komata M, Katou Y, Guan Z, Reis CC, Budd M, Shirahige K,
•• Campbell JL: **Mrc1 and DNA polymerase epsilon function together in linking DNA replication and the S phase checkpoint**. *Mol Cell* 2008, **32**:106-117.
Reports that Mrc1 interacts with the N-terminal and C-terminal half of polymerase ε and that phosphorylation of Mrc1 during S phase checkpoint activation prevents its interaction with N-terminal half of polymerase ε. A model was suggested in which Mrc1 modulates coupling of DNA unwinding and polymerization during replication.

35. Petermann E, Helleday T, Caldecott KW: **Claspin promotes normal replication fork rates in human cells**. *Mol Biol Cell* 2008, **19**:2373-2378.

36. Bjergbaek L, Cobb JA, Tsai-Pflugfelder M, Gasser SM: **Mechanistically distinct roles for Sgs1p in checkpoint activation and replication fork maintenance**. *EMBO J* 2005, **24**:405-417.

37. Zhang W, Durocher D: **Dun1 counts on rad53 to be turned on**. *Mol Cell* 2008, **31**:1-2.

38. Matsuoka S, Ballif BA, Smogorzewska A, McDonald ER 3rd, Hurov KE, Luo J, Bakalarski CE, Zhao Z, Solimini N, Lerenthal Y *et al.*: **ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage**. *Science* 2007, **316**:1160-1166.

39. Shimada M, Niida H, Zineldeen DH, Tagami H, Tanaka M, Saito H, Nakanishi M: **Chk1 is a histone H3 threonine 11 kinase that regulates DNA damage-induced transcriptional repression**. *Cell* 2008, **132**:221-232.

40. Heideker J, Lis ET, Romesberg FE: **Phosphatases, DNA damage checkpoints and checkpoint deactivation**. *Cell Cycle* 2007, **6**:3058-3064.

41. Morin I, Ngo HP, Greenall A, Zubko MK, Morrice N, Lydall D: **Checkpoint-dependent phosphorylation of Exo1 modulates the DNA damage response**. *EMBO J* 2008, **27**:2400-2410.

42. Branzei D, Foiani M: **The Rad53 signal transduction pathway: replication fork stabilization, DNA repair, and adaptation**. *Exp Cell Res* 2006, **312**:2654-2659.

43. Alvino GM, Collingwood D, Murphy JM, Delrow J, Brewer BJ,
• Raghuraman MK: **Replication in hydroxyurea: it's a matter of time**. *Mol Cell Biol* 2007, **27**:6396-6406.
This study questions the current model that Rad53 specifically inhibits late origin firing after replication stress, but suggests a delay of the entire S phase according to the rate of replication progression.

44. Trenz K, Errico A, Costanzo V: **Plx1 is required for chromosomal**
•• **DNA replication under stressful conditions**. *EMBO J* 2008, **27**:876-885.
This study shows that ATR-dependent phosphorylation of MCM2 recruits Polo-like kinase to stalled replication forks leading to origin firing and completion of replication.

45. Szyjka SJ, Aparicio JG, Viggiani CJ, Knott S, Xu W, Tavare S,
• Aparicio OM: **Rad53 regulates replication fork restart after DNA damage in *Saccharomyces cerevisiae***. *Genes Dev* 2008, **22**:1906-1920.
This paper provides a mechanistic link between Rad53 dephosphorylation and replication fork restart. Cells lacking the the Psy2-Pph3 phosphatase show defects in Rad53 dephosphorylation and fork restart after MMS treatment, while deactivation of Rad53 by overerxpression of a dominant-negative kinase-dead variant of Rad53 is sufficient to allow fork restart during recovery.

46. Travesa A, Duch A, Quintana DG: **Distinct phosphatases mediate the deactivation of the DNA damage checkpoint kinase Rad53**. *J Biol Chem* 2008, **283**:17123-17130.

47. O'Neill BM, Szyjka SJ, Lis ET, Bailey AO, Yates JR 3rd, Aparicio OM, Romesberg FE: **Pph3-Psy2 is a phosphatase complex required for Rad53 dephosphorylation and replication fork restart during recovery from DNA damage**. *Proc Natl Acad Sci U S A* 2007, **104**:9290-9295.

48. Leroy C, Lee SE, Vaze MB, Ochsenbien F, Guerois R, Haber JE, Marsolier-Kergoat MC: **PP2C phosphatases Ptc2 and Ptc3 are required for DNA checkpoint inactivation after a double-strand break**. *Mol Cell* 2003, **11**:827-835.

49. van Attikum H, Fritsch O, Gasser SM: **Distinct roles for SWR1 and INO80 chromatin remodeling complexes at chromosomal double-strand breaks**. *EMBO J* 2007, **26**:4113-4125.

50. Papamichos-Chronakis M, Peterson CL: **The Ino80 chromatin-remodeling enzyme regulates replisome function and stability**. *Nat Struct Mol Biol* 2008, **15**:338-345.

51. •• Shimada K, Oma Y, Schleker T, Kugou K, Ohta K, Harata M, Gasser SM: **Ino80 chromatin remodeling complex promotes recovery of stalled replication forks**. *Curr Biol* 2008, **18**:566-575.
This study reports that Ino80 is enriched at stalled forks and shows, together with reference [50], that Ino80 stabilizes stalled replication forks and facilitates fork restart.

52. Morrison AJ, Kim JA, Person MD, Highland J, Xiao J, Wehr TS, Hensley S, Bao Y, Shen J, Collins SR *et al.*: **Mec1/Tel1 phosphorylation of the INO80 chromatin remodeling complex influences DNA damage checkpoint responses**. *Cell* 2007, **130**:499-511.

1.6. Scope of the thesis

S phase is the period of the cell cycle, in which cells duplicate their entire genomic information. A vast amount of complex DNA structures needs to be unpacked, unwound and replicated. During these processes the genome is very vulnerable. Fragile DNA structures are formed which, if not stabilized, result in replication fork collapse and the formation of DNA double strand breaks (DSB). DSB have been shown to be a major reason for gross chromosomal rearrangements and genomic instability, a hallmark of cancer in higher eukaryotes.

Cobb and colleagues have demonstrated that the RecQ helicase Sgs1 and the checkpoint kinase Mec1-Ddc2 contribute synergistically to DNA polymerase stability at stalled replication forks. Interestingly, both pathways for stabilizing the replication fork seemed to converge on replication protein A, (RPA) (Cobb, Schleker et al. 2005). Indeed, RPA is both a target of Mec1 and acts to recruit it to a stalled or damaged replication fork and also to interact with Sgs1 (Zou and Elledge 2003), (Cobb, Bjergbaek et al. 2003).

The goal of this thesis was to understand the molecular mechanism of how Sgs1, RPA and Mec1 stabilize the replisome at a stalled fork and therefore prevent fork collapse. Therefore I aimed to specifically disrupt the interaction site between RPA and Sgs1 *in vivo* and monitor the effect on polymerase stability and intra-S phase checkpoint activation at stalled replication forks. To achieve this, we intended to map the interaction site between Sgs1 and RPA by two hybrid analysis, mutate the binding site and introduce the mutants into their endogenous genomic sites. On one hand, I created a new mutation, *sgs1*-r1, which completely abolished Rpa70 interaction in two hybrid analyses and also affected RPA binding *in vivo*. This mutant was analyzed for its ability to stabilize DNA pol α at stalled replication forks in response to replication stress. Furthermore, we observed an epistatic relationship of *sgs1*-r1 with *mec1*-100. Therefore, we

asked whether Mec1 targets the RPA-interaction site on Sgs1 *in vitro* and investigated the effect on Rad53 activation.

On the other hand, we observed that *rfa1*-t11 affects the interaction with Sgs1 in two hybrid studies. This Rpa70 mutant carries a charge-reversal mutation in the basic cleft of the N-OB fold. *rfa1*-t11 cells were previously described as being proficient for replication under normal conditions, However, we observed that *rfa1*-t11 mutants are HU-sensitive suggesting that they are defective for replication fork stabilization. Unexpectedly, this defect was more pronounced than for *sgs1*Δ cells and highly synergistic with *mec1*-100. Using different techniques such as DNA pol ChIP, DNA combing and 2D gels, we have investigated the effect of *rfa1*-t11 and *rfa1*-t11 *mec1*-100 mutants on replisome stability in response to the replication fork inhibitor HU.

2. The Rpa70 interaction domain of Sgs1 contributes to both replication checkpoint activation and fork stability

A. M. Friedel, B. L. Pike, K. Shimada, (Y. Moriyama, M. Vogel, P. Amsler, N. Thomae, S. Rubin), S. M. Gasser

2.1. Abstract

During S phase, when cells are unwinding and replicating an enormous amount of DNA, the genome is exceptionally sensitive towards any source of DNA damage. Replication stress in particular, bears the high risk of creating double strand breaks, a major thread for genome stability and one main reason for cancer development in higher eukaryotes. Thus, the intra-S phase checkpoint is activated in response to replication stress stabilizing arrested replication forks to prevent potential fork collapse and to regulate cell cycle progression, late origin firing and DNA repair. Previously, the checkpoint kinase Mec1/ATR and the RecQ helicase Sgs1 have been implicated in polymerase stabilization at replication forks stalled by hydroxyurea (HU). It was suggested that both pathways converge on replication protein A (RPA). Indeed, disruption of the RPA-Sgs1 interaction in the *sgs1*-r1 mutant, leads to partial loss of DNA pol α at stalled forks. However, when combined with the S-phase specific *mec1*-100 mutant, *sgs1*-r1 behaves epistatic to *mec1*-100 on HU, suggesting that it acts in the same pathway for replication fork stability. Here we demonstrate that both the RPA-interaction and the helicase function of Sgs1 are necessary to stabilize DNA pol α at HU-arrested replication forks. Furthermore, we show that the same Sgs1 region that binds RPA is also a Mec1/ATR target *in vitro* and is important for Rad53 activation after exposure to HU.

2.2. Introduction

The accurate replication of DNA and its segregation into daughter cells is aided by checkpoint pathways, which slow the cell cycle and activate repair in response to damage. During DNA replication, eukaryotic cells are particularly sensitive to intrinsic sources of damage, such as fork collapse, as well as extrinsic DNA damaging agents. When forks do stall, the checkpoint response must stabilize the replisome components to prevent complete fork collapse.

The checkpoint kinase Mec1-Ddc2 in *S. cerevisiae*, or ATR-ATRIP in humans, plays a critical role in maintaining genome integrity by stabilizing the replisome (for review (Friedel, Pike et al. 2009), (Cimprich and Cortez 2008)). Fork stalling leads to a functional uncoupling of the replicative helicases from the DNA polymerases and the formation of long stretches of single-stranded DNA (ssDNA), which are subsequently coated by replication protein A (RPA) (Aparicio, Stout et al. 1999). It is currently thought that it is these long stretches of RPA coated ssDNA that serve as the signal, conserved from yeast to man, to recruit Mec1-Ddc2. In both budding yeast and mammals, RPA was shown to recruit Mec1/ATR to a stalled or damaged replication fork via its co-factor, Ddc2/ATRIP (Melo, Cohen et al. 2001), (Rouse and Jackson 2002). Furthermore, RPA is itself a target of Mec1/ATR phosphorylation upon checkpoint activation (Zou and Elledge 2003).

How exactly Mec1/ATR gets activated after recruitment remains to be investigated, but it requires additional proteins such as the 9-1-1 checkpoint clamp and Dbp11/TOPBP1 (Majka, Niedziela-Majka et al. 2006), (Navadgi-Patil and Burgers 2008), (Mordes, Glick et al. 2008), (Mordes, Nam et al. 2008). After its activation, Mec1/ATR interacts locally with fork components to stabilize polymerases and activate the intra-S phase checkpoint. This checkpoint is initiated by phosphorylation of numerous targets, including the downstream

effector kinases Chk1 and Rad53/CHK2. The kinases Rad53 in *S. cerevisiae* and CHK1 in man are central to modulating downstream effects of the checkpoint response such as origin firing, DNA repair, fork restart and cell cycle progression (Tourriere and Pasero 2007).

RecQ helicases have also been shown to be important for replication fork stabilization and efficient fork restart in response to hydroxyurea (HU) or aphidicolin (Cobb, Schleker et al. 2005), (Davies, North et al. 2007) , (Pirzio, Pichierri et al. 2008), (Bachrati and Hickson 2008). Sgs1 is the single RecQ homologue in *S. cerevisiae* and its loss destabilizes polymerases stalled by the replication fork inhibitor HU and reduces fork recovery. Deletion of Sgs1 leads to hyper-recombination (Watt, Hickson et al. 1996), spontaneous gross chromosomal rearrangements (GCR) (Myung and Kolodner 2002) and sensitivity to genotoxic agents like HU and methylmethane sulphonate (MMS). Importantly, partial loss of DNA polymerase α (DNA pol α) and DNA polymerase ε (DNA pol ε) was observed, when replication forks were stalled by HU in an *sgs1*Δ strain (Cobb, Bjergbaek et al. 2003), (Cobb, Schleker et al. 2005). The importance of RecQ helicases in maintaining genome stability is demonstrated by the fact that mutations in three of the five known human RecQ genes (BLM, WRN, and RECQ4) are associated with genome instability syndromes (Bloom's, Werner's, and Rothmund-Thompson syndromes) which show a predisposition to cancer.

How Sgs1 acts at the fork to stabilize polymerases in order to restart replication is as yet unclear. No direct interaction between Sgs1 and DNA pol α/primase or DNA pol ε has been reported, but it was shown that Sgs1 interacts with RPA and there is evidence that the helicase activity of Sgs1 is important for DNA pol ε stabilization at the fork (ref Cobb 2003) This has led to the hypothesis that Sgs1 might indirectly stabilize polymerases via its interaction with RPA.

Mec1-Ddc2 also acts at stalled forks to stabilize DNA polymerases. Remarkably, this Mec1-Ddc2 function is largely separable from its role in activating the

downstream checkpoint kinase Rad53. Using a Mec1 mutant, *mec1*-100, which shows compromised intra-S phase checkpoint activation, but normal G2/M checkpoint activation, Cobb *et. al,* observed partial loss of DNA pol α and DNA pol ε from at forks stalled on HU. When the *sgs1*Δ mutant was combined with *mec1*-100, they noticed complete loss of polymerases and RPA from the stalled fork, replication fork collapse and a synergistic increase of GCR (Cobb, Schleker et al. 2005). In contrast, deletion of Rad53 kinase was neither additive with *sgs1*Δ nor did the *rad53*-11 or *rad51*Δ mutations have an effect on polymerase stability. However, significant displacement of the MCM helicases from the stalled fork was observed (Cobb, Bjergbaek et al. 2003; Cobb, Schleker et al. 2005). Since Sgs1 and Mec1 contribute synergistically to replication fork stability and both Mec1-Ddc2 and Sgs1 interact with RPA, it was proposed that both pathways for replisome stabilization might converge on RPA.

To test this model, we examined the role of the Sgs1-RPA interaction in stabilizing stalled replication forks. To better understand the interaction between Sgs1 and RPA, we mapped the regions of Sgs1 required to bind RPA and identified an acidic region N-terminal of the helicase domain that is crucial for binding. Using a Sgs1 mutant, *sgs1*-r1 that lacks this RPA interaction site we show that the Sgs1-RPA interaction together with the helicase activity of Sgs1 is important for stabilizing DNA pol α/primase at the HU-stalled replication fork. Interestingly, in contrast to *sgs1*Δ, the relationship between *sgs1*-r1 and *mec1*-100 mutations is epistatic, placing *sgs1*-r1 downstream of *mec1*-100. Furthermore, we show that Mec1-Ddc2 phosphorylates Sgs1 at the RPA-interaction site *in vitro* and that the same Sgs1 region is important for Rad53 interaction and subsequent activation. Our results provide further insights into how Sgs1 maintains genomic integrity by stabilizing polymerases and suggest a model for how the checkpoint kinase Mec1-Ddc2 modulates Sgs1-RPA interaction after replication fork stalling in order to activate the intra-S phase checkpoint response.

2.3. Materials and methods

2.3.1. Yeast strains and plasmids

S. cerevisiae strains (see Table 3) were derived from W303-1A (*MAT***a** *ade2-1 ura3-1his3-11,15 trp1-1 leu2-3,112 can1-100*). If not stated differently, all strains were cultured at 30 °C in YPAD media. The *sgs1*-r1 allele was generated using pop-in/pop-out mutagenesis as previously described (Tam, Pike et al. 2007). For two hybrid analyses, fragments of Sgs1, Rpa70 and Rpa32 were fused in frame to the B42 activator domain in the pJG46 or the lexA DNA binding domain in the pGAL-lexA vector (Bjergbaek, Cobb et al. 2005).

2.3.2. Survival and drop assays

For liquid survival assays, overnight cultures were diluted to OD_{600} = 0.15 and grown for 3 h, then synchronized with α-factor in G1 and released into 0.2 M HU containing YPAD. After the indicated time points relevant dilutions were plated onto fresh YPAD plates and colonies were counted after 3 to 4 days. Survival is defined as the fraction of indicated doses compared to the untreated control (0h) normalized to the survival of WT cells for each time point. For drop tests, overnight cultures were diluted to a starting density of OD_{600} = 0.5 and 2 µl drops with 4 x 10-fold dilutions were plated on YPD or the appropriate selective medium containing various concentrations of MMS or HU as indicated.

2.3.3. Two-hybrid interaction

Two-hybrid experiments were performed as described (Bjergbaek, Cobb et al. 2005). EGY191 cells (GA-1211) containing the lacZ reporter pSH1834, the bait and the prey were glucose depleted, then 2% galactose added to the exponentially growing culture to induce expression of the fusion proteins. The

quantitative β-galactosidase assay for permeabilised cells was used to detect protein-protein interactions (Adams et al, 1997). Four independent transformants were analysed in at least 2 independent experiments. Expression of the fusion proteins was confirmed by western blot analysis (data not shown) (results). β-galactosidase units are defined as $OD_{420}/(OD_{600}$*dilution*time(min)).

2.3.4. Co-Immunoprecipitation

Yeast strains GA-1759 (Myc-Sgs1, HA-Rpa1) and GA-5316 (Myc-sgs1-r1, HA-Rpa1) were grown to 0.5 x 10^6 cells/ml, arrested with α-factor in G1 and released into YPAD to enter S-phase. After 20-30 min cells were collected by centrifugation, the pellet was resuspended in lysis buffer without detergent (50 mM HEPES pH 8.0, 140 mM NaCl, 1 mM EDTA, with a final concentration of protease inhibitors: 0.8 mM PMSF, 300 mg/ml benzamidine, 1 mg/ml pepstatin, 2 mg/ml antipain, 0.5 mg/ml leupeptin, 100 mg/ml TPCK and 50 mg/ml TLCK), and snap frozen in liquid nitrogen. After bead milling the frozen cell powder was resuspended in an equal volume of cold lysis buffer containing 0.4% Triton X-100 on ice. After centrifugation, the supernatant was incubated with either anti-Myc (9E10)- or anti-HA (Santa Cruz,F-7)-coupled dynabeads (M-450, Dynal A.S., Norway) for 2 h at 4 °C. BSA-coupled dynabeads were used as a negative control. The dynabeads were washed twice with lysis buffer and once with wash buffer (10 mM Tris pH 8.0, 250 mM NaCl, 1 mM EDTA, 1% NP-40, 2.5 mM deoxycholate and indicated protease inhibitors). Bound proteins were eluted by 60 µl SDS sample buffer for 5 min at 95 °C and analysed by SDS-PAGE and western blotting.

2.3.5. ChIP analysis

ChIP experiments were performed as described (Cobb, Bjergbaek et al. 2003), (Cobb, Schleker et al. 2005). Cells were synchronized with α-factor in G1 and

released into 0.2 M HU-containing media at 30 °C and fixed with 1% formaldehyde at the indicated time points. Monoclonal anti-HA (F-7, Santa Cruz) was used to precipitate HA-tagged DNA pol α, and anti-Myc (9E10) to precipitate myc-tagged Ddc2. BSA-saturated dynabeads were coupled to the monoclonal antibodies and incubated with the cell extract for 2 h at 4 °C. BSA-coupled dynabeads without antibody were used as a background control. Amplified DNA regions were quantified by real-time PCR using the Applied Biosystems 7500 Fast Real-time PCR System and software. Sequences for the primers/probes that amplify regions in the *S. cerevisiae* genome correspond to ARS607, a site 14 kb away from ARS607 and ARS501 and are available upon request. The data for each strain are averaged over three independent experiments with real-time PCR performed in duplicate (error bars indicate standard error of the mean). Absolute fold enrichment at ARS607 or ARS501 was calculated as follows. For each time point the signal from the antibody-coupled dynabeads was divided by the signal from the BSA-coated dynabeads, after both signals were first normalized to the signal from the input DNA. Finally, the relative enrichment for ARS607 or ARS501 was obtained by normalizing the absolute enrichment at ARS607 or ARS501 for each timepoint to the absolute enrichment at a locus 14 kb away from ARS607.

2.3.6. Mec1 Immunoprecipitation and kinase assay

Exponentially growing cultures of GA-1456 (Mec1-myc) and GA-426 (non-tagged) were exposed to 0.1% MMS for 1 h at 30 °C. The pellet was lysed by adding 0.4 ml lysis buffer (1 x PBS, 10% glycerol, 0.5% Triton X-100, 5 mM EDTA, 5 mM EGTA, 50 mM NaF, 10 mM ß-glycerophosphate, 5 mM sodium pyrophosphate, 2 mM PMSF, 300 mg/ml benzamidine, 1 mg/ml pepstatin, 2 mg/ml antipain, 0.5 mg/ml leupeptin, 100 mg/ml TPCK and 50 mg/ml TLCK), silica beads and bead beating (3 x 1min, full speed at 4 °C). After centrifugation (full speed, 10 min at 4 °C) per IP 40 µl Protein G Sepharose slurry coupled to 20

µg monoclonal anti-Myc antibody was incubated with the supernatant for 2 h at 4 °C. The beads were washed once with TBS-T and resuspended in 60 µl 1x Kinase buffer (2 mM Tris pH 7.4, 1 mM magnesium acetate, 0.01 mM dithiothreitol, 0.005% (w/v) Tween 20). For the kinase assay and western analysis the beads containing the immunoprecipitates were separated into equal portions. The kinase assay was carried out as described in (Pike, Yongkiettrakul et al. 2004). The reaction was initiated by mixing the beads with 20 ng/µl substrate (Sgs1(404-604aa), GST, PHAS-1) and 20 µl of kinase buffer containing 4 mM manganese chloride, 50 µM ATP and 1 µCi of [γ-32P]ATP. Reactions were terminated after 30 min by boiling in SDS sample buffer. Eluted proteins were analyzed by SDS-PAGE, coomassie staining and audioradiography.

2.3.7. Rad53 phosphorylation

To monitor Rad53 activation by Western blot, exponentially growing cells (GA-1981, GA-5321, GA-5076, GA-5324, GA-1761, GA-2056) were synchronized in G1 with α-factor and released for 1 h into 0.2 M HU containing media at 30 °C. For Western Blot analysis, lysates were prepared using silica beads and urea buffer and subjected to 7.5% SDS-PAGE, transferred onto PVDF membrane and detected with anti-Rad53 antibody (Pike, Yongkiettrakul et al. 2003).

2.3.8. Protein purification

The Rad53(22-162) and Rpa70(1-133 and Rpa70(3-133) constructs were amplified and inserted into a pET15 (pNT62e) derived vector, containing a TEV protease cleavable His_6-tag. After overexpression in *E. coli* BL21 strain, cells were lysed by sonication. The Rad53 and Rpa70 constructs were purified by metal chelate affinity (His-Select Nickel Affinity Gel, Sigma-Aldrich), cation-exchange (Resource 15 S, GE Healthcare), and gel-filtration chromatography

(Superdex S-200, GE Healthcare). The His-tag was removed by proteolytic digestion with 1% TEV after the metal chelate affinity step.

Sgs1 constructs (404-485, 404-560) were amplified and inserted into a pET15 (pNT23e) derived vector, containing a thrombin protease cleavable His_6-tag. After overexpression in *E. coli* BL21 strain, cells were lysed by sonication. Sgs1 constructs were purified by metal chelate affinity (His-Select Nickel Affinity Gel, Sigma-Aldrich), anion-exchange (Resource 15 Q, GE Healthcare), and gel-filtration chromatography (Superdex S-200, GE Healthcare). His-tag was removed by proteolytic digestion with 1% thrombin after the metal chelate affinity step.

2.3.9. ITC

ITC experiments on Sgs1(404-485), Sgs1(404-560) and Rpa70(3-133) were conducted with a MicroCal VP-ITC calorimeter. Proteins were dialyzed overnight prior to the assay in a buffer containing 100 mM NaCl, 10 mM 2-mercaptoethanol, and 50 mM Hepes (pH 7.5). The concentrations of Sgs1(404-485) and Sgs1(404-560) were 0.9 mM and 0.4 mM respectively. The concentration of Rpa70(3-133) ranged from 35 µM to 73 µM. Data were analyzed with the Origin calorimetry software package assuming a one site-binding model. Experiments were repeated twice, and the reported error in Figure 10B-C is the standard deviation of each set of measurements.

For ITC experiments on Sgs1(446-456), and Rad53(22-162), the Sgs1 peptide DDLDPTQDTDY and the corresponding phosphopeptide DDLDP**p**TQDTDY were dissolved in 10mM sodium phosphate buffer (pH 6.9). Prior to the assay, Rad53(22-162) and Sgs1 peptides were dialyzed overnight in a buffer containing 50mM sodium phosphate (pH 7.4). ITC experiments were conducted with a MicroCal VP-ITC calorimeter. The concentrations of Sgs1 peptides and Rad53 (22-162) were 1250 µM and 75 µM, respectively. Data were analyzed with the Origin calorimetry software package assuming a one site-binding model.

2.4. Results

2.4.1. Sgs1 interacts with Rpa70 via the acidic region N-terminal of the helicase domain

To analyze the role of Sgs1 in stabilizing stalled replication forks, we first mapped the Sgs1-RPA interaction site. Sgs1 contains three conserved domains typical for RecQ helicases: a helicase domain characteristic of SF2 helicases, a RQC (RecQ C-terminal) and a HRDC (helicase and RNase D C-terminal) domain (Figure 9A). In addition, a region of unknown structure in the N-terminus of Sgs1 (first 158 aa) has been shown to interact with Top3/Rmi1. (Fricke, Kaliraman et al. 2001), (Bennett, Noirot-Gros et al. 2000), (Chen and Brill 2007), (Weinstein and Rothstein 2008). The Sgs1-Top3-Rmi1 complex is conserved throughout evolution and has been implicated in Holliday Junction dissolution to prevent aberrant homologous recombination events (for review see (Bachrati and Hickson 2008), (Ashton and Hickson), (Wu 2007)) N-terminal of the helicase domain is an acidic region that, according to structure prediction data, is intrinsically disordered in solution. This region was recently implicated in protein-Sgs1 interactions (Bernstein, Shor et al. 2009). We fused fragments containing each of these functional domains of Sgs1 to the Gal4 transactivation domain (Gal4-TAD) for two hybrid analysis (Figure 9A).

RPA is an evolutionarily conserved heterotrimeric protein, consisting of Rpa70, Rpa32 and Rpa14, named according to their molecular weight (or Rpa1, Rpa2 and Rpa3 respectively). While the smallest subunit, Rpa14, is believed to only mediate protein-protein interaction within the RPA complex, Rpa70 and Rpa32 were shown to mediate protein interactions with other proteins and were therefore tested for interaction with Sgs1 (Binz, Sheehan et al. 2004), (Zou, Liu et al. 2006). For this purpose we fused full-length Rpa70 or Rpa32 to the LexA-DNA

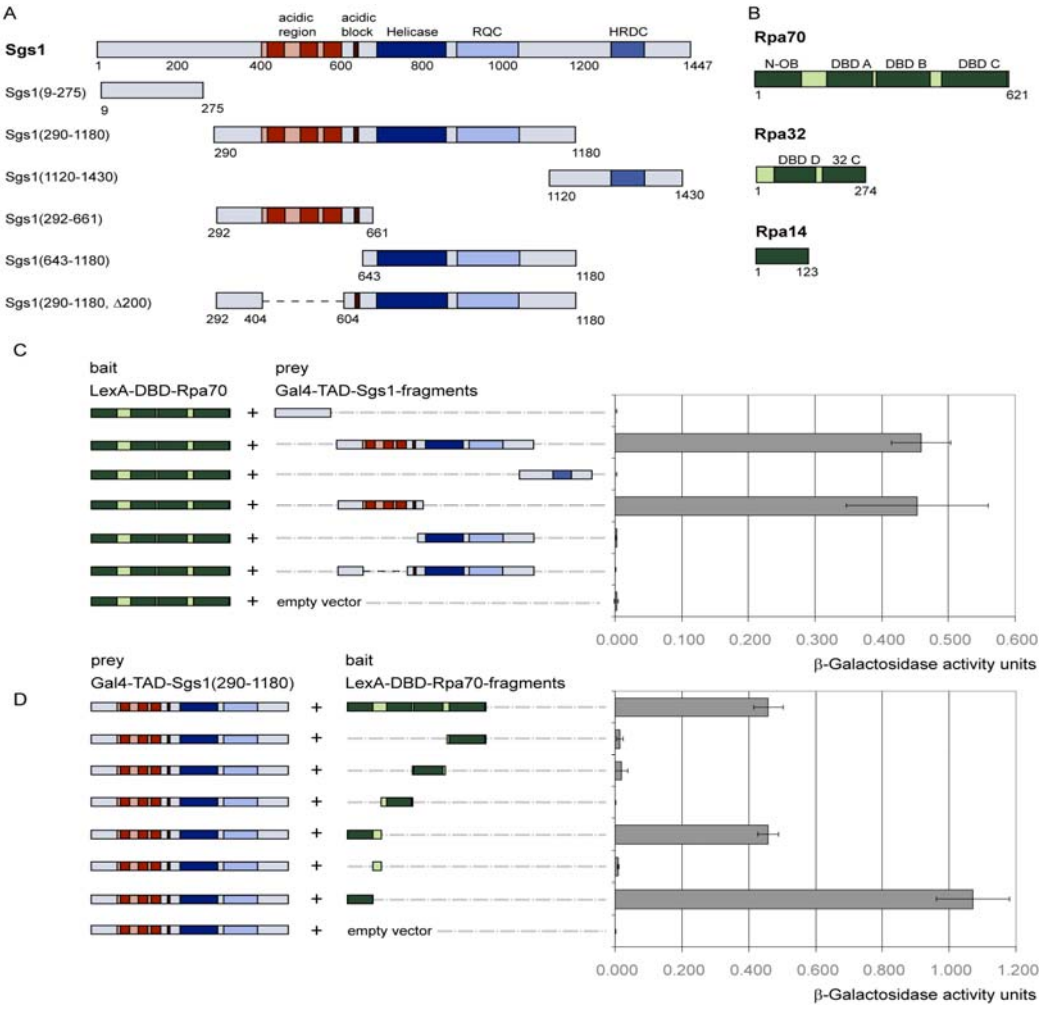


Figure 9 - Mapping the interaction site between Sgs1 and Rpa70. (A) Schematic representation of Sgs1 and its functional domains. Below are the Sgs1 domains used in two hybrid experiments, which were fused to the B42 activator domain in pJG46. The numbers indicate the boundaries of the Sgs1 domains in amino acids. **(B)** Scheme of the RPA subunits with their functional domains. Rpa70 and Rpa32 were fused to the lexA-DNA binding domain (lexA-DBD) in pGAL-lexA and studied in two hybrid analysis. N-OB – N-terminal OB-fold, DBD – DNA binding domain, 32C – Rpa32 C-terminus. **(C)** Two-hybrid assay between Rpa70 fused to lexA-DBD and Sgs1-fragments fused to Gal4-TAD. A region N-terminal of the Sgs1 helicase domain, Sgs1(aa 292-661), showed the highest β-galactosidase activity with Rpa70. This largely disordered region contains three short sequences that are conserved to close homolgoues of

Figure 9 (continued) *S. cerevisiae* (red boxes). Deletion of these three sequences in Sgs1(290-1180, Δ200)-Gal4-TAD abolished the interaction with Rpa70-lexA-DBD completely. **(D)** Two hybrid analysis between Sgs1(290-1180) fused to Gal4-TAD and different Rpa70 fragments fused to lexA-DBD. The highest β-galactosidase activity with Sgs1(290-1180) showed the N-OB fold of Rpa70.

binding domain (LexA-DBD) under a galactose inducible promotor in the two hybrid bait vector (Figure 9B).

A quantitative β-galactosidase assay was used to monitor the interaction between the Sgs1 fragments fused to Gal4-TAD and Rpa70 or Rpa32 fused to LexA-DBD. Rpa32 showed only a very weak β-galactosidase signal in the two-hybrid assay (Figure 16), while the main interaction site was mapped to Rpa70, the largest subunit of RPA (Figure 9C). The region of Sgs1 that led to the highest β-galactosidase activity with Rpa70 was the acidic region N-terminal of the Sgs1 helicase domain (Figure 9C). Within this region, we identified three short sequences (35-41 aa long) that are conserved in close homologues of S. cerevisiae (red boxes Figure 9). To test if these conserved sequences are important for the interaction with Rpa70, we made a Sgs1-Gal4-TAD fusion construct in which we deleted aa 404-604 containing these three conserved sequences. In contrast to the strong interaction observed for the Sgs1(290-1180)-Gal4-TAD construct, the same fusion protein lacking aa 404-604 (Sgs1(290-1180, Δ200)), abolished the interaction with Rpa70 completely. This suggests that this small region of 200 amino acids is the major interaction site with Rpa70. Similarly, we mapped the interaction site on Rpa70. We fused different each Rpa70 DBD to the LexA-DBD and monitored the interaction with the Sgs1(290-1180)-Gal4-TAD fusion. The highest β-galactosidase activity was measured for the N-terminal oligonucleotide binding fold (N-OB) of Rpa70 without the linker region (Figure 9D).

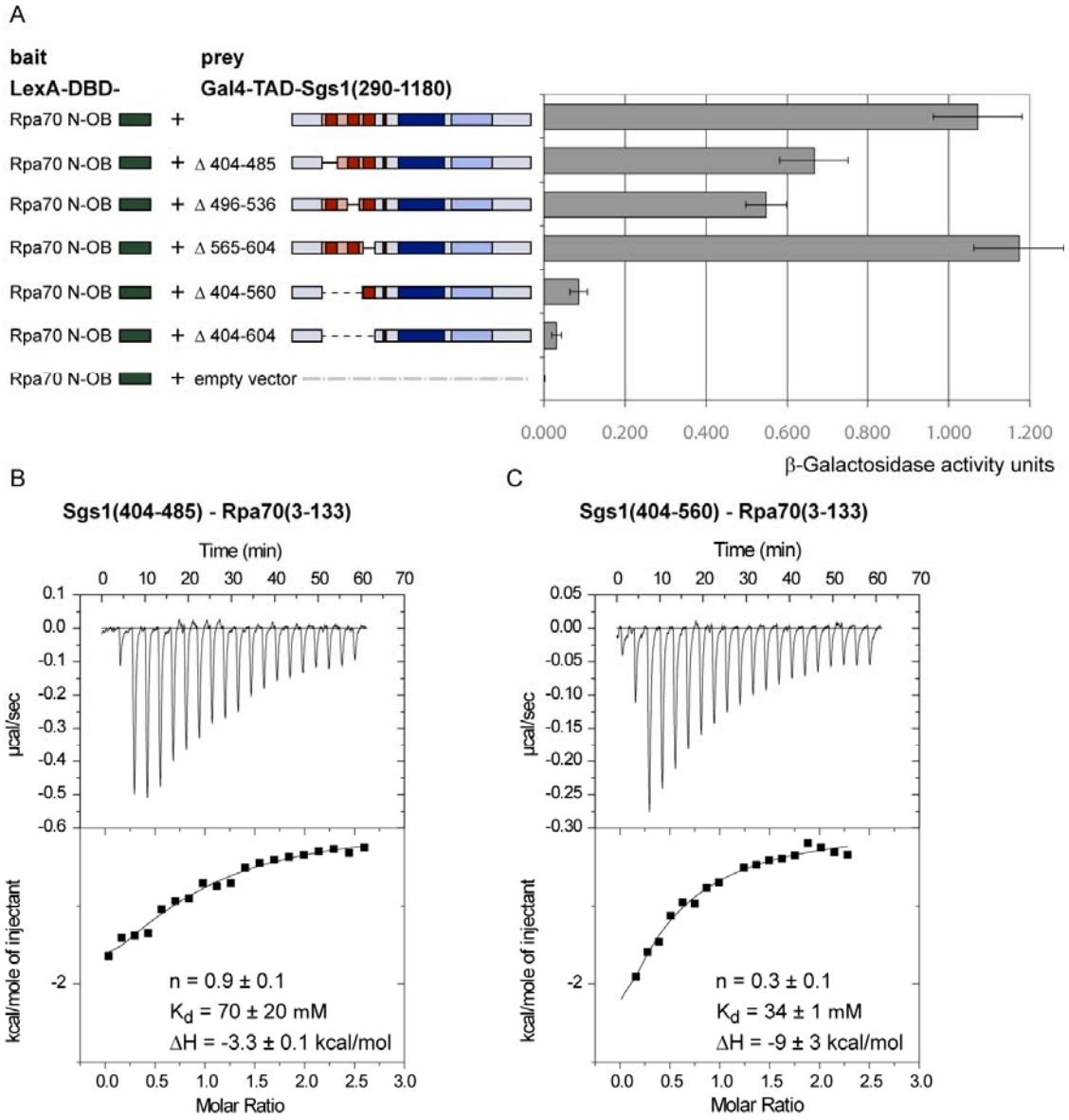


Figure 10 - Sgs1 carries at least two binding sites for Rpa70 N-OB. (A) Two hybrid analysis between Rpa70 N-OB fused to lexA-DBD and Sgs1 fragments fused to Gal4-TAD with different deletions of the three conserved regions within the RPA binding site. **(B, C)** ITC experiment of Rpa70 N-OB (Rpa70(3-133)) with Sgs1(404-485) and Sgs1(404-560). The dissociation constant (K_d), stoichiometry (n) and molar enthalpy (ΔH) are indicated within the figure.

2.4.1.1. Sgs1 carries multiple interaction sites for the Rpa70 N-OB fold

To narrow down the interaction site between Sgs1 and the N-OB fold of Rpa70, we made different Sgs1-Gal4-TAD fusion constructs in which we deleted each of the three conserved sequences (Sgs1(290-1180, Δ404-485), Sgs1(290-1180, Δ496-536) and Sgs1(290-1180, Δ565-604)) and tested them by two hybrid analysis with the Rpa70 N-OB fold (Rpa70(1-133)) fused to LexA-DBD (Figure 10A). Deletion of either the first or second conserved sequence (Sgs1(290-1180, Δ404-485) or Sgs1(290-1180, Δ496-536)) reduced the β-galactosidase signal compared to Sgs1(290-1180) approximately two-fold. In contrast, deletion of the third conserved sequence (Sgs1(290-1180, Δ565-604)) did not affect the interaction to the RPA70 N-OB fold. This suggested that Sgs1 can interact via two sites, aa 404-485 and 496-536, with the RPA70 N-OB fold. To prove this, we made another Sgs1-Gal4-TAD construct in which we deleted aa 404-560 and tested it for Rpa70 binding. Indeed, this construct abolished the interaction with the N-OB fold of Rpa70 almost as efficient as Sgs1(290-1180, Δ200). In addition, we confirmed a direct association between this region of Sgs1 and RPA70 by applying an Isothermal Titration Calorimetry (ITC) assay with purified, recombinant proteins (Figure 10B-C). We found that both Sgs1(404-485) and Sgs1(404-560) bind RPA70(3-133) with similar affinity (K_d = 70 ± 20 μM and K_d = 34 ± 1 μM respectively). Interestingly, the ITC data suggest differences in the complex stoichiometry (n) and molar enthalpy (ΔH) between the two Sgs1 fragments that are consistent with Sgs1(404-560), which contains two conserved repeats, binding more than one RPA70.

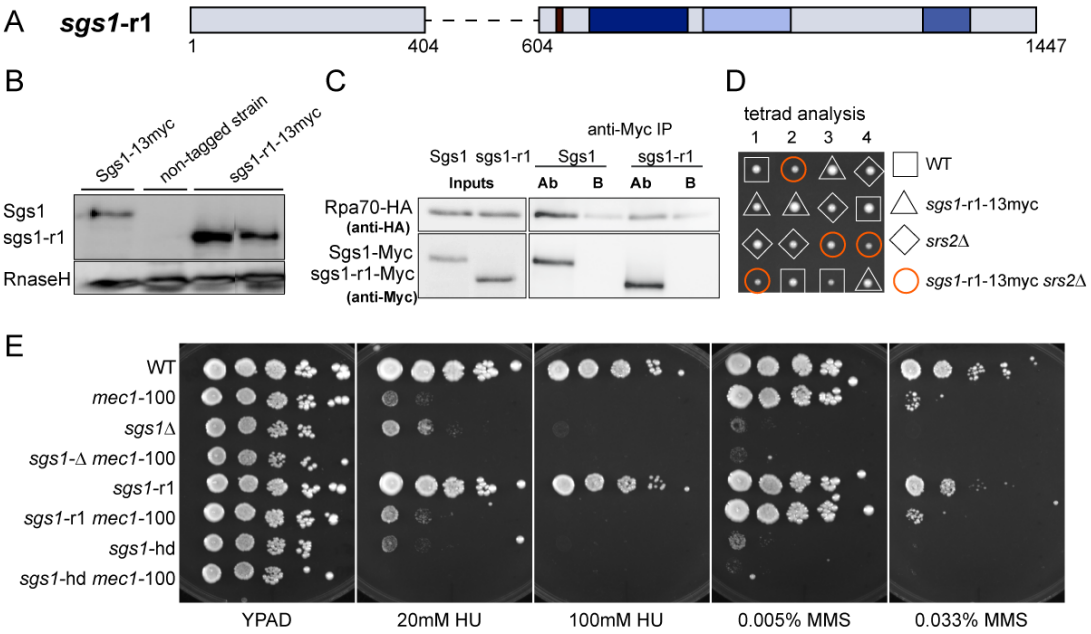


Figure 11 - Disrupting the Sgs1-RPA interaction *in vivo*. The region mapped to be important for RPA-Sgs1 interaction was deleted at the SGS1 native chromosomal locus and called *sgs1*-r1. **(A)** Schematic representation of sgs1-r1. The deleted region is indicated by a horizontal line. Red – acidic block, dark blue – helicase domain, light blue – RQC domain, blue – HRDC domain.

(B) Wild type Sgs1 and sgs1-r1 were 13-Myc-tagged and expression levels analyzed by western blot. RnaseH was used as a loading control.

(C) Co-immunoprecipitation of 13-Myc-tagged Sgs1 or sgs1-r1 and HA-tagged Rpa70. Cells were released from G1 for 1 h into S-phase, collected and precipitated using monoclonal α-Myc antibody coupled to dynabeads (myc). Blots were probed with α-Myc (9E10) for Sgs1 or sgs1-r1 and α-HA (F-7) for Rpa70. Beads without α-Myc antibody, B, were used as a negative control. IN - Input, AB - antibody. Strains used were GA-1759 and GA-5316.

(D) The *sgs1*-r1 mutant (GA-4848) was mated to *srs2*Δ (GA-1805). Diploids were sporulated and analyzed by tetrad dissection. Unlike *sgs1*Δ or *sgs1*-hd, the *sgs1*-r1 mutant does not show synthetic growth defect with *srs2*Δ, suggesting that the helicase activity of sgs1-r1 is functional.

(E) Ten-fold serial dilutions were plated onto YPAD, YPAD with 20 mM HU, 100mM HU, 0.005% MMS or 0.033% MMS. In contrast to *sgs1*Δ or *sgs1*-hd is *sgs1*-r1 not or very little sensitive to HU or MMS and does not display additive sensitivity with *mec1*-100. Strains used were GA-1981, GA-4978, GA-5457, GA-4967, GA-5076, GA-5077, GA-5445, GA-5447 in the same order as in the figure starting from the top.

2.4.2. Deletion of the RPA interaction site on Sgs1 does neither affect protein stability nor helicase activity

To determine whether this Sgs1 region is also important for the interaction with RPA *in vivo*, we deleted amino acids 405-604 within the *SGS1* chromosomal locus using a PCR-based allele-replacement technique. We have called this new allele *sgs1*-r1 (Figure 11A). We checked protein levels of 13-Myc tagged strains by western blot analysis on whole cell extracts. Wild-type Sgs1-13myc was stably expressed when the Myc tag was inserted C-terminal to the endogenous locus (Figure 11B). Similarly, we found that the expression level of 13-Myc tagged *sgs1-r1* was not less than the wild-type *in vivo*. To test if the *sgs1-r1* mutant protein can interact with RPA *in vivo*, we performed co-immunoprecipitation. Strains carrying HA-tagged Rpa70 and either Myc-tagged Sgs1 or Myc-tagged sgs1-r1 were released from G1 phase for 60 min and S phase cells collected. Rpa70-HA and Sgs1-myc were efficiently precipitated as a complex using either anti-Myc antibody (Figure 11C) or anti-HA (Figure 17) In contrast, recovery of Rpa70-HA was reduced after immunoprecipitation of *sgs1-r1*-myc with the anti-Myc antibody (Figure 11C). This confirms that the *sgs1-r1* mutation strongly weakens the interaction with RPA *in vivo.* The residual interaction can be explained either by indirect interaction of *sgs1-r1*-myc and Rpa70-HA with DNA or by additional interaction sites on RPA, such as in Rpa32, which are not affected by the *sgs1*-r1 mutation.

Next we tested if sgs1-r1 retains its helicase activity. Weinstein *et al* have shown that the helicase activity of Sgs1 is important for the synthetic growth defect observed with deletion of the Srs2 helicase (Weinstein and Rothstein 2008). Thus *sgs1-hd srs2Δ* double mutant segregants show the same synthetic sick phenotype as *sgs1Δ srs2Δ*, which often results in inviablity. It was also shown that homologous recombination contributes to this phenotype since the growth defect of both *sgs1Δ srs2Δ* and *sgs1-hd srs2Δ* is alleviated by mutation of Rad51,

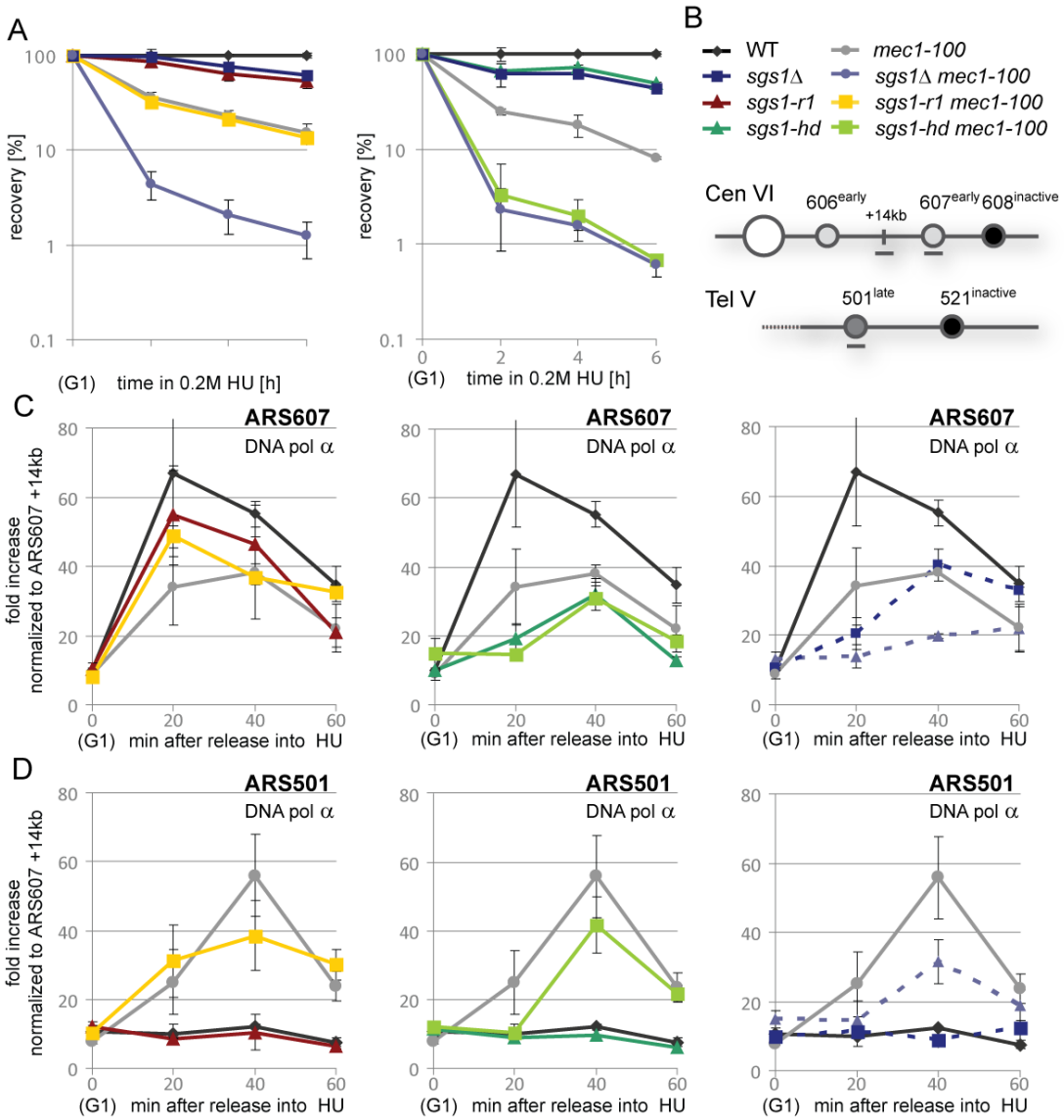


Figure 12 - *sgs1*-r1 partially destabilizes DNA pol α from the HU-stalled replication fork. (A) Recovery from replication fork stalling was monitored as colony outgrowth from in G1 synchronized isogenic strains released into S-phase into 0.2M HU containing YPAD for indicated times. Strains used were GA-1566, GA-880, GA-2478, GA-2514, GA-4502, GA-4504 for A) and GA-1981, GA-4978, GA-5445,GA-5447, GA-5457 and GA-4967 for D). **(B)** Primers (grey bars) used for ChIP that amplify the genomic regions corresponding to the early firing origin ARS607, a region 14 kb away from ARS607 (+14kb) and the late firing origin ARS501 are shown. Legend of the strains used for the recovery assay and ChIP. **(C, D)** ChIP was performed as described under methods. Strains used were GA-4973 (wild type), GA-4974 (*mec1*-100), GA-5055 (*sgs1*-r1), GA-5075 (*sgs1*-r1 *mec1*-100), GA-5449 (*sgs1*-hd), GA-5451 (*sgs1*-hd *mec1*-100). The ChIP data for *sgs1*Δ and *sgs1*Δ *mec1*-100 are taken from Cobb et al 2005 and are shown for comparison (indicated by the dashed lines).

Rad55, or Rad57 (Weinstein and Rothstein 2008), (Gangloff, Soustelle et al. 2000). We sporulated a diploid strain to observe the growth of *sgs1-r1 srs2Δ* double mutants. In contrast to the growth defect observed for *sgs1-hd srs2Δ*, *sgs1-r1 srs2Δ* show normal growth (Figure 11D). This suggests that the helicase activity of *sgs1*-r1 is functional and not significantly influenced by deleting the RPA interaction site.

We tested next whether the *sgs1-r1* allele displays the DNA damage sensitivity phenotypes of *sgs1Δ* in drop assays. This assay monitors the DNA repair ability of cells in response to permanent exposure to different genotoxic drugs, independently of the cell cycle stage. *sgs1Δ* has been shown to be sensitive to

low concentrations of the replication fork inhibitor HU and the alkylating agent methylmethane sulfonate (MMS) on plates, which is dramatically pronounced when *sgs1Δ* is coupled to the S phase specific Mec1 mutant, *mec1*-100. We analyzed *sgs1-r1* for sensitivity to HU and MMS. As described previously, we observed that both *sgs1Δ* and the helicase-dead mutant *sgs1*-hd were sensitive to HU and MMS (Weinstein and Rothstein 2008). However, *sgs1*-r1 was almost as resistant to HU or MMS on plates as the wild-type (Figure 11E). This further confirms that helicase activity is not affected by deleting the RPA interaction site in *sgs1*-r1. Even more interestingly, we found that in contrast to *sgs1*Δ *mec1*-100 or *sgs1*-hd *mec1*-100, combining *sgs1*-r1 with the *mec1*-100 mutation did not result in additive sensitivity to HU or MMS on plates. Instead, *sgs1*-r1 *mec1*-100 cells show the same HU and MMS-sensitivity as cells carrying only the *mec1*-100 mutation, suggesting that either *sgs1*-r1 may act on the same pathway as *mec1*-100 or it has no effect (Figure 11E).

2.4.3. *sgs1*-r1 partially destabilizes polymerase α and acts on the same pathway as *mec1*-100

To look more closely if *sgs1*-r1 is in the same pathway as *mec1*-100 for HU sensitivity, we measured cell survival specifically in response to HU in S phase. G1 phase arrested cells were released into 0.2 M HU-containing YPAD for the indicated time points and consequently plated on YPAD (Figure 12A). In contrast to the lack of sensitivity on HU-containing plates, we observed in this S-phase specific survival assay that *sgs1*-r1 was as HU-sensitive as *sgs1Δ*. However, the double *sgs1*-r1 *mec1*-100 mutant is epistatic with *mec1*-100 and not additive like the *sgs1Δ mec1*-100 or *sgs1*-hd *mec1*-100 double mutants (Figure 12A). This suggests that the interaction between RPA and Sgs1 is important for recovery from HU-induced replication fork stalling during S phase, but that loss or diminished Sgs1-RPA interaction is not the reason for the additive behaviour of *sgs1Δ mec1*-100. These data rather suggest that *sgs1*-r1 acts on the same pathway as *mec1*-100.

To look at whether *sgs1*-r1 affects the stability of replication fork components, we performed Chromatin Immunoprecipitation (ChIP) on DNA polymerases (Bjergbaek, Cobb et al. 2005). For this experiment, we synchronized single and double mutants and released them into S phase in the presence of 0.2 M HU. Over an hour, the abundance of either DNA pol α or ε was studied by real-time PCR at the early firing origin ARS607 and at the late firing origin ARS501 (Figure 12C-D). As a negative control, a locus 14 kb away from ARS607 was analyzed and used to normalize the absolute enrichments at ARS607 or ARS501. Cobb *et al*. had observed that cells carrying the *sgs1Δ* or the *mec1*-100 mutation show partial destabilization of DNA pol α and ε from the HU-stalled replication fork (Figure 12C right panel). This effect is additive when both mutations are combined, resulting in complete loss of DNA pol α and ε from the stalled fork (Figure 12C right panel). However, disruption of the Sgs1-RPA interaction site in

the *sgs1*-r1 mutant does not cause such a dramatic effect when combined with the *mec1*-100 mutation (Figure 12C). DNA pol α is only partially displaced from ARS607 similar to what is observed for *sgs1*-r1 or *mec1*-100 alone.

Because HU-treatment activates the intra-S phase checkpoint response, late firing origins, like ARS501, get repressed. This explains why DNA pol α is not stabilized at ARS501 for wild type cells and why DNA pol α is enriched at ARS501 in the checkpoint mutant *mec1*-100 (Figure 12D). The *sgs1*-r1 mutant alone does not de-repress the late origin ARS501, but when combined with *mec1*-100 DNA pol α is partially lost from ARS501 (Figure 12D). Thus, the Sgs1-RPA interaction is necessary, but not sufficient for stabilization of DNA pol α at the HU-stalled replication fork in the *mec1*-100 background. We also checked if the helicase activity of Sgs1 is responsible for the additive effect in the *sgs1Δ mec1*-100 double mutant (Figure 12C central panel). DNA pol α is also partially displaced from the HU-stalled fork in the *sgs1*-hd mutant. This effect is even more pronounced than either *sgs1*-r1 or *mec1*-100 cells. However, the *sgs1*-hd *mec1*-100 double mutation does not completely displace pol α from the early firing origin ARS607 or the late firing origin ARS501 (Figure 12C-D middle panel). This suggests that both the enzymatic activity of Sgs1 helicase as well as its interaction with RPA contribute to DNA pol α stabilization.

2.4.4. Sgs1 is phosphorylated by Mec1 at the RPA interaction site *in vitro*

The epistatic relationship between *sgs1*-r1 and *mec1*-100 sensitivity in response to HU prompted us to ask if this region of Sgs1 may be a target of Mec1. Activation of the intra-S phase checkpoint by Mec1 occurs via phosphorylation of numerous targets containing SQ/TQ motifs. Previous studies report that Mec1 substrates often contain several closely spaced SQ/TQ motifs, which are also referred to as SQ/TQ cluster domains (SCD) (Traven and Heierhorst 2005). Such

SCDs are defined by at least three SQ/TQ motifs within 100 aa plus additional motifs that are less than 100 aa away. Interestingly, the human Sgs1 homologues BLM and WRN have been shown to be phosphorylated by ATR/ATRIP, the human homologue of Mec1/Ddc2. We investigated the presence of SQ/TQ sites on Sgs1 to ask if Sgs1 could be a target of Mec1. Indeed, we found that Sgs1 contains nine SQ or TQ sites, of which four are located in the region that we mapped as the main interaction site with Rpa70 ($T_{451}Q_{452}$, $S_{470}Q_{471}$, $S_{482}Q_{483}$, $T_{585}Q_{586}$) and one very close to this site ($S_{628}Q_{629}$), (Figure 13A). Thus the Sgs1 region that carries the main Rpa70 interaction site, deleted in *sgs1*-r1, contains a SCD (four SQ/TQ sites within 134 aa) similar to other Mec1 substrates and could be a likely target of Mec1/Ddc2. We performed a Mec1 kinase assay to test if this region of Sgs1 can be phosphorylated by Mec1 *in vitro*. Mec1/Ddc2 was activated by exposing exponentially growing cells to 0.1% MMS for 1 h and Myc-tagged Mec1 was immunoprecipitated using anti-Myc coupled Sepharose beads. A non-tagged yeast strain was used as a negative control (Figure 13B). As shown in Figure 13C, Sgs1(404-604aa) could be efficiently phosphorylated by Mec1-myc *in vitro*. Importantly, the phosphorylation could be reduced to background levels using the Mec1/Tel1 inhibitor caffeine. This suggests that the high level of phosphorylation observed is likely due to Mec1 and not the result of another kinase that can bind unspecifically to myc-tagged Sepharose beads. Mutation of SQ/TQ motifs in this region to either sgs1-4A (Sgs1_404-604aa_T451A_S470A_S482A_T585A), or sgs1-3E (Sgs1_404-604aa_ T451E_S470E_S482E) abolished Sgs1 phosphorylation completely. Since the sgs1-3E mutation is sufficient to abrogate Sgs1 phosphorylation *in vitro*, it is likely that Mec1/Ddc2 targets one or more sites of the following SQ/TQ motifs: $T_{451}Q_{452}$, $S_{470}Q_{471}$, $S_{482}Q_{483}$.

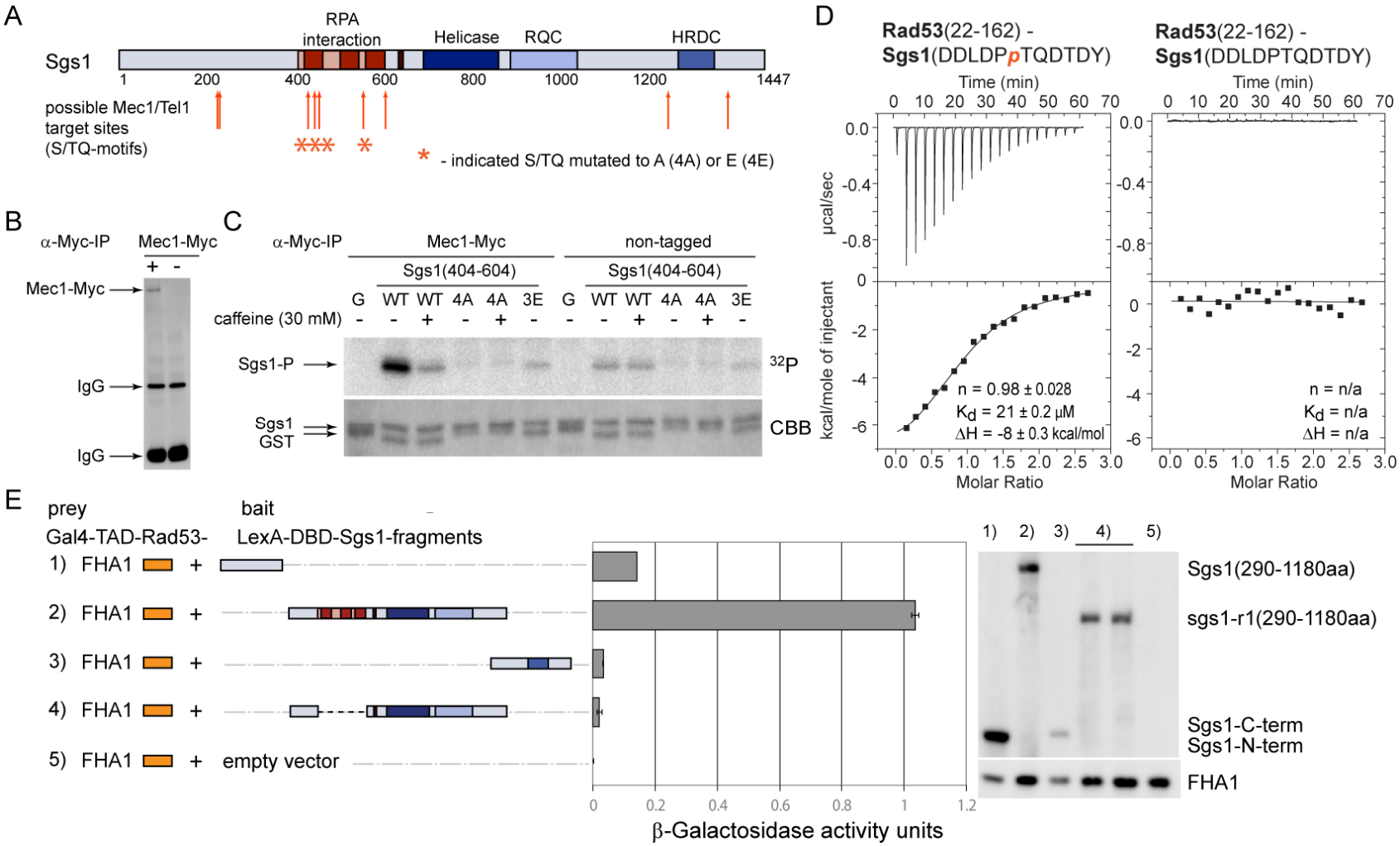

A
RPA interaction
Helicase
RQC
HRDC
Sgs1
1
200
400
600
800
1000
1200
1447
possible Mec1/Tel1 target sites (S/TQ-motifs)
* - indicated S/TQ mutated to A (4A) or E (4E)
B
α-Myc-IP
Mec1-Myc
Mec1-Myc
IgG
IgG
C
α-Myc-IP
Mec1-Myc
non-tagged
Sgs1(404-604)
Sgs1(404-604)
G WT WT 4A 4A 3E G WT WT 4A 4A 3E
caffeine (30 mM)
Sgs1-P
32P
Sgs1 GST
CBB
D
Rad53(22-162) - Sgs1(DDLDPpTQDTDY)
Rad53(22-162) - Sgs1(DDLDPTQDTDY)
Time (min)
μcal/sec
kcal/mole of injectant
n = 0.98 ± 0.028
Kd = 21 ± 0.2 μM
ΔH = -8 ± 0.3 kcal/mol
n = n/a
Kd = n/a
ΔH = n/a
Molar Ratio
E
prey
Gal4-TAD-Rad53-
bait
LexA-DBD-Sgs1-fragments
1) FHA1 +
2) FHA1 +
3) FHA1 +
4) FHA1 +
5) FHA1 + empty vector
β-Galactosidase activity units
Sgs1(290-1180aa)
sgs1-r1(290-1180aa)
Sgs1-C-term
Sgs1-N-term
FHA1

Figure 13 - Mec1 targets the RPA-interacting site on Sgs1 *in vitro*. (A) Schematic representation of Sgs1 with its functional domains drawn to scale. Possible Mec1/Tel1 sites are indicated by the red arrows. There is a cluster of SQ/TQ sites in the region, which was mapped to interact with Rpa70. **(B)** Exponentially growing cells of GA-1456 (Mec1-myc) and GA-426 (non-tagged) were exposed for 1 h with 0.1% MMS. After cell lysis Mec1-myc was precipitated using anti-Myc coupled sepharose beads and used for the Mec1 kinase assay. **(C)** Kinase assay of Mec1-myc or control immunoprecipitates (non-tagged). Sgs1(404-604 aa) , WT, was used as a substrate and efficiently phosphorylated by Mec1-myc in the phosphoimaging autoradiograph (upper panel, ^{32}P). This phosphorylation was reduced to background levels when 30 mM caffeine were added to the reaction (see left panel, non-tagged). Mutation of the SQ/TQ sites on Sgs1 (4A -(Sgs1_404-604aa-T451A-S470A-S482A-T585A), or 3E- (Sgs1_404-604aa-T451E-S470E-S482E)) abolished the Mec1 phosphorylation completely. The loading control is shown in the lower panel (CBB – gel stained with Coomassie brilliant blue). **(D)** ITC assay of the FHA1 domain of Rad53, Rad53(22-162), and two Sgs1(446-456) peptides, Sgs1(DDLDPTQDTDY), encompassing either phosphorylated $T_{451}Q_{452}$ (right panel) or non-phosphorylated $T_{451}Q_{452}$ (left panel). The dissociation constant (K_d), stoichiometry (n) and molar enthalpy (ΔH) are indicated within the figure. **(E)** Two hybrid assay between Rad53-FHA1 domain fused to the Gal4-TAD and Sgs1-fragments fused to the LexA-DBD. Sgs1(290-1180) fused to LexA-DBD shows the highest ß-galactosidase activity with the FHA1 domain of Rad53 (2), which is abolished with sgs1-r1(290-1180) (4). Expression of the fusion proteins was analyzed by western blot (right panel).

Since *sgs1*-r1 is epistatic to *mec1*-100 in survival after HU, and Sgs1(404-604) is phosphorylated by Mec1 *in vitro*, we further examined the interaction of Sgs1 with the checkpoint machinery. Bjergbaek *et al*. published that Sgs1 interacts directly with the major Mec1 target, Rad53 (Bjergbaek, Cobb et al. 2005). They mapped the interaction site by two hybrid assay to the FHA1 domain of Rad53 and a large region of Sgs1 (from 290-1180 aa) that contains the helicase domain, the RQC domain and the N-terminal acidic RPA-interacting region. Therefore, we analyzed if deletion of the Sgs1(404-604) RPA-interaction region from this two-hybrid construct would affect the interaction with Rad53. Indeed, whereas Sgs1(290-1180 aa) interacted as expected with the FHA1 domain of Rad53, the interaction was completely abolished with *sgs1*-r1(290-1180 aa) (Figure 13E). Next, we investigated whether the Rad53-Sgs1 interaction depended on Sgs1 phosphorylation. Therefore, we performed ITC with the FHA1 domain and short Sgs1 peptides encompassing phosphorylated $T_{451}Q_{452}$, $S_{470}Q_{471}$ or $S_{482}Q_{483}$

(Sgs1(446-456), Sgs1(466-475) and Sgs1(478-487) respectively). As a negative control we used unphosphorylated Sgs1(446-456). Only the Sgs1(446-456) peptide containing phosphorylated, but not unphosphorylated $T_{451}Q_{452}$ or the other phosphorylated Sgs1 peptides, showed an interaction with the FHA1 domain of Rad53 (Rad53(22-162), Figure 13D). The affinity of phosphorylated Sgs1(446-456) peptide to the FHA1 domain was with a K_D = 21 ± 0.2 µM in a similar range as observed for Rpa70(3-133) and Sgs1(404-560), (Figure 10C). Thus the data suggest that the RPA-interaction site on Sgs1 is phosphorylated by Mec1 after HU-induced replication fork stalling and that this modification might be necessary for Rad53 interaction..

2.4.5. *sgs1*-r1 cells display a defect in Rad53 activation (*rad24* background)

Previous data have demonstrated that Sgs1 and Rad24 act in parallel pathways to activate Rad53 when cells are exposed to HU during S phase (Bjergbaek, Cobb et al. 2005), (Frei and Gasser 2000). Rad24, the homologue of human RAD17, forms a complex withRfc2-5. Upon accumulation of RPA-coated ssDNA due to fork stalling or DNA damage, Rad24-Rfc2-5 loads the 9-1-1 checkpoint clamp (consisting of RAD9-RAD1-HUS1 in humans and Rad17-Mec3-Ddc1 in budding yeast) onto primer-template junctions adjacent to RPA-coated ssDNA. This can lead to Mec1/Ddc2 activation and subsequently activation of Rad53 and the intra-S phase checkpoint. How Sgs1 stimulates Rad53 is presently not well understood. Bjergkbaek *et al* have shown that Sgs1 interacts with the FHA1 domain of Rad53, but that both the Sgs1 helicase activity and the interaction with Top3 are dispensable for Rad53 activation (Bjergbaek, Cobb et al. 2005).

Therefore, we examined whether the *sgs1*-r1 mutation, which lacks the Mec1-dependent phosphorylation sites and is deficient for Rad53 interaction in two hybrid analysis, has an effect on checkpoint activation. To test this hypothesis,

we released G1 synchronized cells into HU-containing media for 1 h and checked for Rad53 activation by western blot analysis, where hyperphosporylation correlating with activated Rad53 is visible as a slower migrating band (Figure 14A). For all strains, Rad53 is not phosphorylated in G1 phase. After HU-treatment, Rad53 is efficiently phosphorylated in wild type cells, and both *rad24Δ* and *sgs1*-r1 cells also display a Rad53 upshift. In the *sgs1*-r1 *rad24Δ* double mutant however this upshift is significantly reduced, similar to levels observed in *sgs1Δ rad24Δ*, suggesting that *sgs1*-r1 is like *sgs1Δ,* deficient for Rad53 activation. To monitor if the Mec1-phosphorylation motifs in the RPA-interaction site of Sgs1 are necessary for intra-S phase checkpoint activation, we tested the non-phosporylatable *sgs1*-4A and the *sgs1*-4E mutant for Rad53 activation. Figure 14A shows that the Rad53 upshift upon HU-treatment is significantly reduced in both double *sgs1*-4A *rad24Δ* and *sgs1*-4E *rad24Δ* mutants compared to wild type or the single mutants. Next, we tested whether the abrogation of the Mec1-phosphorylation sites on Sgs1 has an effect on DNA damage repair or replisome stability in response to HU. Similar to *sgs1*-r1 cells, non-phosphorylatable *sgs1*-4A and *sgs1*-4E mutants are as resistant to permanent HU-exposure as wild type (Figure 14B). However, in contrast to *sgs1*-r1 or *sgs1*Δ, *sgs1*-4A cells behave like wild type cells and do not comprise recovery after HU-treatment (Figure 14C). This suggests that although Mec1 phosphorylation of the RPA-interaction site on Sgs1 is necessary for Rad53 activation (in the *rad24*Δ background), it does not contribute to replication fork stability in response to replication stress.

2.5. Discussion

In order to explore the effect of RPA-Sgs1 interaction on replisome stability after replication fork stalling, we have identified the main interaction site of Sgs1 helicase with the largest subunit of RPA, Rpa70. This region (aa 404-604) located N-terminal of the helicase domain of Sgs1 is predicted to be structurally

disordered in solution. It contains a high proportion of acidic amino acids and three short sequences conserved in other close homologues of *S. cerevisiae*. Interestingly, Bernstein *et al*. described a separation of function mutant *sgs1*-AR2Δ carrying the deletion of the amino acids 502 – 648, which overlaps with the *sgs1*-r1 mutation characterized in this study (Bernstein, Shor et al. 2009). This *sgs1*-AR2Δ mutant, like *sgs1*Δ, suppresses *top3*Δ slow growth but was shown to be proficient for recombination and was similarly resistant to HU and MMS as wild type Sgs1 on drop tests. A genetic screen isolated a Sgs1 mutant that lost aspartic acid residue 664 (*sgs1*-D664Δ) mimicking the *sgs1*-AR2Δ phenotype. In this point mutant X-structures persisted at damaged replication forks after MMS-treatment, suggesting that *sgs1*-D664Δ as well as the *sgs1*-AR2Δ mutant are incapable of replication-associated repair or stabilization of replisome components, resulting in fork collapse and therefore persistent recombination structures. The *sgs1*-r1 mutant shows a similar phenotype to the *sgs1*-AR2Δ or *sgs1*-D664Δ mutants, with respect to HU and MMS resistance on plates, *top3*Δ slow growth suppression (Figure 18), and in the lack of synthetic sickness when combined with *srs2*Δ.

2.5.1. DNA pol α stability at stalled forks requires RPA-Sgs1 interaction and Sgs1 helicase function

Furthermore, we observed that *sgs1*-r1 partially destabilizes DNA pol α after HU-induced replication fork stalling, indicating that the RPA-Sgs1 interaction promotes the retention of DNA polymerases at stalled forks. The data for the endogenous *sgs1*-hd mutant demonstrates that the helicase activity of Sgs1 also contributes to the maintenance of DNA pol α, in accordance with data from Cobb *et al* 2003, showing that plasmid-born *sgs1*-hd fails to stabilize DNA pol ε in an *sgs1*Δ background (Cobb, Bjergbaek et al. 2003). The exact mechanism of how Sgs1 confers polymerase stabilization at stalled replication forks to ensure proper replication fork restart is still speculative. We propose the following two models.

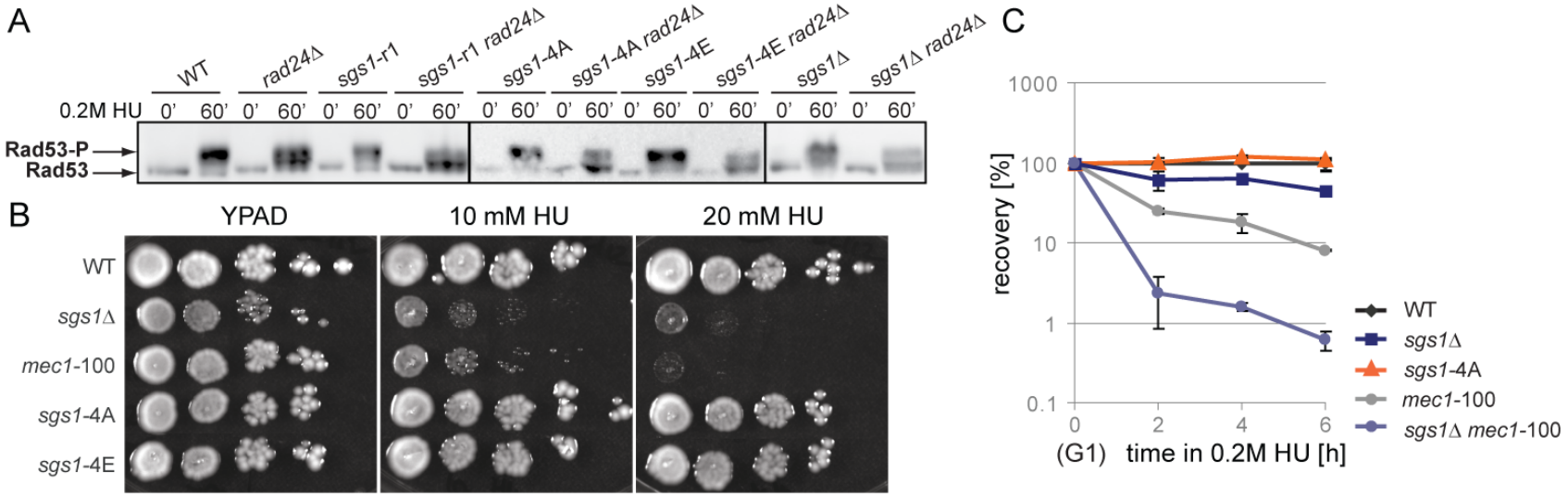


Figure 14 - *sgs1*-r1 cells display a defect in Rad53 activation. (A) Western blot analyses of Rad53 in GA-1981 (wt), GA-5076 (*sgs1*-r1), GA-5321 (*rad24*Δ), GA-5324 (*sgs1*-r1 *rad24*Δ), GA-5932 (*sgs1*-4A), GA-5934 (*sgs1*-4A *rad24*Δ), GA-5845 (*sgs1*-4E), GA-5895 (*sgs1*-4E *rad24*Δ), GA-1761 (*sgs1*Δ) and GA-2056 (*sgs1*Δ *rad24*Δ). Cells were synchronized in G1 and released into S phase for 60 min in the presence of 0.2M HU.

(B) Ten-fold serial dilutions were plated onto YPAD and YPAD with 10mM and 20 mM HU. Strains used were GA-1981, GA-5457, GA-4978, GA-5932 and GA-5845.

(C) Recovery from replication fork arrest was monitored as colony outgrowth from cells synchronized in α-factor and incubated in 0.2 M HU for the indicated time points.

The data for the S-phase specific recovery assay on HU and the DNA polymerase α ChIP of the *sgs1*-r1 and *sgs1*-hd mutant suggest that Sgs1 acts via its interaction with RPA directly at the stalled fork and stabilizes DNA pol α/primase (Figure 11). It is possible that Sgs1 stabilizes pol α/primase by provoking a conformational change in the RPA molecule adjacent pol α/primase. This would require both RPA interaction and Sgs1 helicase activity. Sgs1 would act similarly to Tag helicase in SV40 virus replication (Arunkumar, Klimovich et al. 2005). RPA would change from a elongated form with high affinity for ssDNA into a globular form ('priming mode'), which occludes 8-10 nt instead of 30 nt and has lower affinity for ssDNA (Bochkarev and Bochkareva 2004; Arunkumar, Klimovich et al. 2005). This in turn would make space for pol α/primase to induce primer synthesis and facilitate replication fork restart after stalling or pausing. This model is supported by data from Yodh *et al.* showing that BLM helicase can repetitively unwind and re-anneal a critical length of dsDNA and even displace previously bound hRPA (Yodh, Stevens et al. 2009).

It is also conceivable that Sgs1 stabilizes the replication fork in complex with Top3 and Rmi1 by Holliday junction dissolution (Bjergbaek, Cobb et al. 2005), (Bennett, Keck et al. 1999), (Ashton and Hickson). This function is probably especially important for repair and restart of a collapsed fork after DNA damage, which occurs after persistent exposure with MMS or HU on drop tests. We and others have observed that the helicase function of Sgs1 is crucial for the survival after HU or MMS exposure (Weinstein and Rothstein 2008), (Bernstein, Shor et al. 2009), (Figure 10). Additionally, a *sgs1* mutant, which has lost its interaction site with Top3, *sgs1*-NΔ, shows similar or even more pronounced HU- and MMS-sensitivity as *sgs1*Δ (Weinstein and Rothstein 2008). This suggests that both helicase activity as well as Top3-interaction contribute to DNA repair and survival after exposure to HU or MMS on plates. Rad51 has also been shown to be important for DNA pol ε stabilization at HU-stalled forks (Bjergbaek, Cobb et al. 2005). Homologous recombination (HR) is probably necessary for polymerase stabilization and fork restart, when a lesion at a leading or lagging strand occurs.

The resulting HR-intermediate would be dissolved by the Sgs1-Top3-Rmi1 complex. RPA has been shown to stimulate RecQ helicase activity in human cells (Shen, Lao et al. 2003), (Doherty, Sommers et al. 2005),(Machwe, Lozada et al. 2006), (Yodh, Stevens et al. 2009), (Sowd, Wang et al. 2009). On one hand RPA interacts directly with RecQ helicases and maybe influences enzymatic activity by conformational alterations or changes of the oligomeric state of the RecQ helicase. On the other hand RPA counteracts the annealing activity of the RecQ helicase by stabilizing the ssDNA that occurs from unwinding (Bachrati and Hickson 2008). In contrast to *sgs1*-hd or *sgs1*-NΔ, *sgs1*-r1 cells, which show diminished RPA interaction *in vivo*, are almost as resistant to HU or MMS as wild type cells (Figure 10). This indicates that Sgs1-RPA interaction, although important for stimulation of RecQ helicase activity, is not necessary such that cells are still able to dissolve HR intermediates by the Sgs1-Top3-Rmi1 complex.

2.5.2. Rad53 activation depends partially on Sgs1 phosphorylation at the RPA-interaction site by Mec1-Ddc2

Intra-S phase checkpoint activation is an essential mechanism to deal with DNA damage or replication stress. Previous studies have shown that Sgs1 functions in Rad53 activation in a parallel pathway to Rad24, but epistatic to Mrc1 (Frei and Gasser 2000), (Bjergbaek, Cobb et al. 2005). This function is clearly distinct from polymerase stabilization and relies neither on Sgs1 helicase activity nor the presence of Top3 or Rad51 (Bjergbaek, Cobb et al. 2005). Bjergbaek and coworkers have observed that Sgs1 interacts with the FHA1 domain of Rad53 and proposed a direct recruitment mechanism for Sgs1 and Rad53. In this study, we could demonstrate for the first time that Sgs1 is a target of Mec1-Ddc2 *in vitro* and that it is phosphorylated within its RPA-interaction domain. Furthermore, we could demonstrate that this RPA binding region is important for interaction with the FHA1 domain of Rad53. Mutation of the four SQ/TQ sites to AQ in the Sgs1

RPA-interacting region abolishes Rad53 hyperphosphorylation in a *rad24*Δ background in response to HU to a similar extent as *sgs1*Δ.

Combining our data, we propose the following mechanism for Sgs1 function upon replication stress (Figure 15). In response to replication fork arrest, the checkpoint kinase Mec1-Ddc2 gets activated and recruited to ssDNA coated by RPA. This leads to the phosphorylation of various targets including RPA and Sgs1. The phosphorylation of Sgs1 at the RPA-interaction site provokes a shift in its binding affinity away from RPA towards Rad53, leading to the recruitment of Rad53 kinase. Once in close proximity to Mec1-Ddc2, Rad53 gets phosphorylated and the intra-S phase checkpoint activated. Thus our data suggests that Sgs1 functions as an adaptor between sensor kinase Mec1-Ddc2 and the effector kinase Rad53 in response to replication fork stalling. In addition, Mrc1 has been identified as an adaptor between Mec1-Ddc2 and Rad53 (Alcasabas, Osborn et al. 2001), (Osborn and Elledge 2003). However, no direct interaction between Mrc1 and Rad53 has been described so far and the molecular mechanism as to how hyper-phosphorylated Mrc1 leads to Rad53 activation in response to replication stress is still uncharacterized (Branzei and Foiani 2009). A recent study has demonstrated that Mec1- but not Rad53-dependent phosphorylation of Mrc1 is necessary for the establishment of a positive feedback loop that leads to Mec1 stabilization at stalled replication forks (Naylor, Li et al. 2009). In the absence of Mec1-dependent phosphorylation of Mrc1, Mec1-Ddc2 is not efficiently recruited to stalled replication forks. Thus Mrc1 leads to the accumulation of Mec1-Ddc2 and acts as a platform for the eventual recruitment and phosphorylation of Rad53 (Naylor, Li et al. 2009). Our data suggest that Sgs1 contributes as a scaffold for Rad53 recruitment after its phosphorylation by Mec1-Ddc2.

In human cells, the RecQ helicase BLM has been shown to be a target of ATR in a region N-terminal of its helicase domain (Davies, North et al. 2004). Interestingly, the N-terminal region of BLM (aa) including the ATR-target sites

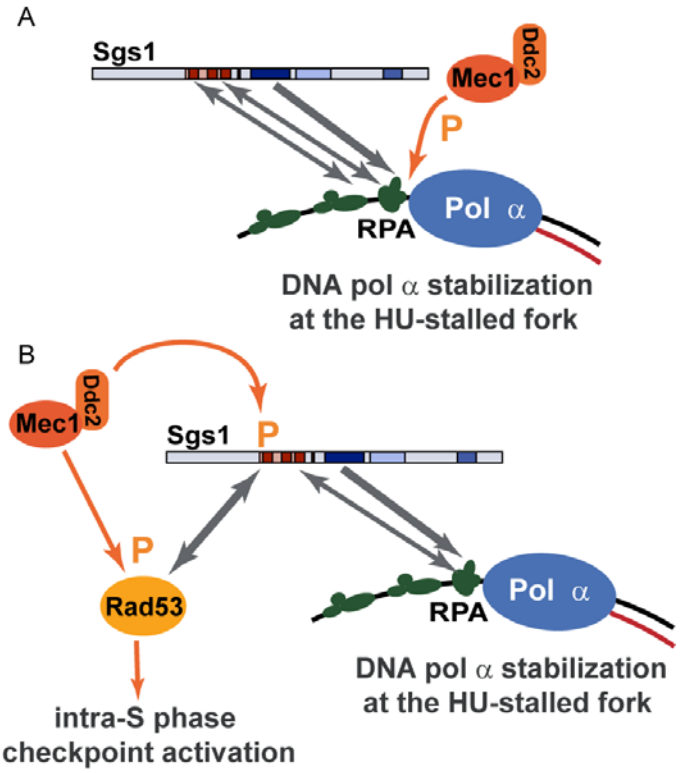


Figure 15 - The Sgs1-RPA-interaction contributes to polymerase stabilization and is a target of Mec1-Ddc2 kinase after replication stress. (A) Sgs1 stabilizes DNA pol α at stalled replication forks. This requires the helicase function of Sgs1 and RPA-interaction. The checkpoint kinase Mec1-Ddc2 is recruited to ssDNA coated by RPA and phosphorylates after its activation a number of downstream targets, including RPA and Sgs1. **(B)** Sgs1 phosphorylation probably leads to a shift of affinity from RPA towards Rad53 and subsequent recruitment of Rad53 to the stalled fork into close proximity of Mec1-Ddc2. Mec1-Ddc2 phosphorylates Rad53, which leads to the activation of the intra-S phase checkpoint response.

has also been shown to bind hRPA *in vitro* (Doherty, Sommers et al. 2005). In contrast to Sgs1, BLM is not a constitutive component of the replisome, but is recruited from PML bodies to sites of stalled replication forks in response to HU (Sengupta, Linke et al. 2003). This re-localisation requires ATR-dependent phosphorylation of BLM (Davalos, Kaminker et al. 2004). It has been suggested that ATR phosphorylation of BLM is required for recovery from HU-mediated replication fork stalling, but not for the recruitment of BLM to damaged forks and its function with hTOPOIIIα-hRMI1-hRMI2 to suppress sister chromatid

exchanges (Davies, North et al. 2004) (Wu 2007). Davies *et al* demonstrated that ATR-dependent phosphorylation of BLM is required for efficient replication fork resumption and suppression of new origin firing after aphidicolin treatment (Davies, North et al. 2007). Furthermore, the presence of BLM at stalled replication forks is required for robust intra-S phase checkpoint activation in human cells (Davalos and Campisi 2003), (Franchitto and Pichierri 2002). However, the molecular mechanism as to how ATR and BLM work together to maintain replisome stability at stalled replication forks and activate the intra-S phase checkpoint are still enigmatic. In this study, we could show the first time that RPA-interaction between the RecQ helicase Sgs1 is on one hand important for polymerase stabilization at stalled replication forks after HU treatment. On the other hand we observe that the RPA-interaction site is a target of Mec1-Ddc2 and is important for intra-S checkpoint activation. This implicates that Mec1-dependent Sgs1 phosphorylation might function as a switch and help to recruit the effector kinase Rad53. Further studies will reveal whether Sgs1, Rad53 and RPA bind in one complex and whether similar molecular mechanisms apply for the human homologues BLM or WRN.

2.6. Supplementary figures

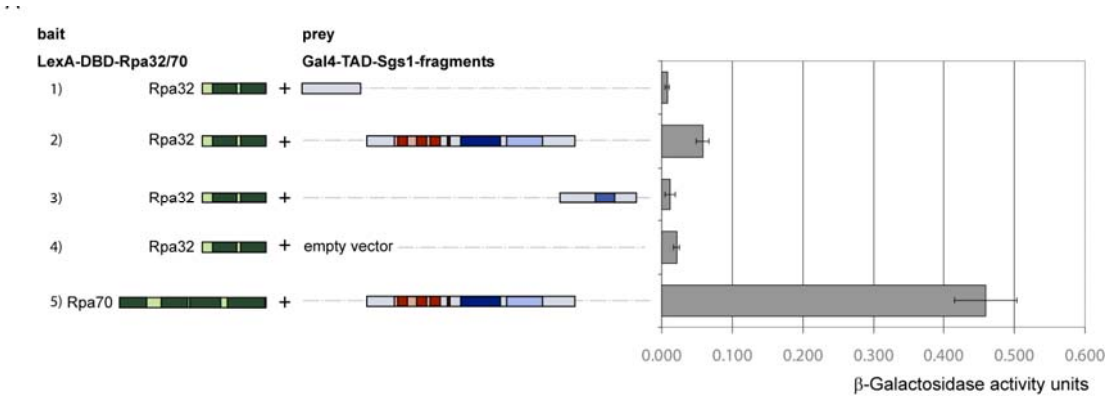


Figure 16 - **Two hybrid analysis between Sgs1 and Rpa32.** Full-length Rpa32 was fused to lexA-DBD and Sgs1-fragments were fused to Gal4-TAD. Rpa70 fused to lexA-DBD was used as a positive control. The β-galactosidase activity for Rpa32 with the different Sgs1-fragments (1-3) was barely above background (4).

.

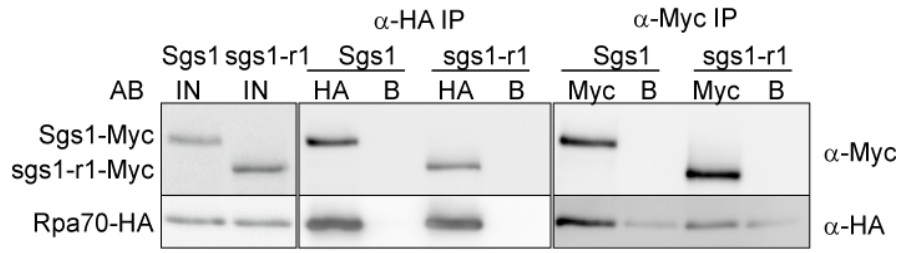


Figure 17 - Co-immunoprecipitation of 13-Myc-tagged Sgs1 or sgs1-r1 and HA-tagged Rpa70. Cells synchronized in G1 were released for 1 h into S-phase, collected and precipitated using monoclonal α-Myc antibody or α-HA antibody coupled to dynabeads (myc, HA). Blots were probed with α-Myc (9E10) for Sgs1 or sgs1-r1 and α-HA (F-7) for Rpa70. Beads without α-Myc antibody, B, were used as a negative control. IN - Input, AB - antibody. Strains used were GA-1759 and GA-5316.

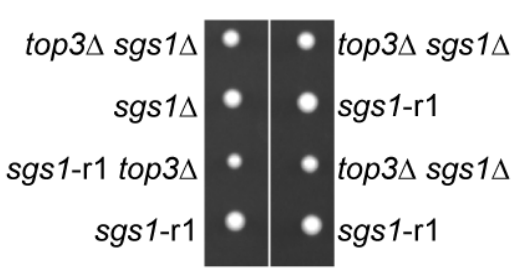


Figure 18 - *sgs1*-r1 suppresses *top3*Δ slow growth. *sgs1*-r1 (GA-4848) was mated to *top3*Δ (GA-731). Diploids were analyzed after sporulation by tetrad dissection. Similar like *sgs1*Δ suppresses the *sgs1*-r1 mutant *top3*Δ slow growth.

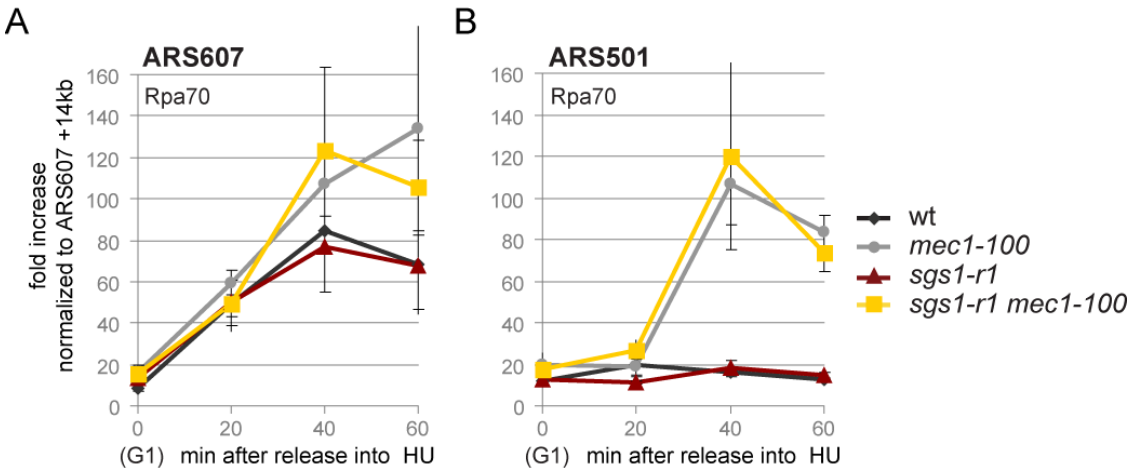


Figure 19 - RPA levels at the fork are not changed in the *sgs1*-r1 or *sgs1*-r1 *mec1*-100 mutant. ChIP of Myc-tagged RPA of GA-5525 (wt), GA-5365 (*sgs1*-r1), GA-5366 (*mec1*-100), GA-5367 (*sgs1*-r1 *mec1*-100) as described under Fig. 3 or under Materials and Methods. **(A)** RPA levels at the early firing origin ARS607 and **(B)** at the late firing origin ARS501

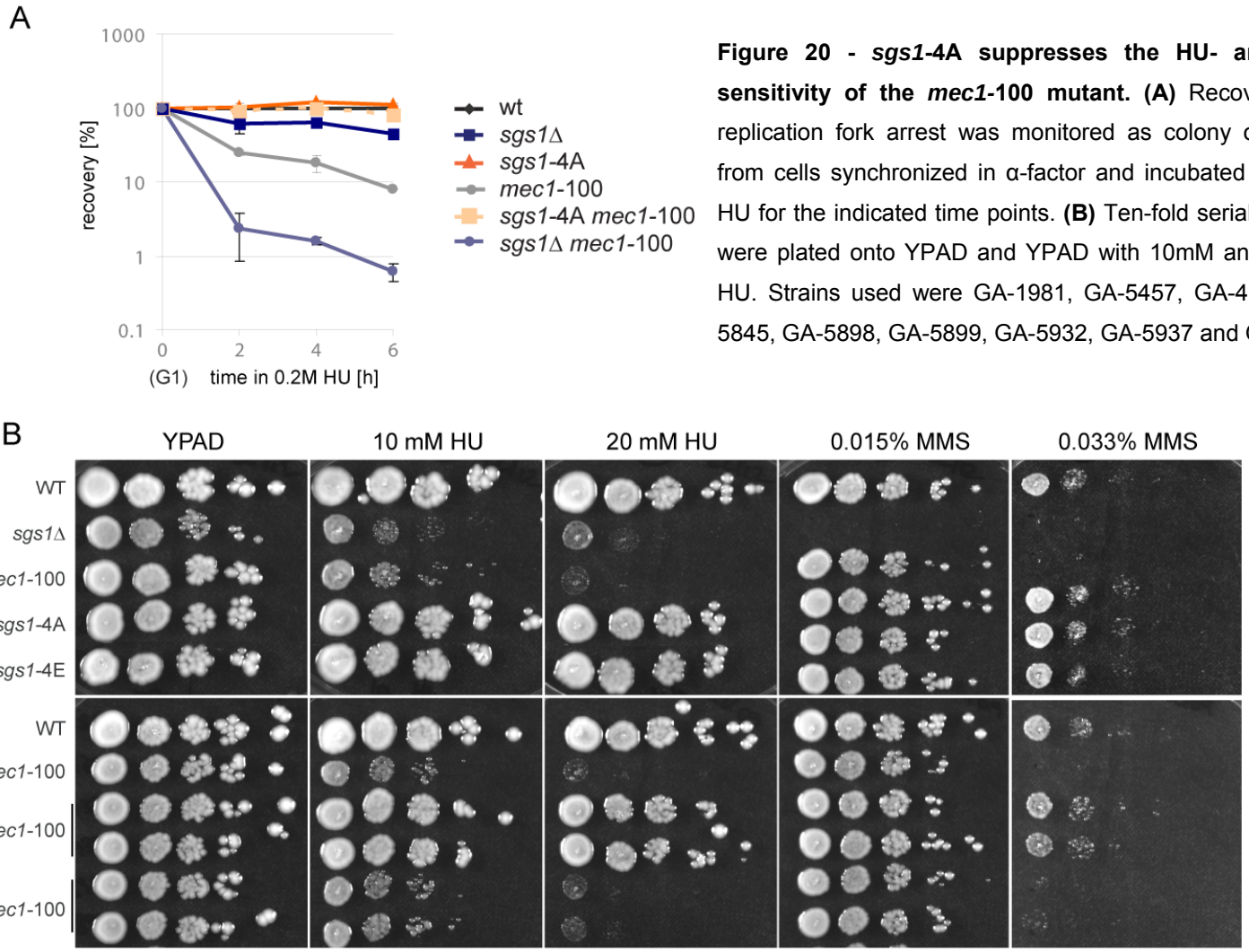


Figure 20 - *sgs1*-4A suppresses the HU- and MMS sensitivity of the *mec1*-100 mutant. (A) Recovery from replication fork arrest was monitored as colony outgrowth from cells synchronized in α-factor and incubated in 0.2 M HU for the indicated time points. **(B)** Ten-fold serial dilutions were plated onto YPAD and YPAD with 10mM and 20 mM HU. Strains used were GA-1981, GA-5457, GA-4978, GA-5845, GA-5898, GA-5899, GA-5932, GA-5937 and GA-5938

Acknowledgements

P. Maillard (initiated the RPA-Sgs1 mapping)

S. Küng (Co-IP)

Sgs1-hd gift from Weinstein et al (Rothstein)

Sgs1(404-604aa) was overexpressed and purified by P. Amsler, M. Vogel

Y. Moriyama helped to construct the *sgs1*-4E and *sgs1*-4A strains

Table 3 - Yeast strains used in this study

Strain	Genotype	Source
GA-180	MAT**a**, *ade2*-1, *trp1*-1, *his3*-11, -15, *ura3*-1, *leu2*-3,-112, *can1*-100 (W303)	S. Elledge
GA-181	MAT**α**, *ade2*-1, *trp1*-1, *his3*-11, -15, *ura3*-1, *leu2*-3,-112, *can1*-100 (W303)	R. Rothstein
GA-426	MATa, *ade2*::hisG, *can1*::hisG, *his3*-11, *leu2*, *trp1*, *ura3*-52, *TelVR::ade2*	H. Renault
GA-880	MAT**a** *his3*, *leu2, ura3, trp1, mec1*-1 *sml1* (A364a background) *sgs1::LEU2*	C. Frei
GA-1211	MATα, his3, trp1, ura3-52, leu2::prolLEU2-lexAop2 (EGY188)	E. Golemis
GA-1456	GA-426 with DIA5-1, *MEC1-myc*	F. Hediger
GA-1759	GA-180 with *pep4::LEU2* ; *SGS1-13Myc::HIS*; *HA-RPA1::URA*	Cobb *et al* 2003
GA-1761	GA-180 with *pep4::LEU2* ; *sgs11-3::TRP* (Sternglanz)	Bjergbaek *et al* 2005
GA-1805	GA-181 with *srs2::HIS*	L. Bjergbaek
GA-1981	MAT**a**, *ade2*-1, *trp1*-1, *his3*-11, -15, *ura3*-1, *leu2*-3,-112, *can1*-100 (W303), RAD5+	H.L. Klein
GA-1982	MAT**α**, *ade2*-1, *trp1*-1, *his3*-11, -15, *ura3*-1, *leu2*-3,-112, *can1*-100 (W303), RAD5+	H.L. Klein
GA-2056	GA-180 with *sgs1::TRP, rad24::URA pep4::LEU2*	Bjergbaek et al 2005
GA-2478	GA-180 with *sml1::KanMX* , *mec1*-100::*LEU2(HIS)*	Cobb *et al* 2005
GA-2514	GA-180 with *mec1*-100::*LEU2 (HIS), sgs1::TRP*	Cobb *et al* 2005
GA-4848	GA-180 with *sgs1*-r1-Myc13::*URA*	This study
GA-4502	GA-180 with *sgs1-r1*	This study
GA-4504	GA-180 with *sml::KanMX* , *mec1*-100::*LEU2(HIS) , sgs1*-r1	This study
GA-4967	GA-1981 with *sgs1::G418, mec1*-100::*LEU2(HIS)*	This study
GA-4973	GA-1981 with *CDC17-3HA*::*TRP1*	This study
GA-4974	GA-1981 with *CDC17-3HA*::*TRP1*, mec1-100::LEU2(HIS)	This study
GA-4978	GA-1981 with *mec1*-100::*LEU2(HIS)*	This study
GA-5055	GA-1981 with *sgs1*-r1-13Myc::*URA, CDC17-3HA*::*TRP1*	This study
GA-5057	GA-1981 with *sgs1*-r1-13Myc::*URA, mec1*-100::*LEU2(HIS)*, *CDC17-3HA*::*TRP1*	This study
GA-5075	GA-1981 with *sgs1*-r1, *mec1*-100::*LEU2(HIS)*, *CDC17-3HA*::*TRP1*	This study
GA-5076	GA-1981 with *sgs1*-r1	This study
GA-5077	GA-1981 with *sgs1*-r1, *mec1*-100::*LEU2(HIS)*	This study
GA-5316	GA-1981 with *sgs1*-r1-13Myc::*URA*, HA-*RPA1*::*URA*, *pep4::LEU2*	This study
GA-5321	GA-1981 with *rad24::TRP*	This study

GA-5324	GA-1981 with *rad24::TRP, sgs1*-r1	This study
GA-5365	GA-1981 with *RPA1*-13Myc::*TRP, Ddc2*-HA::*URA, sgs1*-r1	This study
GA-5366	GA-1981 with *RPA1*-13Myc::*TRP, Ddc2*-HA::*URA*, *mec1*-100::*LEU2(HIS)*	This study
GA-5367	GA-1981 with *RPA1*-13Myc::*TRP*, *Ddc2*-HA::*URA*, *mec1*-100::*LEU2(HIS)*, *sgs1*-r1	This study
GA-5445	GA-1981 with *sgs1*-K706R	R. Rothstein
GA-5447	GA-5445 with *mec1*-100::*LEU2(HIS)*	This study
GA-5449	GA-5445 with *CDC17-3HA*::*TRP1*	This study
GA-5451	GA-5445 with *CDC17-3HA*::*TRP1*, *mec1*-100::*LEU2(HIS)*	This study
GA-5457	GA-1981 with *sgs1::G418*	This study
GA-5525	GA-1981 with *RPA1*-13Myc::*TRP, Ddc2*-HA::*URA*	This study
GA-5845	GA-1981 with *sgs1*-T451E-S470E-S482E-T585E (= *sgs1*-4E)	This study
GA-5895	GA-1981 with *rad24::TRP, sgs1*-4E	This study
GA-5932	GA-1981 with *sgs1*-T451A-S470A-S482A-T585A (= *sgs1*-4A)	This study
GA-5934	GA-1981 with *rad24::TRP, sgs1*-4A	This study

3. *rfa1*-t11 affects the interaction with Sgs1 (and Mre11) & destabilizes replisome components at stalled replication forks

A. M. Friedel, M. Vogel, K. Shimada, P. Amsler, N. Thomae, P. Pasero, S. M. Gasser

3.1. Abstract

Stabiliziation of functional replication forks during S phase is crucial for accurate DNA synthesis to maintain genomic integrity. Recently, it was reported that fork stability in *S. cerevisiae* depends on the checkpoint kinase Mec1 and the RecQ helicase Sgs1. Both pathways were proposed to converge on the single strand binding protein RPA which recruits Mec1-Ddc2 to stalled forks and interacts with Sgs1. To test the effect of the RPA-Sgs1 interaction on replisome stability, we have mapped the binding site to the N-terminal oligonucleotide binding (N-OB) fold of Rpa70 and solved the structure. The crystal structure of budding yeast Rpa70 N-OB displays high 3D conservation with the human homologue, which was shown to mediate different protein interactions via its basic cleft. We made use of the *rfa1*-t11 mutation, which carries a charge reversal mutation pointing towards this cleft. Indeed, *rfa1*-t11 partially disrupts Sgs1 binding as monitored by two-hybrid analysis. Furthermore, we observe that *rfa1*-t11 fails to stabilize DNA pol α in response to the replication inhibitor HU and displays a genome-wide replication defect after recovery from replication stress. All these phenotypes are stronger than for *sgs1*Δ and indicate that only a fraction can be attributed to loss of Sgs1 interaction. In addition, we detect an epistatic relationship between *rfa1*-t11 and *mre11*Δ or *rad51*Δ after HU treatment. This indicates that impaired homologous recombination in *rfa1*-t11 cells might be the reason for the failure to resume DNA synthesis at stalled or collapsed forks.

3.2. Introduction

During S-phase, eukaryotic cells unwind and replicate tremendous amounts of DNA, creating structures that are very sensitive to both intrinsic and extrinsic DNA damaging agents. Thus, to ensure genome integrity, replication forks need to be stabilized to prevent stalling and potential collapse. The intra-S phase checkpoint plays a crucial role in maintaining functional replication forks. It acts directly at the stalled fork, but also activates a global checkpoint response, which regulates DNA repair, late origin firing and cell cycle progression. In addition, there is growing evidence that structural stabilization of the replication fork itself is critical for replication fork restart and progression. Two important factors for the maintenance of stalled replication forks are the conserved checkpoint kinase Mec1-Ddc2 and the RecQ helicase Sgs1 (Cobb, Schleker et al. 2005).

The yeast kinase Mec1-Ddc2 (ATR-ATRIP in man) has been shown to maintain DNA polymerases at stalled replication forks (Cobb, Schleker et al. 2005). Using a partial loss of function mutant, *mec1*-100, which compromises the intra-S phase checkpoint but maintains functional G2/M arrest, it was shown that the function of Mec1 in stabilizing replication forks is largely separable from its role in activating the downstream checkpoint kinase Rad53 (Paciotti, Clerici et al. 2001), (Cobb, Schleker et al. 2005). Indeed, deletion of *RAD53* or strains carrying the kinase-dead *rad53*-11 allele, which does not activate the checkpoint, has little or no effect on DNA polymerase stability (Pellicioli, Lucca et al. 1999), (Cobb, Bjergbaek et al. 2003), (Cobb, Schleker et al. 2005). This suggested that it was not global checkpoint activation but rather the ability of Mec1 to target local replisome components that was important to stabilise the fork by, perhaps, maintaining it in a conformation able to allow efficient restart.

In keeping with the idea that replication fork structure is important, it is interesting that the sole yeast RecQ helicase Sgs1 has been shown to act synergistically with Mec1-Ddc2 in stabilizing stalled replication forks. Deletion of *SGS1* results in

partial destabilization of DNA pol α and DNA pol ε from forks arrested with the replication inhibitor hydroxyurea (HU) (Cobb, Schleker et al. 2005). Complete loss of DNA polymerases and replication protein A (RPA) from stalled forks (as monitored by ChIP) was seen when *sgs1*Δ was combined with *mec1*-100. Additionally, they also observed replication fork collapse and a dramatic increase in gross chromosomal rearrangements (GCR). This role in fork stabilisation is likely conserved since mutations in three of the five human RecQ genes lead to diseases associated with elevated rates of translocations and cancer (Bachrati and Hickson 2008). How Sgs1 acts at the stalled fork to stabilize polymerases was unclear, since no direct interaction between Sgs1 and DNA pol α or DNA pol ε had been reported. One possibility could be via RPA, as it was very recently seen that Sgs1 binds the largest subunit of RPA (Friedel et al, submitted), (Cobb, Schleker et al. 2005).

RPA plays a critical role at the replication fork by preventing intra- and interstrand re-annealing of ssDNA and protecting it from nuclease digestion (Wold 1997). In addition, RPA is thought to promote replication fork stability by several means. Mec1/ATR recruitment to stalled replication forks depends on the interaction between the cofactor Ddc2/ATRIP and RPA (Rouse and Jackson 2002), (Paciotti, Clerici et al. 2000), (Zou and Elledge 2003). Additionally, RPA is necessary for homologous recombination (HR), which has been shown to be critical for replication fork restart upon stalling or collapse (San Filippo, Sung et al. 2008), (Wang, Ira et al. 2004). There is emerging evidence that during replication stress the yeast Mre11-Rad50-Xrs2 (MRX) complex acts as a scaffold to maintain functional replication forks (Tittel-Elmer, Alabert et al. 2009). The MRX complex (or MRN in man) has important functions in homologous recombination, DNA double strand break (DSB) repair and checkpoint activation (Khanna and Jackson 2001), (Tauchi, Kobayashi et al. 2002), (Lavin 2004) (Olson, Nievera et al. 2007). Interestingly, the RAD50 subunit of the MRN complex contains an extended coiled-coil structure with a zinc-hook and belongs to the “structural maintenance of chromosome” (SMC) family of proteins (Hopfner, Craig et al.

2002). Thus Cobb and coworkers suggest that MRX is recruited to stalled forks and functions in a cohesion-like manner to hold the sister chromatids together (Tittel-Elmer, Alabert et al. 2009). In human cells, the recruitment of the MRN complex to stalled replication forks is dependent on RPA (Olson, Nievera et al. 2007).

Given that both Sgs1 and Mec1-Ddc2 bind RPA, we hypothesized that the Sgs1 and Mec1-Ddc2 pathways for maintaining replication fork stability might converge on RPA. To test the effect of the RPA-Sgs1 interaction on replication fork stability, we mapped the region responsible for Sgs1 binding within RPA. We identified the N-OB fold of RPA70 as the main binding site and crystallized this domain, solving the structure with native and selenomethionine labeled Rpa70(3-133) crystals diffracting to a resolution of 2.1 Å and 1.8 Å, respectively. The yeast RPA70 N-OB fold shows a high degree of structural conservation with its human homologue. To study this interaction *in vivo*, we used a RPA70 mutant, *rfa1*-t11, as deletion of the entire N-OB fold is lethal in *S. cerevisiae.* The *rfa1-t11* allele contains a single point mutation (K45E) that is directed towards the putative binding surface of the N-OB fold. (Umezu, Sugawara et al. 1998). Surprisingly, we find that although this mutation destabilizes the interaction with Sgs1 by two hybrid studies, a much higher HU sensitivity for *rfa1*-t11 than for *sgs1*Δ. This suggested that the *rfa1*-t11 mutant, that was previously shown to be proficient for DNA replication under normal conditions (Umezu, Sugawara et al. 1998), shows a strong defect after fork stalling on HU. This defect in stabilizing replisome components was confirmed using DNA pol ChIP, DNA combing and 2D gel analysis. Importantly, it indicated that only a fraction of the *rfa1*-t11 phenotype could be attributed to diminished Sgs1 interaction. We propose that *rfa1*-t11 affects the recruitment of the MRX complex and show epistatic HU sensitivity with HR proteins such as *rad51* and *mre11*. We propose that *rfa1*-t11 destabilizes the structure of the replication fork: it impairs the interaction with Sgs1 but also with the MRX complex thereby affecting replication fork stability and fork restart by homologous recombination.

3.3. Materials and methods

3.3.1. Yeast strains and plasmids

All strains used were derived from W303-1A (*MAT***a** *ade2-1 ura3-1his3-11,15 trp1-1 leu2-3,112 can1-100*) and are listed in Table 4. The *rfa1*-t11 allele was generated by transforming NheI-linearized plasmid pKU2-*rfa1*-t11, kindly provided by Dr. A. Nicolas, into W303-1A (Soustelle, Vedel et al. 2002). Cells were selected for the plasmid-borne *URA3* marker (pop-in) and plated on 5-fluoroorotic acid (5-FOA) (pop-out). Transformants in which *rfa1*-t11 replaced the endogenous *RFA1* allele were identified by sequencing and sensitivity to MMS and HU. For two hybrid analyses, fragments of Sgs1, RPA70, RPA32 and Rad53 were generated by PCR and fused in frame to the B42 activator domain in the pJG46 or the lexA DNA binding domain in the pGAL-lexA vector (Bjergbaek, Cobb et al. 2005). Standard culture conditions for all yeast strains were at 30 °C in YPAD media, unless stated differently.

3.3.2. Drop tests and recovery assays

For drop assays, overnight cultures were diluted to a starting density of OD_{600} = 0.5 and serial 1:10 dilutions were plated on YPD or the appropriate selective medium containing the indicated concentrations of MMS or HU. For liquid recovery or survival assays, overnight cultures were diluted to OD_{600} = 0.15 and grown for 3 h. G1-synchronized cultures were released into YPAD containing 0.2 M HU. After the indicated time points, relevant dilutions were plated onto YPAD and colonies were counted after 3 to 4 days. Recovery in [%] is the fraction of colonies at the indicated doses compared to the untreated control (0 h) normalized to the survival of WT cells for each time point.

3.3.3. Two-hybrid interaction

Two-hybrid analyses were performed as described (Bjergbaek, Cobb et al. 2005). The lacZ reporter pSH1834, the bait and the prey were transformed into EGY191 cells (GA-1211). After glucose depletion, 2% galactose was added to the exponentially growing culture and expression of the fusion proteins induced. The β-galactosidase assay for permeabilized cells was used to detect and quantify protein-protein interactions. Four independent transformants were analyzed in at least two independent experiments. Western blot analysis was used to check the expression of the fusion proteins (data not shown). β-galactosidase units are defined as $OD_{420}/(OD_{600}$*dilution*time(min)).

3.3.4. Protein overexpression and purification

Rpa70 constructs (1-133, 3-133) were amplified and inserted into a pET15 (pNT62e) derived vector, containing a TEV protease cleavable His_6-tag. After overexpression in *E. coli* BL21 strain, cells were lysed by sonication. Rpa70 N-OB constructs were purified by metal chelate affinity (His-Select Nickel Affinity Gel, Sigma-Aldrich), cation-exchange (Resource 15 S, GE Healthcare), and gel-filtration chromatography (Superdex S-200, GE Healthcare). The His-tag was removed by proteolytic digestion with 1% TEV after the metal chelate affinity step. Sgs1 constructs (404-485, 404-560) were amplified and inserted into a pET15 (pNT23e) derived vector, containing a thrombin protease cleavable His_6-tag. After overexpression in *E. coli* BL21 strain, cells were lysed by sonication. Sgs1 constructs were purified by metal chelate affinity (His-Select Nickel Affinity Gel, Sigma-Aldrich), anion-exchange (Resource 15 Q, GE Healthcare), and gel-filtration chromatography (Superdex S-200, GE Healthcare). The His-tag was removed by proteolytic digestion with 1% thrombin after the metal chelate affinity step.

3.3.5. Affinity chromatography

Analytical gel filtration experiments were carried out with purified Rpa70(3-133) and Sgs1(404-485). For complex formation, the Sgs1 peptide was mixed with a six times molar excess of Rpa70(3-133), as determined by UV absorption. The Rpa70/Sgs1 mixture was incubated at RT for 15 min prior to loading on a Superdex-200 gel filtration column (GE Healthcare).

3.3.6. X-ray crystallography

Crystals of Rpa70(3-133) were grown at 4 °C by vapor diffusion as hanging drops prepared by mixing 1 µl of protein (concentration of ≈ 20 mg/ml)/1 µl of the crystallization buffer for native Rpa70(3-133): 100 mM Hepes pH 6.8, 30% PEG 550MME, 150 mM NaCl, 5 mM DTT or 100 mM NH4Ac pH 5.0, 26% PEG 8000, 150 mM NaCl, 5 mM DTT. Indexing of the diffraction patterns from crystals grown under these two conditions identified them as belonging to space group P1 and C2, respectively. Crystals of Se-Met labeled Rpa70(3-133) were also grown at 4 °C by vapor diffusion as hanging drops in a buffer containing 100 mM MES pH 6.0, 1% PEG 8000, 25% PEG 550MME, 150 mM NaCl, 5 mM DTT. These crystals belonged to space group C2. Prior to data collection, crystals were transferred into a cryogenic solution consisting of the reservoir solution plus 10% (v/v) glycerol. Native data sets were collected on a rotating anode equipped with osmic mirrors and a mar345dtb detector. Data collection for the Se-Met labeled protein was carried out at the synchrotron beam line SLS-X10SA (Villigen, Switzerland) equipped with a marCCD225 detector. Images were indexed, integrated and scaled with the HKL-2000 package (Otwinowski and Minor 1997). The resolution of the diffraction data was 2.10 Å for the native protein (P1), 2.08 Å for the native protein (C2) and 1.80Å for the Se-Met labeled protein (C2). The scaled Se-Met peak data set was used to determine the initial phases by single wavelength anomalous dispersion (SAD) as implemented in RANTAN (Yao 1981)

and refinement of heavy atom positions by autosharp (Vonrhein, Blanc et al. 2005). The electron density map of the Se-Met data set was traced using COOT (Emsley and Cowtan 2004) resulting in an initial protein model. This manually built model was compared to the automatic model built by ARP/wARP (Perrakis, Morris et al. 1999). In order to obtain a model for the native protein with sulfur instead of selenium, the Se-Met model was used for molecular replacement as a search model in PHASER (Mccoy, Grosse-Kunstleve et al. 2007).

3.3.7. ChIP analysis

Chromatin immunoprecipitation experiments were performed as described (Cobb, Bjergbaek et al. 2003), (Cobb, Schleker et al. 2005). G1-synchronized cells were released into 0.2 M HU-containing media at 30 °C for approximately 1 h and fixed with 1% formaldehyde at the indicated time points. Monoclonal anti-HA (12CA5, Santa Cruz) was used to precipitate HA-tagged DNA pol α. Cell extracts were incubated with BSA-saturated dynabeads coupled to anti-HA antibody for 2 h at 4 °C. As a background control we used BSA-coupled dynabeads without antibody. Real-time PCR was used for amplification of the precipitated DNA regions. Sequences for the primers/probes that amplify regions in the *S. cerevisiae* genome correspond to ARS607, a site 14 kb away from ARS607 and ARS501 and are available upon request. For quantification, Applied Biosystems 7500 Fast Real-time PCR System and software was used. The data for each strain are the average of 3 independent experiments with real-time PCR performed in duplicate (standard error of the mean is indicated by the error bars). Absolute fold enrichment at ARS607 or ARS501 was calculated for each time point as follows: the signal from the anti-HA-coupled dynabeads was divided by the signal from the BSA-coated dynabeads, after both signals were first normalized to the signal from input DNA. Relative enrichment at ARS607 or ARS501 was obtained by normalizing the absolute enrichment at ARS607 or ARS501 to the absolute enrichment at a locus 14 kb away from ARS607.

3.3.8. 2D gel analysis

Neutral/neutral 2D agarose gels were performed as described (Huberman, Spotila et al. 1987), (Wu and Gilbert 1995). Genomic DNA was isolated from cells at a density of $5x10^6$–$1x10^7$ from GA-4973, GA-5048, GA-4971 and GA-4974 using a G-20 column (QIAGEN) followed by digestion with PstI. Genomic DNA was separated on a 0.4% agarose gel in TBE for 40 h at 0.6 V/cm in the first dimension and on a 1.2% agarose gel in TBE at 3 V/cm for 18 h. Replication intermediates at ARS607 were detected after Southern blotting and hybridization with a DIG-labeled probe. The relative ratio of fork firing is expressed as signal of bubble arc to the amount of 1N linear fragments, normalized to wild-type.

3.3.9. DNA combing

Dynamic molecular combing was performed as described previously (Michalet, Ekong et al. 1997), (Tourriere, Versini et al. 2005). Wild-type (GA-5382), rfa1-t11 (GA-5383), mec1-100 (GA-5385), and rfa1-t11 mec1-100 (GA-5386) were arrested in G1. 20 min before release into S-phase 0.4 mg/ml IdU were added. Cells were incubated for 90 min in YPAD containing 0.2M HU and 0.4 mg/ml IdU, then washed and released into fresh YPAD in presence of 0.4mg/ml CldU for additional 90 min. IdU and CldU were detected with anti-BrdU antibodies (BD44-Becton Dickinson and BU1/75-AbCys, respectively). DNA molecules were counter-stained with an anti-ssDNA antibody (MAB3034, Chemicon) and an anti-mouse IgG coupled to Alexa 647 (Molecular Probes). A Leica DM6000B microscope was used to record the images, which were processed as described (Pasero, Bensimon et al. 2002). DNA fibers from 4 independent experiments were analyzed using MetaMorph. R was used for statistical analysis. Each experiment was checked for batch effects, before all DNA fibers per strain were pooled and analyzed by a paired Wilcox test. DNA fibers analyzed from rfa1-t11,

mec1-100 and rfa1-t11 mec1-100 cells were significantly different from wild-type cells ($P<0.05$).

3.4. Results

3.4.1. The *rfa1*-t11 mutation partially disrupts the interaction with Sgs1 in two-hybrid analysis

The RecQ helicase Sgs1 and the checkpoint kinase Mec1-Ddc2 have been shown to act synergistically in stabilizing polymerases at forks in response to replication stress (Cobb, Schleker et al. 2005). Interestingly, the single strand binding protein RPA has been shown to interact with both Sgs1 and Mec1-Ddc2. On one hand, RPA has been shown to recruit Mec1-Ddc2 to stalled replication forks and to be a target of Mec-Ddc2 phosphorylation, and on the other hand it interacts physically with Sgs1 (Zou and Elledge 2003), (Cobb, Schleker et al. 2005). We investigate in this study whether the pathways converge on RPA. The heterotrimer RPA consists of Rpa70, Rpa32 and Rpa14, named according to their molecular weights and are encoded in yeast by the genes *RFA1*, *RFA2* and *RFA3*, respectively (Figure 21A). Recently, in our studies of the function of Sgs1, we mapped the interaction site between RPA and Sgs1 to the largest subunit of RPA, Rpa70 (Friedel et al, submitted). In this previous study, we focused on narrowing down the interaction site on the 1447 amino acid (aa) Sgs1. We found that the N-terminal oligonucleotide binding (N-OB) fold of Rpa70 interacts with an otherwise uncharacterized acidic region N-terminal to the helicase domain. In contrast, the three conserved RecQ helicase signature domains in Sgs1 (a SF2-type helicase domain, a RQC (RecQ C-terminal) and a HRDC (helicase and RNase D C-terminal) domain) were not important for the interaction with RPA70, nor was a region of unknown structure in the Sgs1 N-terminus that binds Top3/Rmi1.

In this study, we confirmed the interaction of the Rpa70 N-OB fold with the acid region of Sgs1 by affinity chromatography. Figure 21B demonstrates that overexpressed Rpa70 N-OB binds efficiently to an Sgs1(404-485) peptide carrying one RPA interaction site *in vitro*. To look in more detail at the Sgs1-interaction site on Rpa70, we have crystallized the yeast Rpa70 N-OB fold (Figure 21C). Using native and selenomethionine labeled Rpa70(3-133) we solved the structure to a resolution of 2.1 Å and 1.8 Å respectively. In contrast to low sequence conservation between the yeast Rpa70 N-OB and human RPA70 N-OB, both structures display high 3D similarities. The structure consists of a five-stranded β-barrel, capped by short α-helices and a basic cleft in the middle. The loops L12 and L45, which connect the first with the second and the third with the fourth β-strand show the biggest structural difference to those of human RPA70. Thus the RMSD calculated by lsqkab (CCP4 supported program; (Emsley and Cowtan 2004), (Kabsch 1976) including the loops was 7.12 Å. Omitting both loops from the calculation, yielded a much smaller difference in RMSD of 2.12 Å, suggesting that the L12 and L45 loops are structurally not related to those found in the human RPA70 N-OB fold.

In human cells the basic cleft of RPA70 N-OB has been implicated in mediating interactions to other checkpoint or replication proteins, for example p53, ATRIP, RAD9 or MRE11 (Bochkareva, Kaustov et al. 2005), (Ball, Ehrhardt et al. 2007), (Xu, Vaithiyalingam et al. 2008). Therefore, we made use of an existing RPA70 mutant, the *rfa1*-t11 allele (Philipova, Mullen et al. 1996), (Umezu, Sugawara et al. 1998), which carries a charge reversal mutation (K45E) pointing towards the basic cleft of the N-OB (Figure 21C). To test if the *rfa1*-t11 mutation disrupts the interaction with Sgs1, we fused full-length *rfa1*-t11 and an N-OB fragment carrying the *rfa1*-t11 mutation to the LexA-DBD and tested the constructs in two-hybrid analysis with the Gal4-TAD-Sgs1(290-1180) core fragment (Figure 21D). Indeed, in contrast to wild-type Rpa70 N-OB, the N-OB fold carrying the *rfa1*-t11

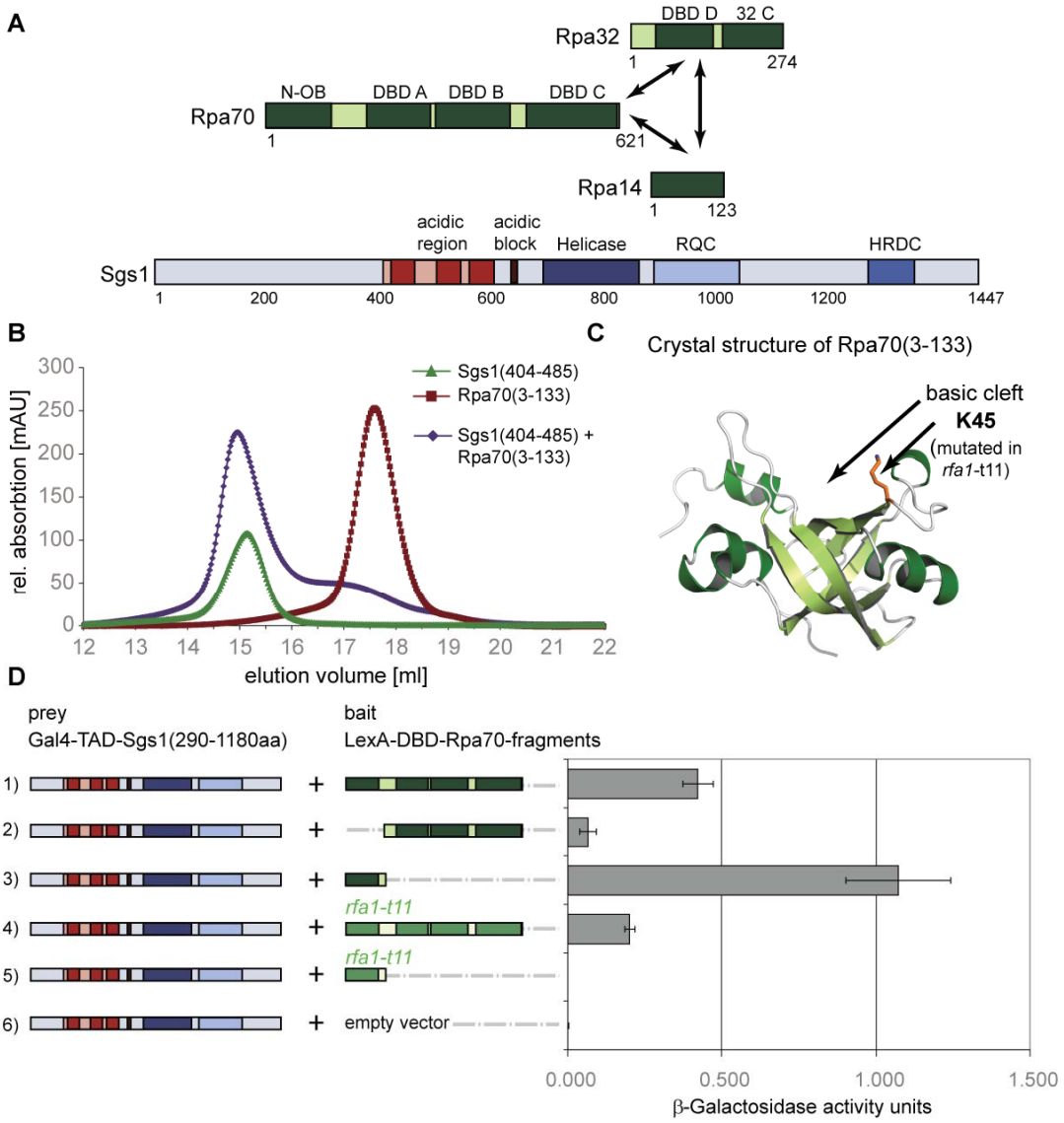


Figure 21 - *rfa1*-t11 partially disrupts the interaction with Sgs1 in two hybrid analysis. A) Schematic representation of RPA and Sgs1 including their functional domains. The trimeric ssDNA binding protein RPA consists of six oligonucleotide binding (OB) folds (N-OB, DBD A-D, RPA14). Sgs1 consists of 1447 amino acids and contains a DExH helicase domain, a RecQ family C-terminal domain (RQC), a Helicase RNase D C-terminal domain (HRDC) and an acidic region N-terminal to the helicase domain (in red), which was shown to be important for RPA70 interaction and intra-S phase checkpoint activation. **B)** Affinity chromatography of Sgs1(404-485) with Rpa70(3-133). The N-terminal OB fold of Rpa70 forms a complex with overexpressed Sgs1(404-485) (blue curve).

Figure 21 (continued) **C)** The crystal structure of the yeast N-terminal OB fold of Rpa70 is very close to the structure of the human N-OB of RPA70, consisting of a five-stranded ß-barrel (light green) capped by short α-helices (dark green) and a basic cleft in the middle. The flexible loops are indicated in grey. The lysine (K45) mutated in the *rfa1*-t11 mutant points towards the basic cleft (highlighted in orange). **D)** Two-hybrid assay between Sgs1(290-1180) fused to the Gal4-TAD and Rpa70 fragments fused to lexA-DBD. The N-terminal OB fold of Rpa70 showed the highest β-galactosidase activity with Sgs1(290-1180). In contrast to the strong interaction observed with the N-OB of Rpa70, the same fusion protein carrying the *rfa1*-t11 (K45E) abolished completely the interaction with Sgs1(aa 290-1180), while full-length Rpa70 carrying the K45E mutation shows a 50% decrease in β-galactosidase activity.

mutation did not give rise to a β-galactosidase signal, suggesting that *rfa1*-t11 completely disrupts the main interaction site of Sgs1. However, full-length *rfa1*-t11 only reduced β-galactosidase activity by 50% compared to the identical fragment without the K45E mutation. Thus, we suspect that there are additional RPA sites that contribute to Sgs1 binding and that the *rfa1*-t11 mutation will not completely disrupt the RPA-Sgs1 interaction *in vivo*.

3.4.2. *rfa1*-t11 and *mec1*-100 are on parallel pathways in response to HU

rfa1-t11 cells were previously shown to be sensitive to the alkylating agent MMS and the replication fork inhibitor HU on plates (Soustelle, Vedel et al. 2002) (Umezu, Sugawara et al. 1998), (Kim and Brill 2001). We used the same NheI-linearized pKU2-*rfa1-t11* plasmid to introduce the *rfa1*-t11 mutation into the endogenous *RFA70* locus in the W303-1A background and confirmed that our *rfa1-t11* mutant strain was sensitive to growth on HU and MMS (Figure 22A). We also found that the HU-sensitivity of *rfa1*-t11 is independent of the growth temperature or whether the DNA repair gene *RAD5* gene is present in its wild-type form or a commonly occurring mutant form in W303, rad5-35 (data not shown). Wild-type growth on HU could be completely restored by complementing the *rfa1*-t11 mutant with WT-RPA70 on a CEN-ARS plasmid (Figure 26).

Cobb and coworkers had reported that *sgs1*Δ cells display additive HU and MMS sensitivity, when combined with the checkpoint mutant *mec1*-100 (Cobb, Schleker et al. 2005). Therefore, we checked whether *rfa1*-t11 is like *sgs1*Δ on a parallel pathway as *mec1*-100 in response to HU or MMS. Interestingly, the *rfa1*-t11 mutant was significantly more sensitive to HU and MMS than a *mec1*-100 mutant and the *rfa1*-t11 *mec1*-100 double mutant seemed to display additive HU-sensitivity (Figure 22A).

To examine if *rfa1*-t11 and *mec1*-100 act also on a parallel pathways for replication fork stabilization, we determined cell survival in response to HU in S-phase (Figure 22B). Remarkably, *rfa1*-t11 showed a strong synergistic effect when combined with *mec1*-100 in response to HU-treatment. This suggests that, like *sgs1*Δ, *rfa1*-t11 acts on a parallel pathway to *mec1*-100. However, the HU-sensitivity of the *rfa1*-t11 *mec1*-100 double mutant is more pronounced than the *sgs1*Δ *mec1*-100 double mutant, and moreover, *rfa1*-t11 HU-sensitivity is additive with *sgs1*Δ, indicating that although a part of the *rfa1*-t11 phenotype might be attributed to diminished Sgs1 interaction, both proteins - RPA as well as Sgs1 - have other functions at a stalled replication fork that don't depend on their interaction (Figure 22B,C).

3.4.3. DNA pol α is displaced from the HU-arrested replication fork in the *rfa1*-t11 and *rfa1*-t11 *mec1*-100 mutants

In response to HU, which provokes depletion of free dNTP levels, early replication forks stall. To prevent fork collapse and to maintain functional replication forks, replisome components such as DNA polymerases need to be stabilized at arrested forks. The checkpoint kinase Mec1 has been reported to prevent replication fork collapse and the S-phase specific *mec1*-100 mutant

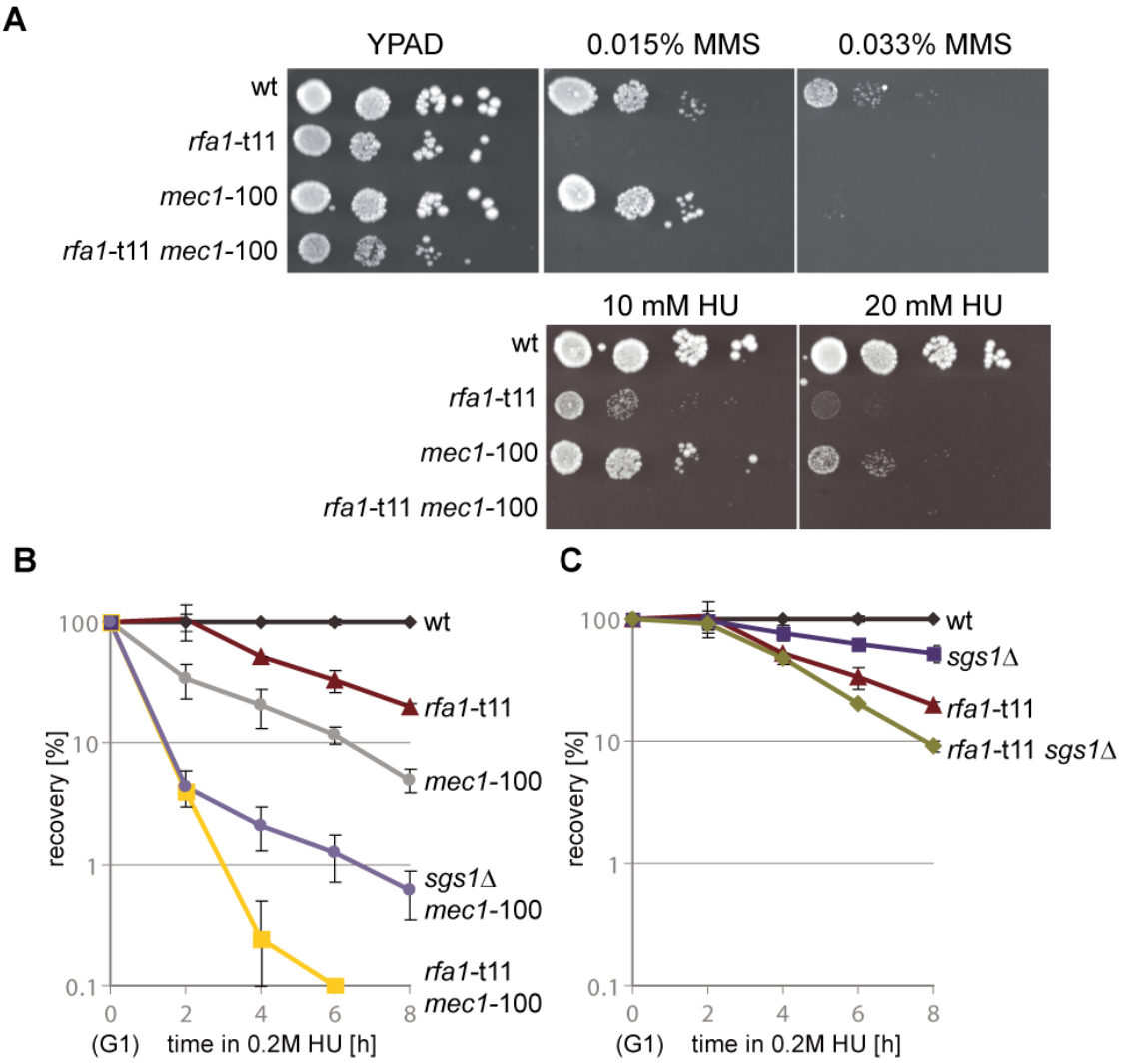


Figure 22 - The *rfa1*-t11 mutant is sensitive to hydroxyurea on plates and in the recovery from S-phase specific HU-arrest. A) Ten-fold serial dilutions were plated onto YPAD, YPAD with 10mM HU, 20mM HU, 0.015% MMS or 0.033% MMS. In contrast to wild-type cells *rfa1*-t11 shows strong sensitivity to both MMS and HU. *rfa1*-t11 together with *mec1*-100 displays additive sensitivity to very low HU concentrations. Strains used were GA-4973, GA-5048, GA-4974 and GA-4971. **B-C)** Recovery from HU-induced replication fork stalling. In G1 synchronized isogenic strains were released into S-phase into 0.2M HU containing YPAD for indicated times. Recovery was monitored as colony outgrowth. Strains used were GA-180 (WT), GA-880 (*sgs1*Δ), GA-4698 (*rfa1*-t11), GA-4704 (*rfa1*-t11 *sgs1*Δ), GA-2478 (*mec1*-100), GA-2514 (*sgs1*Δ *mec1*-100), GA-4702 (*rfa1*-t11 *mec1*-100).

displays a partial loss of both DNA pol α and DNA pol ε in response to HU-induced replication fork stalling (Cobb, Schleker et al. 2005).

The strong HU sensitivity phenotype of *rfa1*-t11 and the synthetic sensitivity of the *rfa1*-t11 *mec1*-100 mutant prompted us to investigate DNA polymerase association after HU-treatment in those mutants by Chromatin immunoprecipitation (ChIP). We synchronized cells in G1 then released them into S phase into YPAD containing 0.2 M HU (Cobb, Bjergbaek et al. 2003), (Bjergbaek, Cobb et al. 2005). We studied the abundance of DNA pol α by real-time PCR at an early firing origin ARS607 and a late firing origin ARS501. As a negative control we examined a locus 14 kb away from ARS607 and used this locus to normalize the absolute enrichments from ARS607 and ARS501 (Figure 23A). As observed previously, *mec1*-100 mutants displayed a partial loss of DNA pol α at the early firing origin ARS607 in response to HU. Interestingly, we noticed an almost complete loss of DNA pol α from ARS607 in the *rfa1*-t11 mutant (Figure 23A, left panel). This very strong effect cannot simply be explained by the loss of interaction with Sgs1, similarly to the data from the recovery assay. *sgs1*Δ alone, or the *sgs1*-r1 mutation that completely disrupts the Sgs1-RPA interaction by two-hybrid, results in only a partial displacement of DNA pol α from a HU-arrested replication fork (Cobb, Schleker et al. 2005), (Friedel et al, submitted). Thus we speculate that the *rfa1*-t11 mutation also affects the binding of another protein, which stabilizes the fork.

In contrast to early firing origins, late firing origins, like ARS501, get repressed in response to HU-treatment by the intra-S checkpoint response and do not fire until the threat is removed. Consequently, DNA pol α is not enriched at ARS501 in wild-type cells, but it is present in *mec1*-100 that cannot signal a checkpoint arrest (Figure 23A, right panel). When *rfa1*-t11 is combined with the *mec1*-100 mutant, DNA pol α is completely displaced from the stalled replication fork. This is true for the early firing origin ARS607 and the late firing origin ARS501,

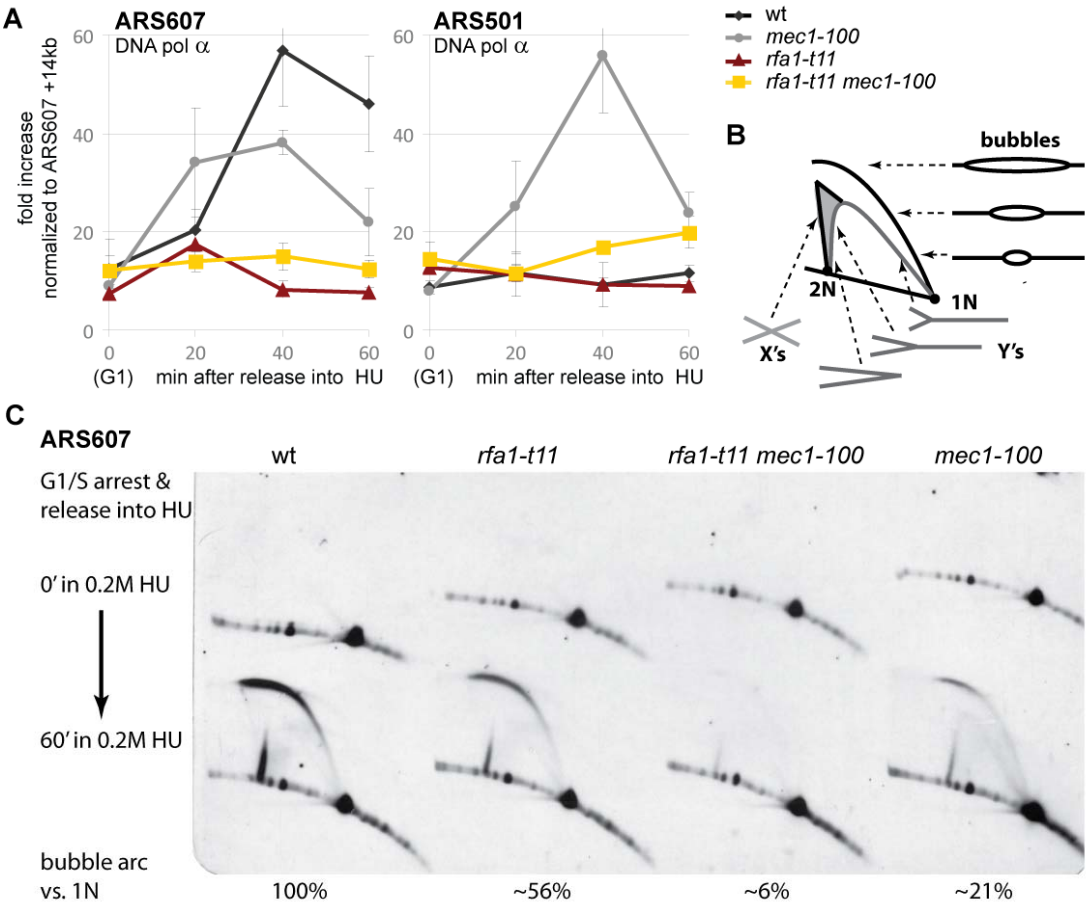


Figure 23 - *rfa1*-t11 completely destabilizes DNA pol α from the HU-stalled replication fork. **A)** DNA pol α ChIP was performed on synchronized cultures, which were released into S phase in the presence of 0.2M HU. Strains used were GA-2238 (wild-type), GA-4974 (*mec1*-100), GA-4802 (*rfa1*-t11) and GA-4800 (*rfa1*-t11 *mec1*-100). The relative enrichment for ARS607 or ARS501 was calculated by normalizing the absolute enrichment at ARS607 or ARS501 for each time point to the absolute enrichment at a locus 14kb away from the early origin ARS607. **B)** Schematic representation of replication intermediates as visualized in 2D gels. 1N – non-replicated fragment, 2N – almost fully replicated fragment just before sister chromatid separation, Y's – Y-structures, X's – recombination structures. **C)** Analysis of replication fork intermediates at the early firing origin ARS607 of wild-type cells (GA-4973), *rfa1*-t11 (GA-5048), *rfa1*-t11 *mec1*-100 (GA-4971) and *mec1*-100 (GA-4974) cells. G1-synchronized cells were released into S-phase in HU-containing media for 1 h. The DNA from the indicated strains was digested by PstI separated on 2D gels and analyzed by Southern blot using a DIG-labeled probe against ARS607. The relative ratio of fork firing was obtained by normalizing the ratio of bubble arc signal over 1N signal to wild-type.

indicating that either the origins don't fire or they collapse and resulting in polymerase displacement.

In addition to DNA pol ChIP, we examined the replication intermediates of *rfa1*-t11 and *rfa1*-t11 *mec1*-100 cells in response to HU by 2D-gel analysis. We arrested cells in G1 and released them into S-phase in the presence of HU. After 60 min we collected the cells and prepared genomic DNA, which was digested by PstI and subsequently separated by agarose gel electrophoresis according to its molecular weight and shape. At 0 min in HU all cells were synchronized in G1 as can be seen by the lack of replication intermediates on the gel and the strong signal for unreplicated 1N DNA (Figure 23C). An origin that fires efficiently and is digested symmetrically gives rise to a bubble arc as monitored for wild-type (Figure 23B, C). Interestingly, the *rfa1*-t11 mutant is also able to fire the early firing origin ARS607, however compared to wild-type only 50% of the bubble arc signal is observed (Figure 23C). This implies that either ARS607 fires less efficiently in the *rfa1*-t11 mutant or that the origin fires and replication forks collapse at an early timepoint. The phenotype of the *rfa1*-t11 *mec1*-100 double mutant is even more pronounced, so that only 6% of the bubble arc was measured compared to wild-type. Thus consistent with the data for DNA pol α ChIP there are less replication intermediates observed at the early firing origin ARS607 for *rfa1*-t11 and *rfa1*-t11 *mec1*-100 cells after exposure to the replication fork inhibitor HU.

3.4.4. *rfa1*-t11 and *rfa1*-t11 *mec1*-100 cells show a genome-wide defect in recovery from HU-arrest

To address the question, whether the *rfa1*-t11 mutation causes a genome-wide replication defect in response to replication stress, we chose to do single molecule analysis by DNA combing of mutant and wild-type cells after recovery from HU treatment (Tourriere, Versini et al. 2005). We arrested G1 synchronized cells in YPAD containing 0.2 M HU and 0.4 mg/ml IdU (Figure 24A). After 90 min,

we released the cells from HU-arrest and incubated them in fresh YPAD in presence of 0.4mg/ml CldU. After preparation of genomic DNA, the IdU/CldU-substituted ssDNA fibers were combed onto silanized coverslips and immunodetected using primary antibodies against IdU/CldU and ssDNA. Due to cross-reaction of the IdU- and CldU-specific antibodies, both channels are shown together as “replicated fiber fraction” (green/white channels) (Figure 24C). As previously described by Tourriere *et al*, wild-type cells recover quickly from HU-arrest (Tourriere, Versini et al. 2005). This is consistent with our data, where DNA fibers from wild-type cells are almost completely replicated with few unreplicated gaps detected after 90 min recovery (Figure 24B,C). All mutant cells, *rfa1*-t11, *mec1*-100 and *rfa1*-t11 *mec1*-100, display defects in recovery from HU treatment, as can be noticed by significantly longer unreplicated regions and a higher number of gaps compared to wild-type. This effect is strongest in the *rfa1*-t11 *mec1*-100 double mutant, where less than 20% of the fiber fractions are completely replicated in contrast to 50% in wild-type cells (Figure 24B). Thus our data demonstrate that *rfa1*-t11 cells, especially when combined with the *mec1*-100 mutation, show strong defects in recovery from HU-mediated replication fork arrest, which is most likely due to replication fork collapse after stalling.

3.4.5. *rfa1*-t11, *mre11*Δ and *rad51*Δ act on the same pathway after replication fork stalling

Homologous recombination (HR) is important for the recovery of stalled or collapsed replication forks and efficient resumption of DNA synthesis after HU-mediated replication fork stalling (Wang, Ira et al. 2004), (Alabert, Bianco et al. 2009). We therefore investigated, whether the lack of recovery from HU-stalling observed in the *rfa1*-t11 mutant could be due to impaired homologous recombination. Previous studies have shown that *rfa1*-t11 is deficient in recombination, mating type switching and single strand annealing after induction of a DNA double strand break (Umezu, Sugawara et al. 1998), (Kantake, Sugiyama et al. 2003), (Wang and Haber 2004). We combined *rfa1*-t11 with

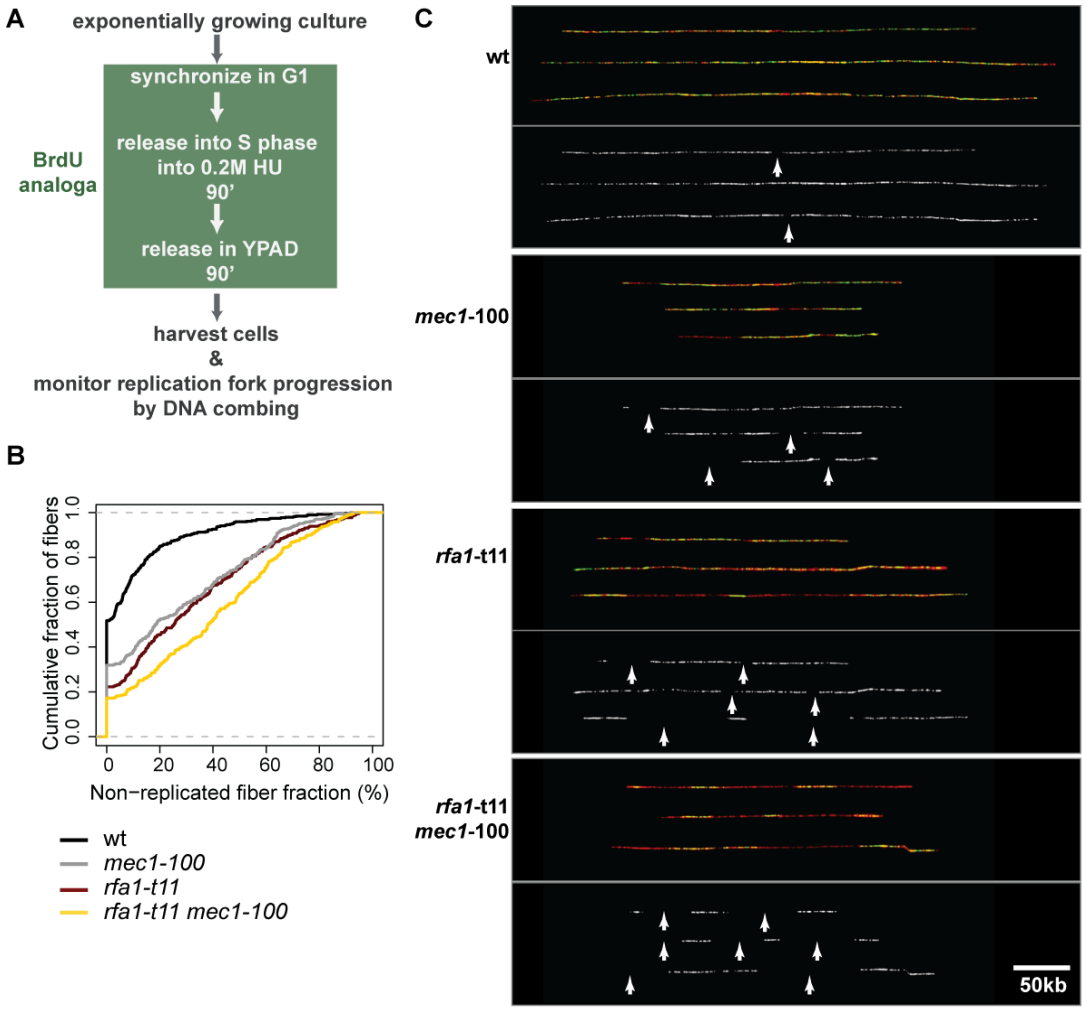


Figure 24 - Genome wide analysis of replication fork recovery after HU-stalling by DNA combing. A) Schematic representation of experimental procedure for DNA combing. Wild-type (GA-5382), *rfa1*-t11 (GA-5383), *mec1*-100 (GA-5385), and *rfa1*-t11 *mec1*-100 (GA-5386) cells were synchronized in G1 and released into S-phase into media containing 0.2M HU and 0.4 mg/ml IdU. After 90 min, cells were washed and resuspended in fresh YPAD containing 0.4mg/ml CldU for additional 90 min. DNA fibers were analyzed by DNA combing as described under materials and methods. **B)** Quantitative analyses of the replication status of all DNA fibers for each strain. The cumulative fiber fraction was plotted over the non-replicated fiber fraction. 50% of the wild-type DNA fibers were completely replicated in comparison to less than 20% of the *rfa1*-t11 *mec1*-100 double mutant. **C)** Representative DNA fibers after 90 min recovery from HU-stalling. The replicated fiber fraction (IdU and CldU channel together) is shown separately for clarity. The arrows indicate the gaps, the bar represents 50kb. red channel – ssDNA; green, white channel – IdU/BrdU substituted DNA.

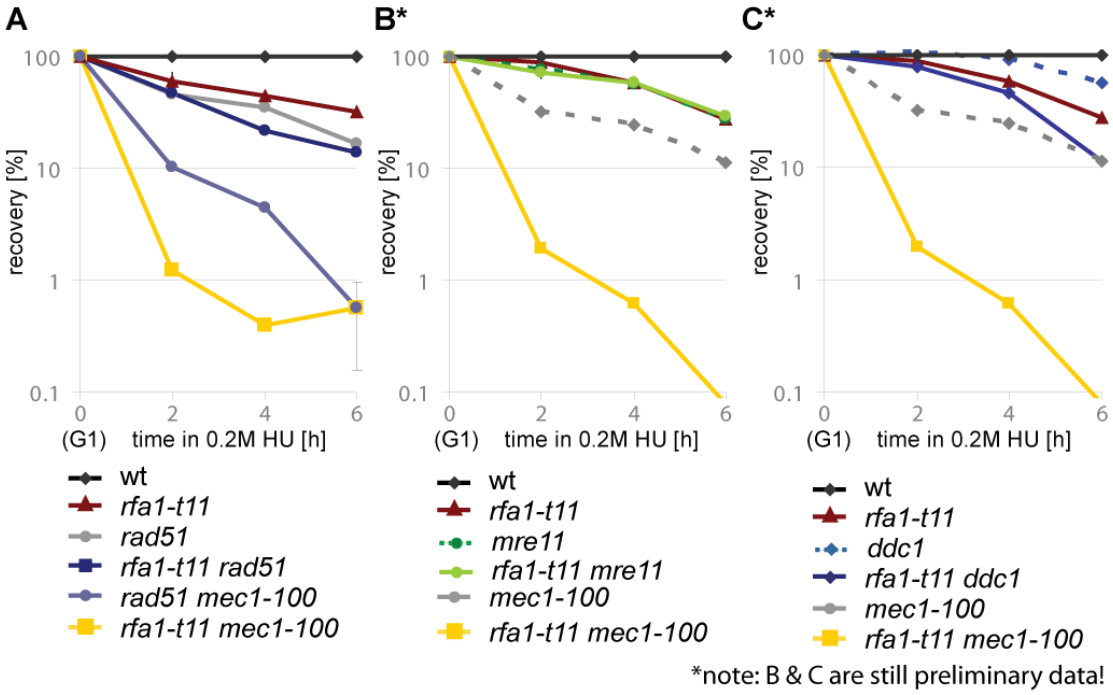
*rad51*Δ. Rad51 is a recombinase, which binds ssDNA coated by RPA and mediates the strand invasion step during HR (San Filippo, Sung et al. 2008). In the recovery assay, *rad51*Δ is slightly more sensitive to HU than *rfa1*-t11 (Figure 25A). The double mutant displays almost the same recovery efficiency from HU as *rad51*Δ alone, suggesting that *rfa1*-t11 functions on the same pathway as *rad51*Δ.

The N-terminal OB fold of human RPA70 has important roles in mediating protein-protein interactions. Olson and coworkers have shown that it mediates interaction between human RPA and the Mre11/Rad50/Nbs1 (MRN) complex via direct interaction with Mre11 (Olson, Nievera et al. 2007). Mre11 is the core component of the Mre11/Rad50/Xrs2 (MRX) complex in *S. cerevisiae* and interacts with both, Rad50 and Nbs2/Xrs2 (Hopfner, Craig et al. 2002), (Williams, Dodson et al. 2009). Mre11 binds various types of duplexed DNA, ends of ssDNA and possesses 3'-5' exonuclease and endonuclease activity (Paull and Gellert 1998). MRX in yeast and the MRN in man are highly conserved and participate in multiple pathways to maintain genomic integrity. In addition to its well studied function in DSB repair by homologous recombination, MRN/MRX has been implicated in non-homologous end-joining, the DNA-damage and intra-S phase checkpoint and in stabilizing the replisome during replication stress (Khanna and Jackson 2001), (Tauchi, Kobayashi et al. 2002), (Lavin 2004) (Olson, Nievera et al. 2007), (Zha, Boboila et al. 2009), (Tittel-Elmer, Alabert et al. 2009).

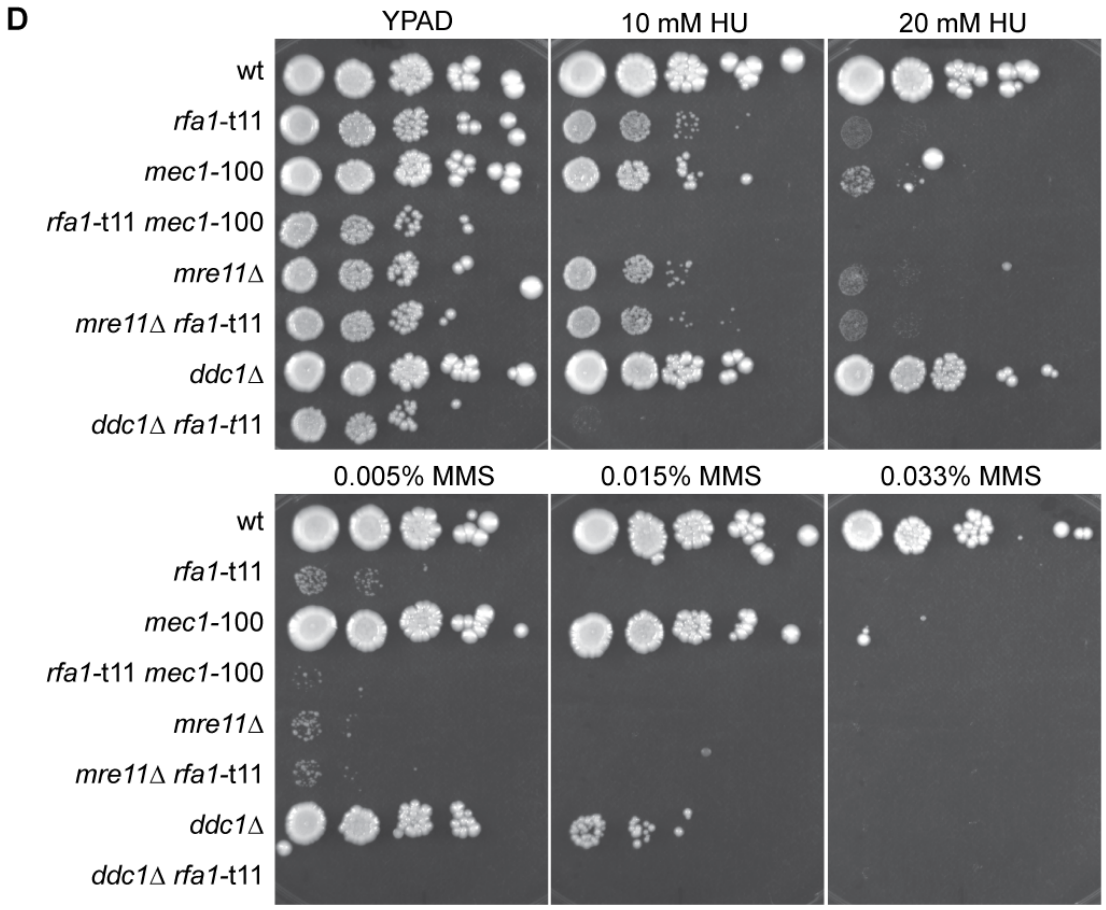
Here we show that deletion of *mre11* phenocopies the HU and MMS sensitivity of the *rfa1*-t11 mutant on both drop tests and S-phase specific recovery from HU-arrest (Figure 25B,D). Consistently, the HU/MMS phenotype of the *rfa1*-t11 *mre11*Δ double mutant is exactly the same as the phenotypes detected for either single mutant, suggesting an epistatic relationship between *mre11*Δ and *rfa1*-t11 (Figure 25B,D).

Kanoh *et al* reported that the *rfa1*-t11 mutation reduces the recruitment of the DNA damage checkpoint sensor Ddc1-Rad17-Mec3 (9-1-1) to HU-stalled replication forks (Kanoh, Tamai et al. 2006). Furthermore, it was shown that RAD9 a component of the human 9-1-1 complex (RAD9-HUS1-RAD1) interacts with the N-terminal OB-fold of human RPA70. We therefore addressed whether *rfa1*-t11 and 9-1-1 act genetically on the same pathway in *S. cerevisiae*. In contrast to Mre11, deletion of Ddc1, a component of the 9-1-1 complex that is important for Mec1-Ddc2 activation after DNA damage, results in additive HU and MMS sensitivity with *rfa1*-t11 (Figure 25C,D). This suggests that *rfa1*-t11 and 9-1-1 act on parallel pathways and that the reduced recruitment of 9-1-1 to stalled replication forks noticed by Kanoh and coworkers does not explain the *rfa1*-t11 phenotype in response to HU.

Figure 25 (following page)- Synthetic interactions of *rfa1*-t11 with *rad51*Δ, *mre11*Δ and *ddc1*Δ. A - C) Recovery from HU-induced fork stalling was monitored as colony outgrowth from in G1 synchronized strains, which were released into S-phase into 0.2M HU containing YPAD for indicated times. *rfa1*-t11 *rad51*Δ shows almost the same HU-sensitivity as the *rad51*Δ single mutant. *rfa1*-t11 *mre11*Δ and *rfa1*-t11 *mre11*Δ mutants display exactly the same HU-sensitivity, while *ddc1*Δ and *rfa1*-t11 show additive behaviour. **D)** Drop assay of the indicated strains. Ten-fold serial dilutions were plated onto YPAD, YPAD with 10 mM HU, 20 mM HU, 0.015% MMS or 0.033% MMS. Strains used were GA-1981, GA-4968, GA-4978, GA-4980, GA-5908, GA-5914, GA-5940 and GA-5942.



*note: B & C are still preliminary data!



3.5. Discussion

3.5.1. The binding mode for the RPA70 N-OB to different replication and checkpoint proteins is conserved from yeast to man

This study initially aimed to clarify which impact the RPA-Sgs1 interaction has on replisome stabilization. We crystallized the yeast N-terminal OB-fold of RPA70 and observed strong structural conservation with the human homologue. Previously, the N-terminal OB-fold has been implicated in various protein-protein interactions. Bochkareva and coworkers monitored that the transactivation domain of p53, which is normally disordered in solution, undergoes a conformational change upon RPA binding in human cells (Bochkareva, Kaustov et al. 2005). Two amphiphatic helices are formed which bind to the basic cleft of the N-OB fold of RPA70. Similar binding modes have been proposed for ATRIP, RAD9 and MRE11 involving a conserved DDXD/E motif (Ball, Ehrhardt et al. 2007), (Xu, Vaithiyalingam et al. 2008). The Sgs1 region that binds yeast N-OB is also predicted to be structurally disordered in solution and contains two DDXD/E motifs, thus we suggest that Sgs1 binds the basic cleft of the RPA70 N-OB (Friedel et al, submitted).

To test this hypothesis, we used the *rfa1*-t11 mutant, which carries a charge reversal mutation pointing towards the basic cleft of the N-OB of Rpa70. The *rfa1*-t11 mutant was shown to disrupt the main interaction between Rpa70 and Sgs1 in two hybrid, indicating that indeed a general binding mechanism between RPA and other checkpoint and replication proteins exists, which is conserved from yeast to man. However our two hybrid data also suggest that additional interaction sites exist outside the N-OB fold. Thus it is likely that the Sgs1-rfa1-t11 interaction is only to some extend affected *in vivo*. Therefore, the phenotype observed for the *rfa1*-t11 mutant can only be partially ascribed to loss of the Sgs1 mutation.

3.5.2. *rfa1*-t11 destabilizes replication fork components and displays strong defects after recovery from HU-induced replication fork arrest

We observe a very strong HU phenotype for *rfa1*-t11 on plates and in the S-phase specific recovery assay. This is in agreement with data reported from Kim et al, who monitored no growth of *rfa1*-t11 cells on 50 mM HU (Kim and Brill 2001). However, Kanoh et al does not detect HU sensitivity for *rfa1*-t11 in the recovery assay in reponse to 200 mM HU (Kanoh, Tamai et al. 2006). This might be explained by different experimental settings, eg. lower culturing temperature (23 °C instead of 30 °C) and the shorter exposure time to HU before plating on YPAD (only 4h). Alternatively, it could be attributed to the differences of the yeast backgrounds (BY4741 vs. W303-1a).

The HU sensitivity of the *rfa1*-t11 mutant suggests that it destabilizes replisome components after fork stalling. Consistently, we observe for *rfa1*-t11 cells almost complete displacement of DNA pol α from the stalled replication fork and 50% less replication intermediates at ARS607 one hour after HU treatment. In addition, cells carrying the *rfa1*-t11 mutation display strong defects in recovery from HU-induced replication fork arrest. Taken together, our data suggest that in response to HU either origin firing is reduced or replication forks collapse in *rfa1*-t11 cells. Different groups demonstrated that *rfa1*-t11 is proficient for replication under normal conditions, but is defective for meiotic and homologous recombination (Umezu, Sugawara et al. 1998), (Soustelle, Vedel et al. 2002), (Kantake, Sugiyama et al. 2003), (Wang and Haber 2004). Other studies have shown that HR itself is crucial for the maintenance of replication forks and to restart DNA synthesis after fork collapse (San Filippo, Sung et al. 2008). Therefore we propose that the HU phenotype of *rfa1*-t11 results mainly from fork collapse, although we can not exclude the possibility of reduced origin firing.

Combination of *rfa1*-t11 with *mec1*-100 results in synergistic sensitivity in response to HU. DNA pol α is completely displaced from the early and late firing origins, ARS607 and ARS501 respectively. Concordantly, almost no bubble arc is detected at ARS607 one hour after release into HU and *rfa1*-t11 *mec1*-100 double mutants seem to recover from fork stalling even worse than *rfa1*-t11 or *mec1*-100 as monitored by DNA combing. This indicates that *rfa1*-t11 and *mec1*-100 act on parallel pathways in replication fork stabilization. In addition, all those phenotypes for *rfa1*-t11 and *rfa1*-t11 *mec1*-100 in response to HU are far stronger than observed for *sgs1*Δ or *sgs1*-r1, suggesting that the *rfa1*-t11 phenotype can not be explained by a mere loss of Sgs1-RPA interaction. We therefore suspect that *rfa1*-t11 disrupts the binding of another important checkpoint or replication protein, which affects replisome stability.

3.5.3. *rfa1*-t11 might affect the interaction with MRX and therefore impairs replication fork restart by HR leading to fork collapse

To test if compromised HR might be the reason for defective replication fork stabilization of *rfa1*-t11, we investigated whether *rfa1*-t11 acts on the same pathway as the HR mutant, *rad51*Δ. Indeed, *rfa1*-t11 *rad51*Δ double mutants show almost the same HU sensitivity as the *rad51*Δ single mutant. In addition, it was reported that the human MRN complex is important for proper HR and that its recruitment to sites at or adjacent to origins depends on RPA (Tauchi, Kobayashi et al. 2002), (Olson, Nievera et al. 2007). In this study, we show that *rfa1*-t11 acts in the same pathway as *mre11*Δ. Tittel-Elmer *et al* have demonstrated that MRX is recruited to stalled replication forks in yeast (Tittel-Elmer, Alabert et al. 2009) and that MRX acts as a scaffold at stalled sites and stabilizes the replisome, independent of its nuclease activity. Similar to what we detect for *rfa1*-t11, deletion of *MRE11* leads to displacement of DNA pol α and DNA pol ε from HU-arrested replication forks, correlating with significantly shorter BrdU tracks as observed by DNA combing (Tittel-Elmer, Alabert et al. 2009).

Therefore, we propose that *rfa1*-t11 affects the recruitment of the MRX complex to stalled forks.

Human data suggest that MRX suppresses late origin firing. Olson et al used cell lines with hypomorphic mutations of *MRE11* and enriched them in S phase by a double thymidine block (Olson, Nievera et al. 2007). When they complemented the cells with MRE11-mutants defective for RPA70 interaction, they observe ATM, NBS and CHK2 phosphorylation similar to wild-type MRE11 in response to IR. Interestingly, they monitored an increase in new origin firing after IR for the MRE11-mutants. The authors suggest active suppression of late origin firing by the MRN complex, which is tethered through its interaction with RPA to replication-proximal sites. However, it is also possible that due to replication fork inhibition or collapse, dormant origins within the active replicon clusters got activated (Blow and Ge 2009). These dormant origins are licensed by MCM2-7 and not used during normal S-phase, but get replicated passively from adjacent origins (Woodward, Gohler et al. 2006), (Ge, Jackson et al. 2007).

When we combine *rfa1*-t11 with *ddc*1Δ, we see enhanced HU sensitivity for the double mutant, while the *ddc1*Δ single mutant is almost as resistant as wild-type. Ddc1 is a subunit of the 9-1-1 complex and is especially important for checkpoint activation in response to DNA damage (Navadgi-Patil and Burgers 2008). In contrast to MMS, exposure to HU leads mainly to inhibition of DNA synthesis and not to DNA damage. Therefore, checkpoint activation by 9-1-1 is likely not required and deletion of *ddc1* without major consequences. The fact that *ddc1*Δ increases *rfa1*-t11 sensitivity towards HU or MMS, suggests that in *rfa1*-t11 cells DNA structures accumulate, which lead to replication fork collapse and DNA damage.

3.5.4. *rfa1-t11* acts with *mec1*-100 on parallel pathways to stabilize the replisome

In response to HU, no defect or a small delay in Rad53 phosphorylation was observed for *rfa1*-t11 cells (Kanoh, Tamai et al. 2006), (Lucca, Vanoli et al. 2004). Kanoh *et al* observed no difference for Ddc2 recruitment to HU-arrested replication forks by ChIP-chip analysis in *rfa1*-t11 cells compared to wild-type (Kanoh, Tamai et al. 2006). Consistently, they showed that *rfa1*-t11 does not compromise Ddc2 binding in S phase and Rad53 is activated normally in presence of 0.2 M HU. This indicates that *rfa1*-t11 is proficient for intra-S phase checkpoint activation. In contrast Lucca *et al* failed to detect Ddc2 at ARS305 under similar conditions (Lucca, Vanoli et al. 2004). However, both studies report reduced Ddc1 recruitment to stalled replication forks, suggesting that *rfa1*-t11 is mainly defective for the DNA damage checkpoint (Lucca, Vanoli et al. 2004), (Kanoh, Tamai et al. 2006). Furthermore, *rfa1*-t11 has been shown to rescue several adaptation mutants, which can not inactivate the checkpoint due to continuous activity of Mec1 and Rad53 kinase (Pellicioli, Lee et al. 2001), (Lee, Moore et al. 1998), (Lee, Pellicioli et al. 2003). This is in agreement with the observation that *rfa1*-t11 diminishes Ddc2-recruitment to HO-induced DSBs *in vivo* and is defective for Ddc2 recruitment *in vitro* (Zou and Elledge 2003).

In agreement with Kanoh *et al*, we did not observe a significant difference for Ddc2 interaction with wild-type Rpa70 or *rfa1*-t11 in two-hybrid analysis (data not shown). This indicates that upon DNA damage Ddc2-Mec1 recruitment might be regulated via different mechanisms than under normal or stress conditions. However, when we combined *rfa1*-t11 with the S-phase specific kinase mutant, *mec1*-100, we observed synergistic HU sensitivity. This suggests that both proteins act on parallel pathways in fork stabilization. It is currently not clear, what proteins *mec1*-100 targets or fails to phosphorylate compared to wild-type Mec1. However, recent studies imply that RPA phosphorylation by Mec1/ATR regulates DNA synthesis and fork stability under replication stress (Vassin,

Anantha et al. 2009), (Shi, Feng et al.). Hence it is also possible that aberrant or missing *rfa1*-t11 phosphorylation in *mec1*-100 cells further deregulates the interaction with other stabilizing checkpoint or repair proteins and therefore provokes replication fork collapse. Future work will address how the binding between the N-OB of Rpa70 with different proteins is regulated in order to maintain replisome stability in response to stress.

Acknowledgements:

B. L. Pike, H. Ferreira

J. Bianco (Pasero lab for help, DNA combing) and the rest of the Pasero lab

M. Stadler (for statistical analysis of the DNA combing data)

L Gelman (Imaging facility of the FMI)

C. Soustelle, A. Nicolas, Genetics, 2002 (for *rfa1*-t11 plasmid)

3.6. Supplementary Figures

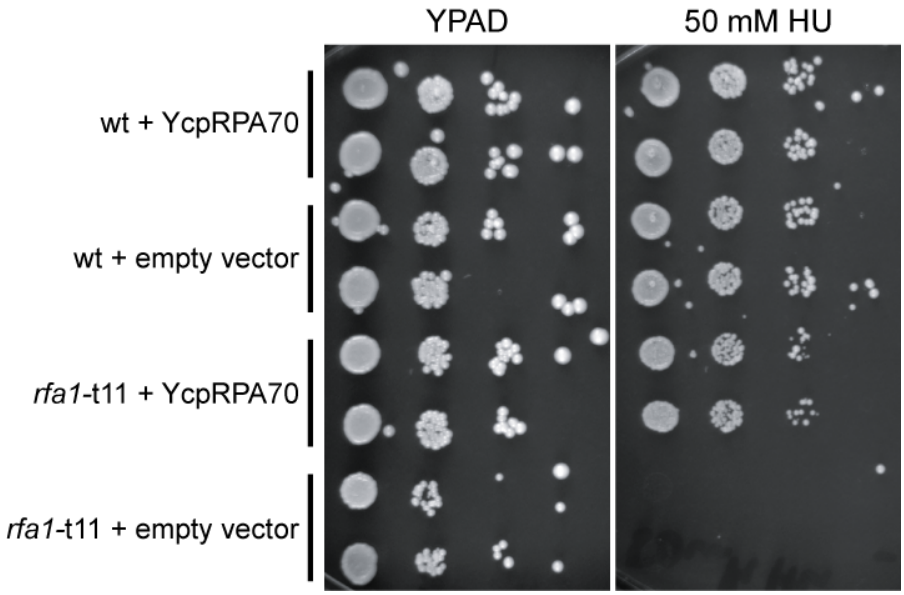


Figure 26 - Wild-type RPA70 suppresses the HU-sensitivity phenotype of *rfa1*-t11. A) Wild-type (GA-2238) and *rfa1*-t11 (GA-4802) cells were transformed with a CEN-ARS-plasmid containing WT-RPA70 (YcpRPA70), or an empty CEN-ARS-plasmid. Two clones each were analyzed in drop tests, where ten fold serial dilutions were plated onto YPAD or YPAD with 50 mM HU. *rfa*-t11 cells containing YcpRPA70 are completely restored for growth on 50 mM HU in contrast to *rfa1*-t11 cells containing the empty vector control.

Figure 27 (following page)- Genetic interactions between *rfa1*-t11, *mre11*Δ, *rad51*Δ and *mec1*-100. Ten fold serial dilutions of GA-1981 (wt) , GA-4968 (*rfa1*-t11) , GA-5919 (*rad51*Δ) , GA-5908 (*mre11*Δ), GA-5914 (*mre11*Δ *rfa1*-t11), GA-6025 (*mre11*Δ *rad51*Δ), GA-5926 (*rfa1*-t11 *rad51*Δ), GA-4978 (*mec1*-100), GA-4980 (*rfa1*-t11 *mec1*-100), GA-5922 (*rad51*Δ *mec1*-100), GA-6032 (*mre11*Δ *mec1*-100) were plated on YPAD, YPAD with 10 mM HU, 20 mM HU, 0.0025% MMS, 0.005% MMS and 0.015% MMS. **A)** *rfa1*-t11, *mre11*Δ and *mre11*Δ *rfa1*-t11 show the same phenotype for HU and MMS. Similar HU- and MMS-phenotype is observed for the double mutant *rfa1*-t11 *rad51*Δ and *mre11*Δ *rad51*Δ. **B)** *rfa1*-t11, *rad51*Δ and *mre11*Δ cells display synergistic genetic interaction with *mec1*-100 on HU-containing YPAD plates

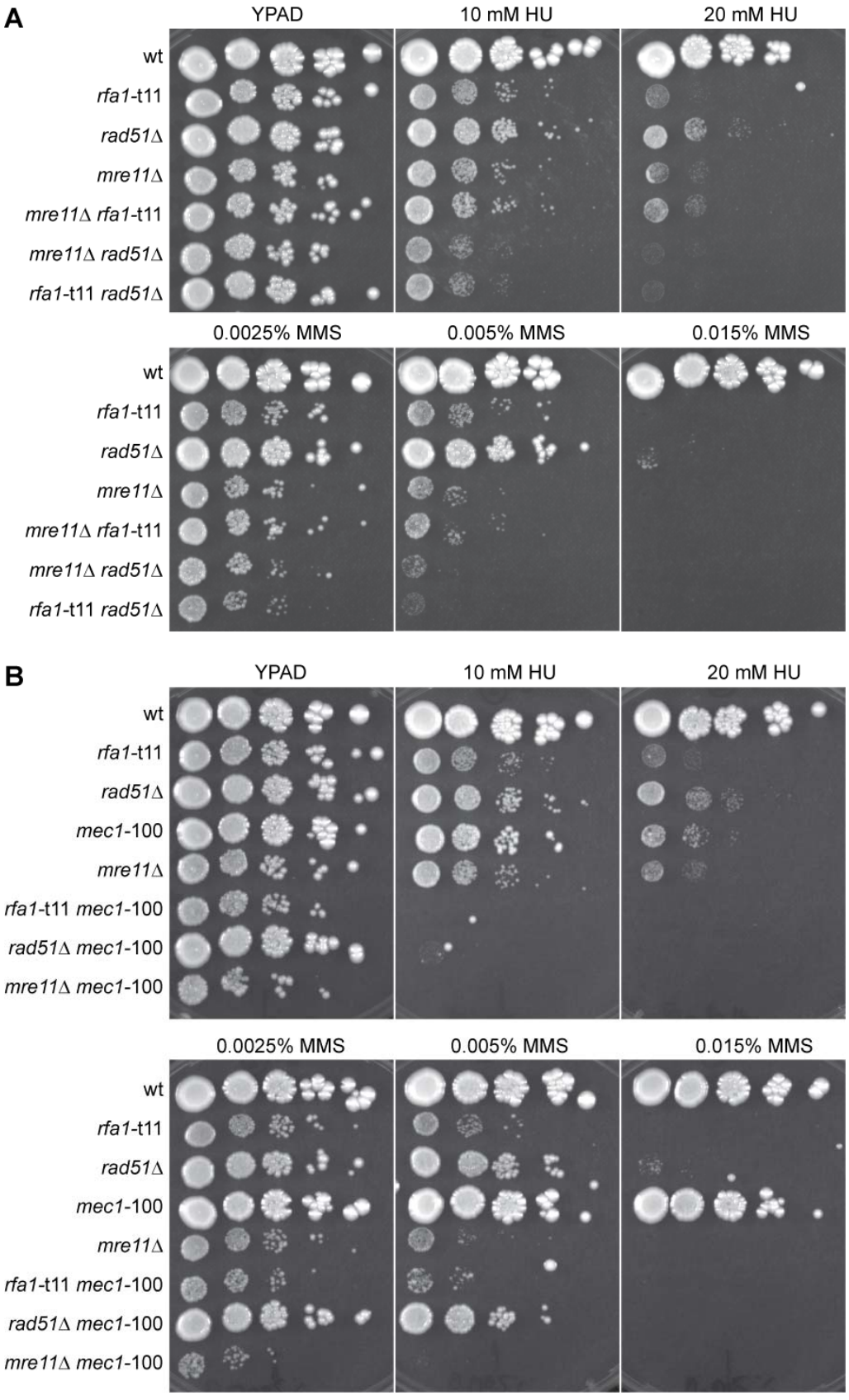

A
YPAD
10 mM HU
20 mM HU
wt
rfa1-t11
rad51Δ
mre11Δ
mre11Δ rfa1-t11
mre11Δ rad51Δ
rfa1-t11 rad51Δ
0.0025% MMS
0.005% MMS
0.015% MMS
wt
rfa1-t11
rad51Δ
mre11Δ
mre11Δ rfa1-t11
mre11Δ rad51Δ
rfa1-t11 rad51Δ
B
YPAD
10 mM HU
20 mM HU
wt
rfa1-t11
rad51Δ
mec1-100
mre11Δ
rfa1-t11 mec1-100
rad51Δ mec1-100
mre11Δ mec1-100
0.0025% MMS
0.005% MMS
0.015% MMS
wt
rfa1-t11
rad51Δ
mec1-100
mre11Δ
rfa1-t11 mec1-100
rad51Δ mec1-100
mre11Δ mec1-100

Table 4 - Yeast strains used in this study

Strain	Genotype	Source
GA-180	MAT**a**, *ade2*-1, *trp1*-1, *his3*-11, -15, *ura3*-1, *leu2*-3,-112, *can1*-100 (W303-1a)	S. Elledge
GA-880	MAT**a** his3, leu2, ura3, trp1, mec1-1 sml1 (A364a background) sgs1::LEU2	C. Frei
GA-1211	MATα, his3, trp1, ura3-52, leu2::proLEU2-lexAop2 (EGY188)	E. Golemis
GA-1981	MAT**a**, *ade2*-1, *trp1*-1, *his3*-11, -15, *ura3*-1, *leu2*-3,-112, *can1*-100 (W303), RAD5+	H.L. Klein
GA-2238	GA-180 with Pol alpha-HA::TRP	Cobb 2005
GA-2478	GA-180 with sml1::KanMX , mec1-100::LEU2(HIS)	Cobb 2005
GA-2514	GA-180 with mec1-100::LEU2 (HIS), sgs1::TRP	Cobb 2005
GA-4800	GA-180 with *mec1-100::LEU2 (HIS) rfa1-t11, Pol alpha-HA::TRP*	This study
GA-4802	GA-180 with rfa1-t11, Pol alpha-HA::TRP, sml1::G418	This study
GA-4920	GA-180 with mec1-100::LEU2 (HIS), Pol alpha-HA::TRP	This study
GA-4968	GA-1981 with rfa1-t11	This study
GA-4971	GA-1981 with mec1-100::LEU2(HIS), rfa1-t11, Pol alpha-HA::TRP	This study
GA-4973	GA-1981 with Pol alpha-HA::TRP	Sgs1-r1 study
GA-4974	GA-1981 with Pol alpha-HA::TRP, mec1-100::LEU2(HIS)	Sgs1-r1 study
GA-4978	GA-1981 with mec1-100::LEU2(HIS)	Sgs1-r1 study
GA-4980	GA-1981 with mec1-100::LEU2(HIS), rfa1-t11	This study
GA-5048	GA-1981 with Pol alpha-HA::TRP, rfa1-t11	This study
GA-5382	GA-1981 with his3::hENT1:HSV-TK-HIS3 ura3::TK7x-URA3	This study
GA-5383	GA-5382 with rfa1-t11	This study
GA-5385	GA-5382 with mec1-100::LEU2(HIS)	This study
GA-5386	GA-5382 with rfa1-t11, mec1-100::LEU2(HIS)	This study
GA-5908	GA-1981 with mre11::NAT	This study
GA-5914	GA-1981 with mre11::NAT, rfa1-t11	This study
GA-5919	GA-1981 with rad51::URA	This study
GA-5922	GA-1981 with rad51::URA, mec1-100::LEU2(HIS)	This study
GA-5926	GA-1981 with rad51::URA, rfa1-t11	This study
GA-5940	GA-1981 with ddc1::G418	This study
GA-5942	GA-1981 with ddc1::G418, rfa1-t11	This study
GA-6025	GA-1981 with mre11::NAT, rad51::URA	This study
GA-6032	GA-5445 with mre11::NAT, mec1-100::LEU2(HIS)	This study

4. General conclusions

In this study, we aimed to investigate the impact of the Sgs1-RPA interaction on stabilizing stalled replication forks in *S. cerevisiae*. We mapped the interaction site between Sgs1 and RPA. On one hand, we have created a new *sgs1* mutant, *sgs1*-r1, which completely disrupts Rpa70 binding in two-hybrid analysis. On the other hand, we have used a previously described Rpa70 mutant, *rfa1*-t11, which only partially disrupts Sgs1 binding in two-hybrid studies. We used both *sgs1*-r1 and *rfa1*-t11 mutants to dissect the functions of Sgs1 and RPA at the replication fork in response to replication stress. However, we found that although *rfa1*-t11 only partially affects Sgs1 interaction, it displays a stronger replication defect in response to HU than *sgs1*-r1 or *sgs1*Δ. This discrepancy suggests that only a fraction of the *rfa1*-t11 phenotype might be attributed to loss of Sgs1 interaction.

Previously, it was shown that the RecQ helicase Sgs1 and the checkpoint kinase Mec1 act on parallel pathways to maintain fork stability and it was proposed that both pathways might converge on RPA (Cobb, Schleker et al. 2005). Surprisingly, we realized that disruption of the Sgs1-RPA interaction in the *sgs1*-r1 *mec1*-100 double mutant did not lead to synergistic HU-sensitivity in the recovery assay unlike monitored for *sgs1*Δ *mec1*-100 or *sgs1*-hd *mec1*-100. Consistently, DNA pol α was only partially displaced from HU-arrested forks in the *sgs1*-r1 mutant or the *sgs1*-r1 *mec1*-100 double mutant. This implied that although Sgs1-RPA-binding contributes to replication fork stability, it is not sufficient to maintain functional forks in response to HU. Furthermore, our data suggest that in addition to the Sgs1-RPA interaction the helicase activity of Sgs1 is necessary for stable DNA pol α association. This is consistent with previous studies, which have reported that full DNA pol ε association with stalled replication forks depends on both Sgs1 helicase activity and Top3 function (Cobb, Bjergbaek et al. 2003), (Bjergbaek, Cobb et al. 2005).

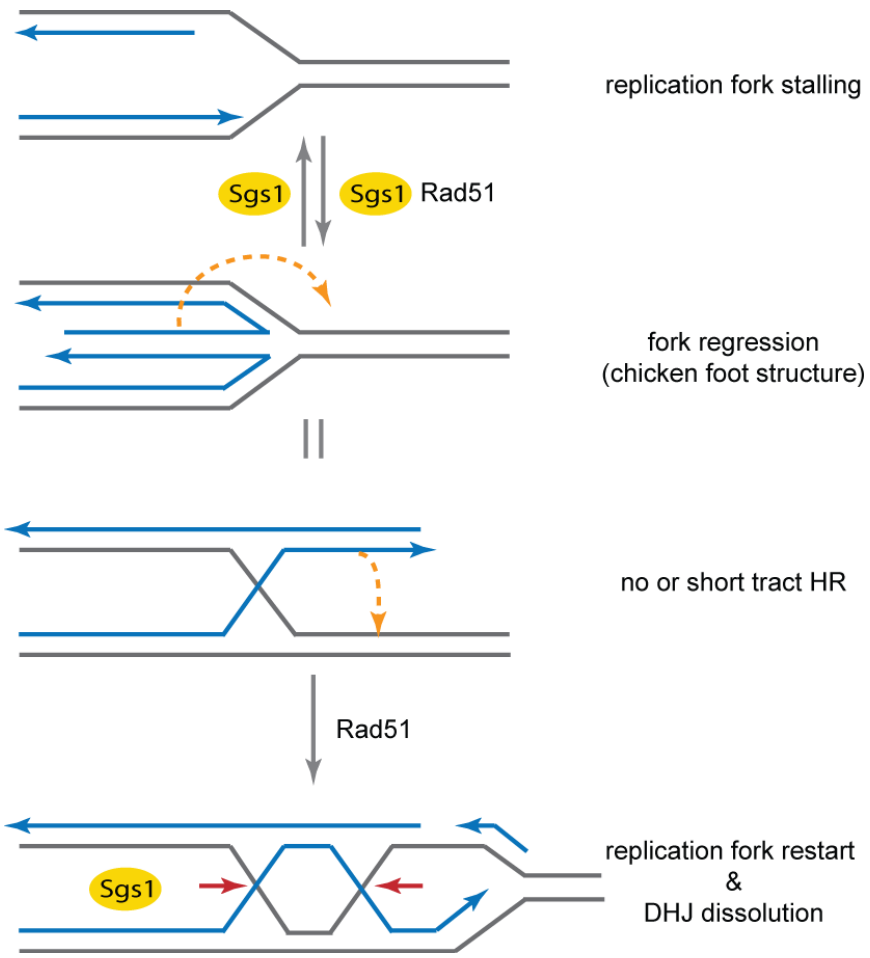


Figure 28 - Sgs1 might facilitate replication fork restart during a pathway involving HR. In response to replication fork stalling, Sgs1-Top3-Rmi1 can promote or prevent fork regression. Rad51-facilitates replication fork restart by recombination over a short tract. The resulting double holliday junction (DHJ) is dissolved by the Sgs1-Top3-Rmi1 complex.

How does Sgs1 stabilize DNA pol α and DNA pol ε at replication forks in response to replication stress? We suggest the following two mechanisms, which might both apply: The first model involves Holliday junction dissolution or the antirecombinase activity by the Sgs1-Top3-Rmi1 (RTR) complex (see Figure 28). The RTR complex has been proposed to promote or prevent the nascent DNA strands from pairing, leading to so called chicken-foot structures (Cobb and Bjergbaek 2006). In response to DNA damage, the formation of a chicken-foot structure allows DNA synthesis past the lesion by usage of the nascent strand as

the template (Wu 2007), (Ashton and Hickson). Replication fork restart could be then mediated by regression of the chicken foot structure by the RTR complex or by HR (Ralf, Hickson et al. 2006), (Alabert, Bianco et al. 2009). Recently, it has been proposed that two distinct types of Rad51-mediated pathways for replication restart and repair exist in response to HU (Petermann, Orta et al.). Petermann and coworkers show by DNA fiber spreading of human cells that most replication forks resume DNA synthesis after short HU blocks (1-2 h) however they become inactivated after long exposure (24 h) to HU (Petermann, Orta et al.). Furthermore, they report that replication fork restart in response to short HU exposure depends on RAD51, which might facilitate the formation of a Holliday junction intermediate and recombination over a short tract. After formation of the DHJ, the RTR complex mediates strand migration and Holliday junction resolution. In contrast, long exposure to HU, leads to replication fork collapse and DSB formation. Global replication is rescued by activation of new origins and subsequent long tract HR repair involving also RAD51 (Petermann, Orta et al.).

The second model of DNA pol α stabilization at HU-stalled forks also depends on the Sgs1 helicase activity and the ability of Sgs1 to bind RPA, but does not necessarily involve Top3-Rmi1. It is possible that Sgs1 stabilizes DNA pol α/ primase by similar mechanisms as T-antigen helicase during SV40 virus replication (Arunkumar, Klimovich et al. 2005), (Figure 29). The Sgs1-RPA interaction might change the conformation of RPA: from an elongated high affinity mode to a more globular low affinity mode. This would make space for DNA pol α/ primase loading and facilitate the resumption of DNA synthesis. Like T-antigen helicase, Sgs1 can interact with the biggest subunit of RPA, Rpa70 and also shows some weak affinity for the second subunit Rpa32. Additionally, it was shown that the human RecQ helicase BLM is able to displace RPA during repetitive unwinding and re-annealing of short dsDNA (Yodh, Stevens et al. 2009). However, another open question remains: in contrast to T-antigen, no direct interaction between DNA pol α/ primase and Sgs1 has been reported so

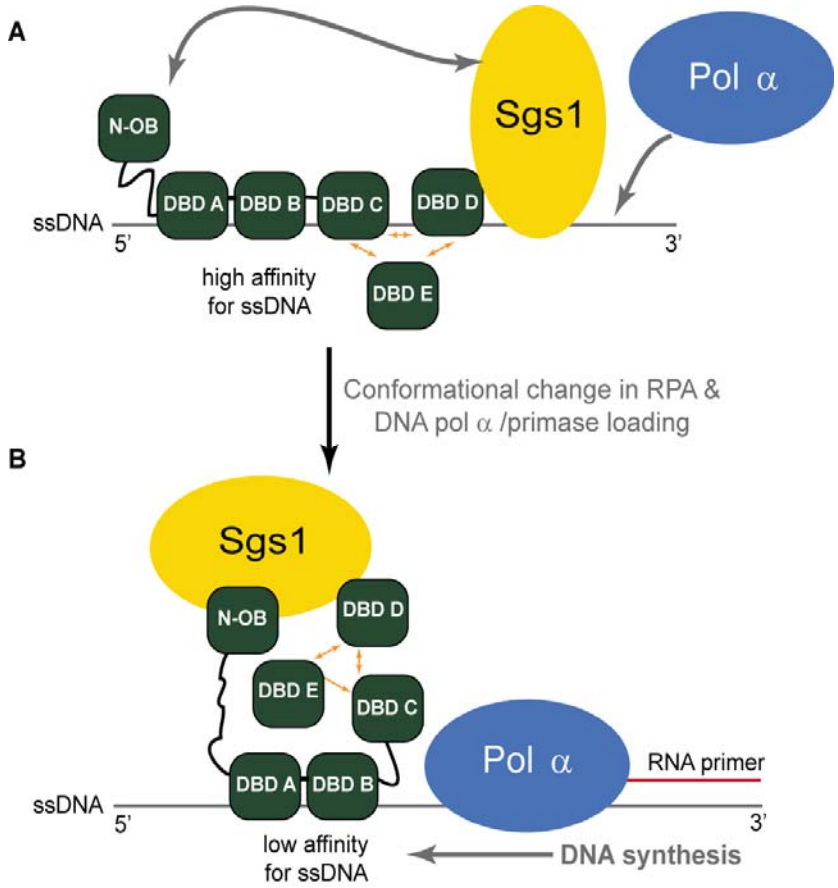


Figure 29 - Model of how Sgs1 stimulates primer synthesis by DNA pol α/primase. This hypothesis is adapted from a model proposed for T-antigen helicase mediated primer synthesis in SV40 virus replication (Arunkumar *et al.*). Sgs1-dependent interaction might remodel RPA from an elongated high affinity mode to a more globular conformation, which is easily displaced during DNA synthesis. The resulting free stretch of ssDNA can be bound by DNA pol α/primase, which initiates an RNA primer, followed by DNA synthesis.

far. Thus, it is not clear how DNA pol α/ primase would be loaded onto ssDNA to resume DNA synthesis.

Furthermore, we show the first time that Sgs1 is phosphorylated by the checkpoint kinase Mec1 *in vitro* and that Mec1-dependent phosphorylation of Sgs1 is necessary for full Rad53 activation in response to HU. Interestingly, Mec1 targets a SQ/TQ cluster domain (SCD) within the Rpa70 binding site of

Sgs1, which then probably binds the FHA1 domain of Rad53. In section 2, we propose a model how Sgs1 might act as a scaffold to facilitate Rad53 activation in a parallel pathway to Rad24. However, it seems that although Mec1 targets the RPA interaction site within Sgs1, replication fork stabilization is separable from checkpoint activation. Deletion of the RPA-interaction site in *sgs1*-r1 leads to HU sensitivity in the replication fork recovery assay and compromised checkpoint activation (in absence of *RAD24*), while non-phosphorylatable *sgs1*-4A mutants do not compromise recovery after HU-treatment. This could imply that Sgs1 still binds RPA, while it mediates Rad53 activation in response to replication stress. This idea is supported by the fact that Sgs1 has two binding sites for the N-OB of Rpa70 and that the entire SCD ($T_{451}Q_{452}$, $S_{470}Q_{471}$, $S_{482}Q_{483}$) is only located in the first Rpa70 binding site of Sgs1.

Surprisingly, we observe that sensitivity of *mec1*-100 cells towards HU and MMS can be completely suppressed by *sgs1*-4A (see Figure 20). This suppression is specific for *sgs1*-4A and can not be detected for the *sgs1*-4E mutant or for another Mec1 mutant, *mec1*-101 (data not shown). To our knowledge, *sgs1*-4A is the first suppressor of *mec1*-100 sensitivity; however the molecular mechanism by which *sgs1*-4A re-establishes Mec1 function are still enigmatic. Segurado *et al.* have reported that deletion of the flap-endonuclease *EXO1* suppresses *rad53*Δ sensitivity to DNA damaging agents such as MMS, UV and IR, but not HU (Segurado and Diffley 2008). This *EXO1*-dependent suppression is specific to *rad53*Δ and was not monitored for *mec1*Δ. It was suggested that Rad53 directly inhibits negative Exo1 functions at damaged replication forks. Alternatively, Rad53 could be implicated in the stabilization of some replisome components, such as the MCM helicase, and loss of *RAD53* would expose the replication fork to degradation by Exo1 (Cobb, Schleker et al. 2005), (Segurado and Diffley 2008).

Similar mechanisms might apply for *mec1*-100 and *sgs1*-4A. It was shown that DNA polymerases are partially destabilized in the *mec1*-100 mutant (Cobb,

Schleker et al. 2005), which might also expose the replication fork to degradation by nucleases. The *sgs1*-4A mutant might stabilize those replication fork components, thereby preventing nuclease access and degradation. Alternatively, it is possible that *sgs1*-4A specifically interacts with *mec1*-100 which changes the conformation of the kinase and restores Mec1 function. Therefore, the next experiments will address whether deletion of nucleases such as *DNA2* or *EXO1* suppresses *mec1*-100 HU-sensitivity or whether HU-induced Rad53 activation is restored in the *mec1*-100 *sgs1*-4A double mutant. In any case, understanding the mechanism by which *sgs1*-4A suppresses the HU-sensitivity of *mec1*-100, will shed new light on how Mec1 maintains functional forks in response to replication stress.

Another important question to comprehend Mec1 function is the identification of the differences in protein phosphorylation by *mec1*-100 in comparison to wild-type Mec1. It is unknown if and which replication fork components *mec1*-100 phosphorylates in response to replication stress. However, this would probably help to understand why *mec1*-100 and *sgs1*Δ display synergistic HU-sensitivity and a synergistic increase in GCR rates, when combined.

Interestingly, *mec1*-100 is even more HU-sensitive in combination with *rfa1*-t11. We show by different methods including DNA pol ChIP, 2D gel analysis and DNA combing that *rfa1*-t11 displays a strong replication defect in response to replication stress. Our genetic data with *rad51* and *mre11* suggest that this replication defect is caused by impaired homologous recombination. We detect that *mre11*Δ phenocopies the HU and MMS sensitivity of *rfa1*-t11 and therefore suspect that *rfa1*-t11 disrupts MRX recruitment to stalled forks, which has been proposed to stabilize the arrested fork in a conformation competent for replication fork restart by HR (Tittel-Elmer, Alabert et al. 2009), (Figure 30). In human cells, MRN is recruited to replication foci via an RPA-dependent mechanism in absence or presence of replication stress (Robison, Elliott et al. 2004), (Olson, Nievera et al. 2007). MRE11 was shown to bind to the N-OB fold of human

RPA70 (Xu, Vaithiyalingam et al. 2008), (Oakley, Tillison et al. 2009), the same OB-fold which carries the charge reversal mutation of *rfa1*-t11 in yeast. Therefore we speculate that *S. cerevisiae* Rpa70 might employ a similar binding mode for yeast Mre11 and that *rfa1*-t11 might disrupt this interaction. In addition, previous studies have reported that *rfa1*-t11 mutants are defective for meiotic and homologous recombination (Soustelle, Vedel et al. 2002), (Kantake, Sugiyama et al. 2003), (Wang and Haber 2004).

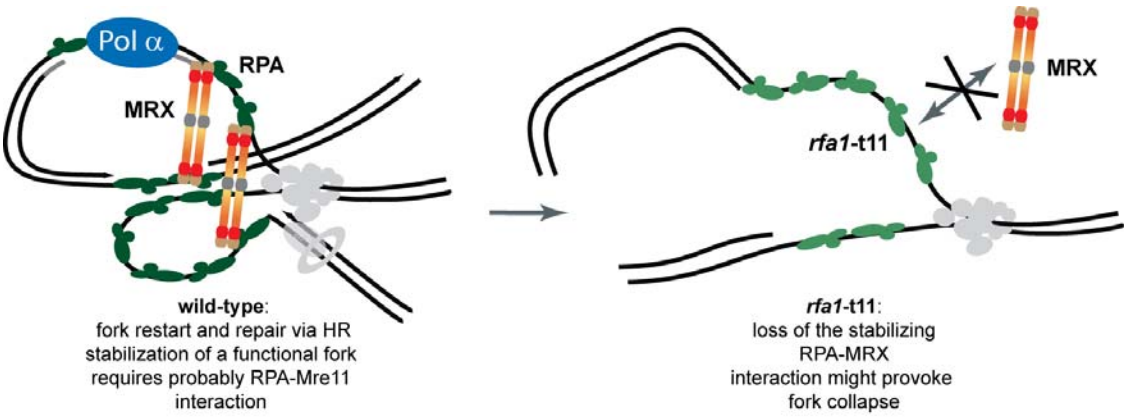


Figure 30 - RPA-dependent recruitment of the MRX complex after fork stalling. In response to replication stress, replication forks arrest and long stretches of ssDNA coated by RPA are formed. In wild-type cells, this signal might recruit the MRX complex, which stabilizes the fragile structure and allows efficient replication fork restart by HR. In rfa1-t11 cells, MRX recruitment might be impaired resulting in replication fork collapse and the dissociation of replisome components (such as DNA pol α/primase).

Why are *rfa1*-t11 *mec1*-100 double mutants highly synergistic in response to HU? This is an open question that remains to be elucidated. There is growing evidence that Mec1/ATR-dependent RPA hyperphosphorylation regulates replication fork restart of HU-arrested forks by HR in human cells (Vassin, Anantha et al. 2009), (Shi, Feng et al.). Furthermore, it was reported that a RPA32 peptide that mimics hyperphosphorylation, which occurs in response to DNA damage or replication stress, can compete with a p53 peptide bound to the N-OB fold of RPA70 *in vitro* (Bochkareva, Kaustov et al. 2005). This implies that hyperphosphorylation of the RPA32 N-terminus by Mec1/ATR might regulate the

interaction between RPA and other proteins such as p53. Thus, *mec1*-100 could fail to phosphorylate RPA in yeast thereby aggravating the replication defect of *rfa1*-t11 in response to HU. Alternatively, inappropriate or lacking phosphorylation events in *mec1*-100 cells might deregulate other protein-interactions at the stalled fork, which are crucial for replication fork maintenance.

Taken together, this study provides new insights how Sgs1 and RPA function at stalled replication forks to maintain genome integrity. We pin-point the molecular mechanism by which Sgs1 contributes to intra-S checkpoint activation in response to replication stress (when *RAD24* is absent) and demonstrate that Sgs1-RPA interaction is necessary for replication fork stability. Furthermore, our data suggests that both Sgs1 and RPA are involved in replication fork restart probably by employing HR. Sgs1 might facilitate restart by promoting fork regression and during a later step DHJ dissolution or by removing RPA and thereby facilitating DNA pol α loading. In addition to its known functions during replication, checkpoint signaling and recombination, we suggest that *S. cerevisiae* RPA maintains HU-arrested forks by recruiting the MRX complex. This might stabilize the fragile DNA structure and therefore prevent fork collapse.

References

Alabert, C., J. N. Bianco, et al. (2009). "Differential regulation of homologous recombination at DNA breaks and replication forks by the Mrc1 branch of the S-phase checkpoint." EMBO J **28**(8): 1131-41.

Alcasabas, A. A., A. J. Osborn, et al. (2001). "Mrc1 transduces signals of DNA replication stress to activate Rad53." Nat Cell Biol **3**(11): 958-65.

Aparicio, O. M., A. M. Stout, et al. (1999). "Differential assembly of Cdc45p and DNA polymerases at early and late origins of DNA replication." Proc Natl Acad Sci U S A **96**(16): 9130-5.

Arunkumar, A. I., V. Klimovich, et al. (2005). "Insights into hRPA32 C-terminal domain--mediated assembly of the simian virus 40 replisome." Nat Struct Mol Biol **12**(4): 332-9.

Ashton, T. M. and I. D. Hickson "Yeast as a model system to study RecQ helicase function." DNA Repair (Amst).

Azvolinsky, A., S. Dunaway, et al. (2006). "The S. cerevisiae Rrm3p DNA helicase moves with the replication fork and affects replication of all yeast chromosomes." Genes Dev **20**(22): 3104-16.

Bachrati, C. Z. and I. D. Hickson (2008). "RecQ helicases: guardian angels of the DNA replication fork." Chromosoma **117**(3): 219-33.

Ball, H. L., M. R. Ehrhardt, et al. (2007). "Function of a conserved checkpoint recruitment domain in ATRIP proteins." Mol Cell Biol **27**(9): 3367-77.

Ballabeni, A., M. Melixetian, et al. (2004). "Human geminin promotes pre-RC formation and DNA replication by stabilizing CDT1 in mitosis." EMBO J **23**(15): 3122-32.

Bando, M., Y. Katou, et al. (2009). "Csm3, Tof1, and Mrc1 form a heterotrimeric mediator complex that associates with DNA replication forks." J Biol Chem **284**(49): 34355-65.

Bell, S. P. and A. Dutta (2002). "DNA replication in eukaryotic cells." Annu Rev Biochem **71**: 333-74.

Bennett, R. J., J. L. Keck, et al. (1999). "Binding specificity determines polarity of DNA unwinding by the Sgs1 protein of S. cerevisiae." J Mol Biol **289**(2): 235-48.

Bennett, R. J., M. F. Noirot-Gros, et al. (2000). "Interaction between yeast sgs1 helicase and DNA topoisomerase III." J Biol Chem **275**(35): 26898-905.

Bernstein, D. A. and J. L. Keck (2005). "Conferring substrate specificity to DNA helicases: role of the RecQ HRDC domain." Structure **13**(8): 1173-82.

Bernstein, K. A., E. Shor, et al. (2009). "Sgs1 function in the repair of DNA replication intermediates is separable from its role in homologous recombinational repair." EMBO J **28**(7): 915-25.

Binz, S. K., A. M. Sheehan, et al. (2004). "Replication protein A phosphorylation and the cellular response to DNA damage." DNA Repair (Amst) **3**(8-9): 1015-24.

Bjergbaek, L., J. A. Cobb, et al. (2005). "Mechanistically distinct roles for Sgs1p in checkpoint activation and replication fork maintenance." Embo J **24**(2): 405-17.

Blow, J. J. and A. Dutta (2005). "Preventing re-replication of chromosomal DNA." Nat Rev Mol Cell Biol **6**(6): 476-86.

Blow, J. J. and X. Q. Ge (2009). "A model for DNA replication showing how dormant origins safeguard against replication fork failure." EMBO Rep **10**(4): 406-12.

Bochkarev, A. and E. Bochkareva (2004). "From RPA to BRCA2: lessons from single-stranded DNA binding by the OB-fold." Curr Opin Struct Biol **14**(1): 36-42.

Bochkareva, E., L. Kaustov, et al. (2005). "Single-stranded DNA mimicry in the p53 transactivation domain interaction with replication protein A." Proc Natl Acad Sci U S A **102**(43): 15412-7.

Boddy, M. N., P. H. Gaillard, et al. (2001). "Mus81-Eme1 are essential components of a Holliday junction resolvase." Cell **107**(4): 537-48.

Branzei, D. and M. Foiani "Maintaining genome stability at the replication fork." Nat Rev Mol Cell Biol **11**(3): 208-19.

Branzei, D. and M. Foiani (2009). "The checkpoint response to replication stress." DNA Repair (Amst) **8**(9): 1038-46.

Brush, G. S. and T. J. Kelly (2000). "Phosphorylation of the replication protein A large subunit in the Saccharomyces cerevisiae checkpoint response." Nucleic Acids Res **28**(19): 3725-32.

Brush, G. S., D. M. Morrow, et al. (1996). "The ATM homologue MEC1 is required for phosphorylation of replication protein A in yeast." Proc Natl Acad Sci U S A **93**(26): 15075-80.

Buongiorno-Nardelli, M., G. Micheli, et al. (1982). "A relationship between replicon size and supercoiled loop domains in the eukaryotic genome." Nature **298**(5869): 100-2.

Burgers, P. M. (2009). "Polymerase dynamics at the eukaryotic DNA replication fork." J Biol Chem **284**(7): 4041-5.

Byun, T. S., M. Pacek, et al. (2005). "Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint." Genes Dev **19**(9): 1040-52.

Calzada, A., B. Hodgson, et al. (2005). "Molecular anatomy and regulation of a stable replisome at a paused eukaryotic DNA replication fork." Genes Dev **19**(16): 1905-19.

Cayrou, C., P. Coulombe, et al. "Programming DNA replication origins and chromosome organization." Chromosome Res.

Chen, C. F. and S. J. Brill (2007). "Binding and activation of DNA topoisomerase III by the Rmi1 subunit." J Biol Chem **282**(39): 28971-9.

Chen, S., M. A. de Vries, et al. (2007). "Orc6 is required for dynamic recruitment of Cdt1 during repeated Mcm2-7 loading." Genes Dev **21**(22): 2897-907.

Chilkova, O., P. Stenlund, et al. (2007). "The eukaryotic leading and lagging strand DNA polymerases are loaded onto primer-ends via separate

mechanisms but have comparable processivity in the presence of PCNA." Nucleic Acids Res **35**(19): 6588-97.
Cimprich, K. A. and D. Cortez (2008). "ATR: an essential regulator of genome integrity." Nat Rev Mol Cell Biol **9**(8): 616-27.
Cobb, J. A. and L. Bjergbaek (2006). "RecQ helicases: lessons from model organisms." Nucleic Acids Res **34**(15): 4106-14.
Cobb, J. A., L. Bjergbaek, et al. (2003). "DNA polymerase stabilization at stalled replication forks requires Mec1 and the RecQ helicase Sgs1." Embo J **22**(16): 4325-36.
Cobb, J. A., T. Schleker, et al. (2005). "Replisome instability, fork collapse, and gross chromosomal rearrangements arise synergistically from Mec1 kinase and RecQ helicase mutations." Genes Dev **19**(24): 3055-69.
Cotta-Ramusino, C., D. Fachinetti, et al. (2005). "Exo1 processes stalled replication forks and counteracts fork reversal in checkpoint-defective cells." Mol Cell **17**(1): 153-9.
Courbet, S., S. Gay, et al. (2008). "Replication fork movement sets chromatin loop size and origin choice in mammalian cells." Nature **455**(7212): 557-60.
Czornak, K., S. Chughtai, et al. (2008). "Mystery of DNA repair: the role of the MRN complex and ATM kinase in DNA damage repair." J Appl Genet **49**(4): 383-96.
Davalos, A. R. and J. Campisi (2003). "Bloom syndrome cells undergo p53-dependent apoptosis and delayed assembly of BRCA1 and NBS1 repair complexes at stalled replication forks." J Cell Biol **162**(7): 1197-209.
Davalos, A. R., P. Kaminker, et al. (2004). "ATR and ATM-dependent movement of BLM helicase during replication stress ensures optimal ATM activation and 53BP1 focus formation." Cell Cycle **3**(12): 1579-86.
Davies, S. L., P. S. North, et al. (2004). "Phosphorylation of the Bloom's syndrome helicase and its role in recovery from S-phase arrest." Mol Cell Biol **24**(3): 1279-91.
Davies, S. L., P. S. North, et al. (2007). "Role for BLM in replication-fork restart and suppression of origin firing after replicative stress." Nat Struct Mol Biol **14**(7): 677-9.
Delacroix, S., J. M. Wagner, et al. (2007). "The Rad9-Hus1-Rad1 (9-1-1) clamp activates checkpoint signaling via TopBP1." Genes Dev **21**(12): 1472-7.
Dionne, I., R. K. Nookala, et al. (2003). "A heterotrimeric PCNA in the hyperthermophilic archaeon Sulfolobus solfataricus." Mol Cell **11**(1): 275-82.
Doherty, K. M., J. A. Sommers, et al. (2005). "Physical and functional mapping of the replication protein a interaction domain of the werner and bloom syndrome helicases." J Biol Chem **280**(33): 29494-505.
Dua, R., D. L. Levy, et al. (1999). "Analysis of the essential functions of the C-terminal protein/protein interaction domain of Saccharomyces cerevisiae pol epsilon and its unexpected ability to support growth in the absence of the DNA polymerase domain." J Biol Chem **274**(32): 22283-8.
Dubrana, K., H. van Attikum, et al. (2007). "The processing of double-strand breaks and binding of single-strand-binding proteins RPA and Rad51

modulate the formation of ATR-kinase foci in yeast." J Cell Sci **120**(Pt 23): 4209-20.

Dunderdale, H. J., F. E. Benson, et al. (1991). "Formation and resolution of recombination intermediates by E. coli RecA and RuvC proteins." Nature **354**(6354): 506-10.

Dupre, A., L. Boyer-Chatenet, et al. (2006). "Two-step activation of ATM by DNA and the Mre11-Rad50-Nbs1 complex." Nat Struct Mol Biol **13**(5): 451-7.

Emili, A. (1998). "MEC1-dependent phosphorylation of Rad9p in response to DNA damage." Mol Cell **2**(2): 183-9.

Emsley, P. and K. Cowtan (2004). "Coot: model-building tools for molecular graphics." Acta Crystallogr D Biol Crystallogr **60** (Pt 12 Pt 1): 2126-32.

Fanning, E., V. Klimovich, et al. (2006). "A dynamic model for replication protein A (RPA) function in DNA processing pathways." Nucleic Acids Res **34**(15): 4126-37.

Fien, K., Y. S. Cho, et al. (2004). "Primer utilization by DNA polymerase alpha-primase is influenced by its interaction with Mcm10p." J Biol Chem **279**(16): 16144-53.

Foss, E. J. (2001). "Tof1p regulates DNA damage responses during S phase in Saccharomyces cerevisiae." Genetics **157**(2): 567-77.

Franchitto, A. and P. Pichierri (2002). "Bloom's syndrome protein is required for correct relocalization of RAD50/MRE11/NBS1 complex after replication fork arrest." J Cell Biol **157**(1): 19-30.

Frei, C. and S. M. Gasser (2000). "The yeast Sgs1p helicase acts upstream of Rad53p in the DNA replication checkpoint and colocalizes with Rad53p in S-phase-specific foci." Genes Dev **14**(1): 81-96.

Fricke, W. M. and S. J. Brill (2003). "Slx1-Slx4 is a second structure-specific endonuclease functionally redundant with Sgs1-Top3." Genes Dev **17**(14): 1768-78.

Fricke, W. M., V. Kaliraman, et al. (2001). "Mapping the DNA topoisomerase III binding domain of the Sgs1 DNA helicase." J Biol Chem **276**(12): 8848-55.

Friedel, A. M., B. L. Pike, et al. (2009). "ATR/Mec1: coordinating fork stability and repair." Curr Opin Cell Biol **21**(2): 237-44.

Friedman, K. L., J. D. Diller, et al. (1996). "Multiple determinants controlling activation of yeast replication origins late in S phase." Genes Dev **10**(13): 1595-607.

Gambus, A., R. C. Jones, et al. (2006). "GINS maintains association of Cdc45 with MCM in replisome progression complexes at eukaryotic DNA replication forks." Nat Cell Biol **8**(4): 358-66.

Gambus, A., F. van Deursen, et al. (2009). "A key role for Ctf4 in coupling the MCM2-7 helicase to DNA polymerase alpha within the eukaryotic replisome." EMBO J **28**(19): 2992-3004.

Gangloff, S., C. Soustelle, et al. (2000). "Homologous recombination is responsible for cell death in the absence of the Sgs1 and Srs2 helicases." Nat Genet **25**(2): 192-4.

Gao, H., R. B. Cervantes, et al. (2007). "RPA-like proteins mediate yeast telomere function." Nat Struct Mol Biol **14**(3): 208-14.

Garg, P., C. M. Stith, et al. (2004). "Idling by DNA polymerase delta maintains a ligatable nick during lagging-strand DNA replication." Genes Dev **18**(22): 2764-73.

Ge, X. Q., D. A. Jackson, et al. (2007). "Dormant origins licensed by excess Mcm2-7 are required for human cells to survive replicative stress." Genes Dev **21**(24): 3331-41.

Gilbert, C. S., C. M. Green, et al. (2001). "Budding yeast Rad9 is an ATP-dependent Rad53 activating machine." Mol Cell **8**(1): 129-36.

Gilbert, D. M. (2004). "In search of the holy replicator." Nat Rev Mol Cell Biol **5**(10): 848-55.

Gottifredi, V. and C. Prives (2005). "The S phase checkpoint: when the crowd meets at the fork." Semin Cell Dev Biol **16**(3): 355-68.

Gravel, S., J. R. Chapman, et al. (2008). "DNA helicases Sgs1 and BLM promote DNA double-strand break resection." Genes Dev **22**(20): 2767-72.

Haring, S. J., T. D. Humphreys, et al. "A naturally occurring human RPA subunit homolog does not support DNA replication or cell-cycle progression." Nucleic Acids Res **38**(3): 846-58.

Harrison, J. C. and J. E. Haber (2006). "Surviving the breakup: the DNA damage checkpoint." Annu Rev Genet **40**: 209-35.

Hartwell, L. H., J. Culotti, et al. (1974). "Genetic control of the cell division cycle in yeast." Science **183**(4120): 46-51.

Hartwell, L. H., J. Culotti, et al. (1970). "Genetic control of the cell-division cycle in yeast. I. Detection of mutants." Proc Natl Acad Sci U S A **66**(2): 352-9.

Heo, S. J., K. Tatebayashi, et al. (1999). "Bloom's syndrome gene suppresses premature ageing caused by Sgs1 deficiency in yeast." Genes Cells **4**(11): 619-25.

Hoadley, K. A. and J. L. Keck "Werner helicase wings DNA binding." Structure **18**(2): 149-51.

Hodgson, B., A. Calzada, et al. (2007). "Mrc1 and Tof1 regulate DNA replication forks in different ways during normal S phase." Mol Biol Cell **18**(10): 3894-902.

Hoege, C., B. Pfander, et al. (2002). "RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO." Nature **419**(6903): 135-41.

Hopfner, K. P., L. Craig, et al. (2002). "The Rad50 zinc-hook is a structure joining Mre11 complexes in DNA recombination and repair." Nature **418**(6897): 562-6.

Huberman, J. A., L. D. Spotila, et al. (1987). "The in vivo replication origin of the yeast 2 microns plasmid." Cell **51**(3): 473-81.

Im, J. S., S. H. Ki, et al. (2009). "Assembly of the Cdc45-Mcm2-7-GINS complex in human cells requires the Ctf4/And-1, RecQL4, and Mcm10 proteins." Proc Natl Acad Sci U S A **106**(37): 15628-32.

Ip, S. C., U. Rass, et al. (2008). "Identification of Holliday junction resolvases from humans and yeast." Nature **456**(7220): 357-61.

Ira, G., A. Malkova, et al. (2003). "Srs2 and Sgs1-Top3 suppress crossovers during double-strand break repair in yeast." Cell **115**(4): 401-11.

Ishibashi, T., S. Kimura, et al. (2006). "A higher plant has three different types of RPA heterotrimeric complex." J Biochem **139**(1): 99-104.

Jin, Y. H., R. Ayyagari, et al. (2003). "Okazaki fragment maturation in yeast. II. Cooperation between the polymerase and 3'-5'-exonuclease activities of Pol delta in the creation of a ligatable nick." J Biol Chem **278**(3): 1626-33.

Kabsch, W. (1976). "Solution for Best Rotation to Relate 2 Sets of Vectors." Acta Crystallographica Section **A 32**: 922-923.

Kanemaki, M. and K. Labib (2006). "Distinct roles for Sld3 and GINS during establishment and progression of eukaryotic DNA replication forks." EMBO J **25**(8): 1753-63.

Kanemaki, M., A. Sanchez-Diaz, et al. (2003). "Functional proteomic identification of DNA replication proteins by induced proteolysis in vivo." Nature **423**(6941): 720-4.

Kang, Y. H., C. H. Lee, et al. "Dna2 on the road to Okazaki fragment processing and genome stability in eukaryotes." Crit Rev Biochem Mol Biol **45**(2): 71-96.

Kanoh, Y., K. Tamai, et al. (2006). "Different requirements for the association of ATR-ATRIP and 9-1-1 to the stalled replication forks." Gene **377**: 88-95.

Kantake, N., T. Sugiyama, et al. (2003). "The recombination-deficient mutant RPA (rfa1-t11) is displaced slowly from single-stranded DNA by Rad51 protein." J Biol Chem **278**(26): 23410-7.

Kao, H. I., J. Veeraraghavan, et al. (2004). "On the roles of Saccharomyces cerevisiae Dna2p and Flap endonuclease 1 in Okazaki fragment processing." J Biol Chem **279**(15): 15014-24.

Keshav, K. F., C. Chen, et al. (1995). "Rpa4, a homolog of the 34-kilodalton subunit of the replication protein A complex." Mol Cell Biol **15**(6): 3119-28.

Kesti, T., K. Flick, et al. (1999). "DNA polymerase epsilon catalytic domains are dispensable for DNA replication, DNA repair, and cell viability." Mol Cell **3**(5): 679-85.

Khanna, K. K. and S. P. Jackson (2001). "DNA double-strand breaks: signaling, repair and the cancer connection." Nat Genet **27**(3): 247-54.

Killoran, M. P. and J. L. Keck (2006). "Sit down, relax and unwind: structural insights into RecQ helicase mechanisms." Nucleic Acids Res **34**(15): 4098-105.

Kim, H. S. and S. J. Brill (2001). "Rfc4 interacts with Rpa1 and is required for both DNA replication and DNA damage checkpoints in Saccharomyces cerevisiae." Mol Cell Biol **21**(11): 3725-37.

Kitano, K., S. Y. Kim, et al. "Structural basis for DNA strand separation by the unconventional winged-helix domain of RecQ helicase WRN." Structure **18**(2): 177-87.

Krejci, L., S. Van Komen, et al. (2003). "DNA helicase Srs2 disrupts the Rad51 presynaptic filament." Nature **423**(6937): 305-9.

Kumagai, A. and W. G. Dunphy (2000). "Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in Xenopus egg extracts." Mol Cell **6**(4): 839-49.

Kumagai, A. and W. G. Dunphy (2003). "Repeated phosphopeptide motifs in Claspin mediate the regulated binding of Chk1." Nat Cell Biol **5**(2): 161-5.

Kunkel, T. A. and P. M. Burgers (2008). "Dividing the workload at a eukaryotic replication fork." Trends Cell Biol **18**(11): 521-7.

Lavin, M. F. (2004). "The Mre11 complex and ATM: a two-way functional interaction in recognising and signaling DNA double strand breaks." DNA Repair (Amst) **3**(11): 1515-20.

Lee, C., I. Liachko, et al. "Alternative mechanisms for coordinating polymerase alpha and MCM helicase." Mol Cell Biol **30**(2): 423-35.

Lee, S. E., J. K. Moore, et al. (1998). "Saccharomyces Ku70, mre11/rad50 and RPA proteins regulate adaptation to G2/M arrest after DNA damage." Cell **94**(3): 399-409.

Lee, S. E., A. Pellicioli, et al. (2003). "Yeast Rad52 and Rad51 recombination proteins define a second pathway of DNA damage assessment in response to a single double-strand break." Mol Cell Biol **23**(23): 8913-23.

Leman, A. R., C. Noguchi, et al. "Human Timeless and Tipin stabilize replication forks and facilitate sister-chromatid cohesion." J Cell Sci **123**(Pt 5): 660-70.

Li, A. and J. J. Blow (2005). "Cdt1 downregulation by proteolysis and geminin inhibition prevents DNA re-replication in Xenopus." EMBO J **24**(2): 395-404.

Liu, E., X. Li, et al. (2004). "Cyclin-dependent kinases phosphorylate human Cdt1 and induce its degradation." J Biol Chem **279**(17): 17283-8.

Liu, Y. and S. C. West (2008). "More complexity to the Bloom's syndrome complex." Genes Dev **22**(20): 2737-42.

Lou, H., M. Komata, et al. (2008). "Mrc1 and DNA polymerase epsilon function together in linking DNA replication and the S phase checkpoint." Mol Cell **32**(1): 106-17.

Lucca, C., F. Vanoli, et al. (2004). "Checkpoint-mediated control of replisome-fork association and signalling in response to replication pausing." Oncogene **23**(6): 1206-13.

Machwe, A., E. M. Lozada, et al. (2006). "Competition between the DNA unwinding and strand pairing activities of the Werner and Bloom syndrome proteins." BMC Mol Biol **7**: 1.

Majka, J., A. Niedziela-Majka, et al. (2006). "The checkpoint clamp activates Mec1 kinase during initiation of the DNA damage checkpoint." Mol Cell **24**(6): 891-901.

Mankouri, H. W., T. J. Craig, et al. (2002). "SGS1 is a multicopy suppressor of srs2: functional overlap between DNA helicases." Nucleic Acids Res **30**(5): 1103-13.

Marahrens, Y. and B. Stillman (1992). "A yeast chromosomal origin of DNA replication defined by multiple functional elements." Science **255**(5046): 817-23.

Marheineke, K. and O. Hyrien (2004). "Control of replication origin density and firing time in Xenopus egg extracts: role of a caffeine-sensitive, ATR-dependent checkpoint." J Biol Chem **279**(27): 28071-81.

Masumoto, H., A. Sugino, et al. (2000). "Dpb11 controls the association between DNA polymerases alpha and epsilon and the autonomously replicating sequence region of budding yeast." Mol Cell Biol **20**(8): 2809-17.

Maya-Mendoza, A., C. W. Tang, et al. (2009). "Mechanisms regulating S phase progression in mammalian cells." Front Biosci **14**: 4199-213.

Mayer, M. L., I. Pot, et al. (2004). "Identification of protein complexes required for efficient sister chromatid cohesion." Mol Biol Cell **15**(4): 1736-45.

Mccoy, A. J., R. W. Grosse-Kunstleve, et al. (2007). "Phaser crystallographic software." Journal of Applied Crystallography **40**(Pt 4): 658-674.

McFarlane, R. J., S. Mian, et al. "The many facets of the Tim-Tipin protein families' roles in chromosome biology." Cell Cycle **9**(4): 700-5.

Melo, J. A., J. Cohen, et al. (2001). "Two checkpoint complexes are independently recruited to sites of DNA damage in vivo." Genes Dev **15**(21): 2809-21.

Michalet, X., R. Ekong, et al. (1997). "Dynamic molecular combing: stretching the whole human genome for high-resolution studies." Science **277**(5331): 1518-23.

Mimitou, E. P. and L. S. Symington (2008). "Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing." Nature **455**(7214): 770-4.

Misteli, T. and E. Soutoglou (2009). "The emerging role of nuclear architecture in DNA repair and genome maintenance." Nat Rev Mol Cell Biol **10**(4): 243-54.

Mohanty, B. K., N. K. Bairwa, et al. (2006). "The Tof1p-Csm3p protein complex counteracts the Rrm3p helicase to control replication termination of Saccharomyces cerevisiae." Proc Natl Acad Sci U S A **103**(4): 897-902.

Mordes, D. A., G. G. Glick, et al. (2008). "TopBP1 activates ATR through ATRIP and a PIKK regulatory domain." Genes Dev **22**(11): 1478-89.

Mordes, D. A., E. A. Nam, et al. (2008). "Dpb11 activates the Mec1-Ddc2 complex." Proc Natl Acad Sci U S A **105**(48): 18730-4.

Morgan, D. O. (2007). The Cell Cycle, Oxford University Press.

Myung, K. and R. D. Kolodner (2002). "Suppression of genome instability by redundant S-phase checkpoint pathways in Saccharomyces cerevisiae." Proc Natl Acad Sci U S A **99**(7): 4500-7.

Naiki, T., T. Wakayama, et al. (2004). "Association of Rad9 with double-strand breaks through a Mec1-dependent mechanism." Mol Cell Biol **24**(8): 3277-85.

Nakada, D., K. Matsumoto, et al. (2003). "ATM-related Tel1 associates with double-strand breaks through an Xrs2-dependent mechanism." Genes Dev **17**(16): 1957-62.

Navadgi-Patil, V. M. and P. M. Burgers (2008). "Yeast DNA Replication Protein Dpb11 Activates the Mec1/ATR Checkpoint Kinase." J Biol Chem **283**(51): 35853-9.

Naylor, M. L., J. M. Li, et al. (2009). "Mrc1 phosphorylation in response to DNA replication stress is required for Mec1 accumulation at the stalled fork." Proc Natl Acad Sci U S A **106**(31): 12765-70.

Nguyen, V. Q., C. Co, et al. (2001). "Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms." Nature **411**(6841): 1068-73.

Nick McElhinny, S. A., D. A. Gordenin, et al. (2008). "Division of labor at the eukaryotic replication fork." Mol Cell **30**(2): 137-44.

Nishitani, H., S. Taraviras, et al. (2001). "The human licensing factor for DNA replication Cdt1 accumulates in G1 and is destabilized after initiation of S-phase." J Biol Chem **276**(48): 44905-11.

Nuss, J. E., S. M. Patrick, et al. (2005). "DNA damage induced hyperphosphorylation of replication protein A. 1. Identification of novel sites of phosphorylation in response to DNA damage." Biochemistry **44**(23): 8428-37.

Oakley, G. G., K. Tillison, et al. (2009). "Physical interaction between replication protein A (RPA) and MRN: involvement of RPA2 phosphorylation and the N-terminus of RPA1." Biochemistry **48**(31): 7473-81.

Olson, E., C. J. Nievera, et al. (2007). "The Mre11 complex mediates the S-phase checkpoint through an interaction with replication protein A." Mol Cell Biol **27**(17): 6053-67.

Osborn, A. J. and S. J. Elledge (2003). "Mrc1 is a replication fork component whose phosphorylation in response to DNA replication stress activates Rad53." Genes Dev **17**(14): 1755-67.

Otwinowski, Z. and W. Minor (1997). "Processing of X-ray diffraction data collected in oscillation mode." Macromolecular Crystallography **Pt A** (276): 307-326.

Paciotti, V., M. Clerici, et al. (2000). "The checkpoint protein Ddc2, functionally related to S. pombe Rad26, interacts with Mec1 and is regulated by Mec1-dependent phosphorylation in budding yeast." Genes Dev **14**(16): 2046-59.

Paciotti, V., M. Clerici, et al. (2001). "Characterization of mec1 kinase-deficient mutants and of new hypomorphic mec1 alleles impairing subsets of the DNA damage response pathway." Mol Cell Biol **21**(12): 3913-25.

Pasero, P., A. Bensimon, et al. (2002). "Single-molecule analysis reveals clustering and epigenetic regulation of replication origins at the yeast rDNA locus." Genes Dev **16**(19): 2479-84.

Pasero, P., B. P. Duncker, et al. (1999). "A role for the Cdc7 kinase regulatory subunit Dbf4p in the formation of initiation-competent origins of replication." Genes Dev **13**(16): 2159-76.

Paull, T. T. and M. Gellert (1998). "The 3' to 5' exonuclease activity of Mre 11 facilitates repair of DNA double-strand breaks." Mol Cell **1**(7): 969-79.

Paulsen, R. D. and K. A. Cimprich (2007). "The ATR pathway: fine-tuning the fork." DNA Repair (Amst) **6**(7): 953-66.

Pavlov, Y. I., C. Frahm, et al. (2006). "Evidence that errors made by DNA polymerase alpha are corrected by DNA polymerase delta." Curr Biol **16**(2): 202-7.

Pellicioli, A., S. E. Lee, et al. (2001). "Regulation of Saccharomyces Rad53 checkpoint kinase during adaptation from DNA damage-induced G2/M arrest." Mol Cell **7**(2): 293-300.

Pellicioli, A., C. Lucca, et al. (1999). "Activation of Rad53 kinase in response to DNA damage and its effect in modulating phosphorylation of the lagging strand DNA polymerase." EMBO J **18**(22): 6561-72.

Perrakis, A., R. Morris, et al. (1999). "Automated protein model building combined with interactive structure refinement." Nature Structural Biology **6**: 458-463.

Petermann, E., M. L. Orta, et al. "Hydroxyurea-stalled replication forks become progressively inactivated and require two different RAD51-mediated pathways for restart and repair." Mol Cell **37**(4): 492-502.

Philipova, D., J. R. Mullen, et al. (1996). "A hierarchy of SSB protomers in replication protein A." Genes Dev **10**(17): 2222-33.

Pichierri, P., F. Rosselli, et al. (2003). "Werner's syndrome protein is phosphorylated in an ATR/ATM-dependent manner following replication arrest and DNA damage induced during the S phase of the cell cycle." Oncogene **22**(10): 1491-500.

Pike, B. L., S. Yongkiettrakul, et al. (2003). "Diverse but overlapping functions of the two forkhead-associated (FHA) domains in Rad53 checkpoint kinase activation." J Biol Chem **278**(33): 30421-4.

Pike, B. L., S. Yongkiettrakul, et al. (2004). "Mdt1, a novel Rad53 FHA1 domain-interacting protein, modulates DNA damage tolerance and G(2)/M cell cycle progression in Saccharomyces cerevisiae." Mol Cell Biol **24**(7): 2779-88.

Pirzio, L. M., P. Pichierri, et al. (2008). "Werner syndrome helicase activity is essential in maintaining fragile site stability." J Cell Biol **180**(2): 305-14.

Pursell, Z. F., I. Isoz, et al. (2007). "Yeast DNA polymerase epsilon participates in leading-strand DNA replication." Science **317**(5834): 127-30.

Raghuraman, M. K., B. J. Brewer, et al. (1997). "Cell cycle-dependent establishment of a late replication program." Science **276**(5313): 806-9.

Raghuraman, M. K., E. A. Winzeler, et al. (2001). "Replication dynamics of the yeast genome." Science **294**(5540): 115-21.

Ralf, C., I. D. Hickson, et al. (2006). "The Bloom's syndrome helicase can promote the regression of a model replication fork." J Biol Chem **281**(32): 22839-46.

Randell, J. C., J. L. Bowers, et al. (2006). "Sequential ATP hydrolysis by Cdc6 and ORC directs loading of the Mcm2-7 helicase." Mol Cell **21**(1): 29-39.

Remus, D., F. Beuron, et al. (2009). "Concerted loading of Mcm2-7 double hexamers around DNA during DNA replication origin licensing." Cell **139**(4): 719-30.

Ricke, R. M. and A. K. Bielinsky (2004). "Mcm10 regulates the stability and chromatin association of DNA polymerase-alpha." Mol Cell **16**(2): 173-85.

Robison, J. G., J. Elliott, et al. (2004). "Replication protein A and the Mre11.Rad50.Nbs1 complex co-localize and interact at sites of stalled replication forks." J Biol Chem **279**(33): 34802-10.

Rossi, M. L., A. K. Ghosh, et al. "Roles of Werner syndrome protein in protection of genome integrity." DNA Repair (Amst) **9**(3): 331-44.

Rouse, J. and S. P. Jackson (2002). "Lcd1p recruits Mec1p to DNA lesions in vitro and in vivo." Mol Cell **9**(4): 857-69.

San Filippo, J., P. Sung, et al. (2008). "Mechanism of eukaryotic homologous recombination." Annu Rev Biochem **77**: 229-57.

Santocanale, C. and J. F. Diffley (1998). "A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication." Nature **395**(6702): 615-8.

Sawyer, S. L., I. H. Cheng, et al. (2004). "Mcm10 and Cdc45 cooperate in origin activation in Saccharomyces cerevisiae." J Mol Biol **340**(2): 195-202.

Schwartz, M. F., J. K. Duong, et al. (2002). "Rad9 phosphorylation sites couple Rad53 to the Saccharomyces cerevisiae DNA damage checkpoint." Mol Cell **9**(5): 1055-65.

Sclafani, R. A. and T. M. Holzen (2007). "Cell cycle regulation of DNA replication." Annu Rev Genet **41**: 237-80.

Segurado, M., A. de Luis, et al. (2003). "Genome-wide distribution of DNA replication origins at A+T-rich islands in Schizosaccharomyces pombe." EMBO Rep **4**(11): 1048-53.

Segurado, M. and J. F. Diffley (2008). "Separate roles for the DNA damage checkpoint protein kinases in stabilizing DNA replication forks." Genes Dev **22**(13): 1816-27.

Segurado, M. and J. A. Tercero (2009). "The S-phase checkpoint: targeting the replication fork." Biol Cell **101**(11): 617-27.

Sengupta, S., S. P. Linke, et al. (2003). "BLM helicase-dependent transport of p53 to sites of stalled DNA replication forks modulates homologous recombination." Embo J **22**(5): 1210-22.

Shechter, D., V. Costanzo, et al. (2004). "ATR and ATM regulate the timing of DNA replication origin firing." Nat Cell Biol **6**(7): 648-55.

Shechter, D. and J. Gautier (2005). "ATM and ATR check in on origins: a dynamic model for origin selection and activation." Cell Cycle **4**(2): 235-8.

Shen, J. C., Y. Lao, et al. (2003). "The N-terminal domain of the large subunit of human replication protein A binds to Werner syndrome protein and stimulates helicase activity." Mech Ageing Dev **124**(8-9): 921-30.

Sheu, Y. J. and B. Stillman "The Dbf4-Cdc7 kinase promotes S phase by alleviating an inhibitory activity in Mcm4." Nature **463**(7277): 113-7.

Shi, W., Z. Feng, et al. "The Role of RPA2 Phosphorylation in Homologous Recombination in Response to Replication Arrest." Carcinogenesis.

Shimada, K., P. Pasero, et al. (2002). "ORC and the intra-S-phase checkpoint: a threshold regulates Rad53p activation in S phase." Genes Dev **16**(24): 3236-52.

Shirahige, K., Y. Hori, et al. (1998). "Regulation of DNA-replication origins during cell-cycle progression." Nature **395**(6702): 618-21.

Singh, T. R., A. M. Ali, et al. (2008). "BLAP18/RMI2, a novel OB-fold-containing protein, is an essential component of the Bloom helicase-double Holliday junction dissolvasome." Genes Dev **22**(20): 2856-68.

Soustelle, C., M. Vedel, et al. (2002). "Replication protein A is required for meiotic recombination in Saccharomyces cerevisiae." Genetics **161**(2): 535-47.

Sowd, G., H. Wang, et al. (2009). "Replication protein a stimulates the werner syndrome protein branch migration activity." J Biol Chem **284**(50): 34682-91.

Sweeney, F. D., F. Yang, et al. (2005). "Saccharomyces cerevisiae Rad9 acts as a Mec1 adaptor to allow Rad53 activation." Curr Biol **15**(15): 1364-75.

Syljuasen, R. G., C. S. Sorensen, et al. (2005). "Inhibition of human Chk1 causes increased initiation of DNA replication, phosphorylation of ATR targets, and DNA breakage." Mol Cell Biol **25**(9): 3553-62.

Takayama, Y., Y. Kamimura, et al. (2003). "GINS, a novel multiprotein complex required for chromosomal DNA replication in budding yeast." Genes Dev **17**(9): 1153-65.

Tam, A. T., B. L. Pike, et al. (2007). "Telomere-related functions of yeast KU in the repair of bleomycin-induced DNA damage." Biochem Biophys Res Commun **357**(3): 800-3.

Tanaka, S., T. Umemori, et al. (2007). "CDK-dependent phosphorylation of Sld2 and Sld3 initiates DNA replication in budding yeast." Nature **445**(7125): 328-32.

Tanaka, T. and K. Nasmyth (1998). "Association of RPA with chromosomal replication origins requires an Mcm protein, and is regulated by Rad53, and cyclin- and Dbf4-dependent kinases." EMBO J **17**(17): 5182-91.

Tauchi, H., J. Kobayashi, et al. (2002). "Nbs1 is essential for DNA repair by homologous recombination in higher vertebrate cells." Nature **420**(6911): 93-8.

Tercero, J. A., K. Labib, et al. (2000). "DNA synthesis at individual replication forks requires the essential initiation factor Cdc45p." EMBO J **19**(9): 2082-93.

Tercero, J. A., M. P. Longhese, et al. (2003). "A central role for DNA replication forks in checkpoint activation and response." Mol Cell **11**(5): 1323-36.

Tishkoff, D. X., A. L. Boerger, et al. (1997). "Identification and characterization of Saccharomyces cerevisiae EXO1, a gene encoding an exonuclease that interacts with MSH2." Proc Natl Acad Sci U S A **94**(14): 7487-92.

Tittel-Elmer, M., C. Alabert, et al. (2009). "The MRX complex stabilizes the replisome independently of the S phase checkpoint during replication stress." EMBO J **28**(8): 1142-56.

Tourriere, H. and P. Pasero (2007). "Maintenance of fork integrity at damaged DNA and natural pause sites." DNA Repair (Amst) **6**(7): 900-13.

Tourriere, H., G. Versini, et al. (2005). "Mrc1 and Tof1 promote replication fork progression and recovery independently of Rad53." Mol Cell **19**(5): 699-706.

Tran, P. T., N. Erdeniz, et al. (2002). "Characterization of nuclease-dependent functions of Exo1p in Saccharomyces cerevisiae." DNA Repair (Amst) **1**(11): 895-912.

Traven, A. and J. Heierhorst (2005). "SQ/TQ cluster domains: concentrated ATM/ATR kinase phosphorylation site regions in DNA-damage-response proteins." Bioessays **27**(4): 397-407.

Tuduri, S., L. Crabbe, et al. (2009). "Topoisomerase I suppresses genomic instability by preventing interference between replication and transcription." Nat Cell Biol **11**(11): 1315-24.

Uhlmann, F. (2009). "A matter of choice: the establishment of sister chromatid cohesion." EMBO Rep **10**(10): 1095-102.

Umezu, K., N. Sugawara, et al. (1998). "Genetic analysis of yeast RPA1 reveals its multiple functions in DNA metabolism." Genetics **148**(3): 989-1005.

Usui, T., S. S. Foster, et al. (2009). "Maintenance of the DNA-damage checkpoint requires DNA-damage-induced mediator protein oligomerization." Mol Cell **33**(2): 147-59.

Vassin, V. M., R. W. Anantha, et al. (2009). "Human RPA phosphorylation by ATR stimulates DNA synthesis and prevents ssDNA accumulation during DNA-replication stress." J Cell Sci **122**(Pt 22): 4070-80.

Vaziri, C., S. Saxena, et al. (2003). "A p53-dependent checkpoint pathway prevents rereplication." Mol Cell **11**(4): 997-1008.

Veaute, X., J. Jeusset, et al. (2003). "The Srs2 helicase prevents recombination by disrupting Rad51 nucleoprotein filaments." Nature **423**(6937): 309-12.

Vonrhein, C., E. Blanc, et al. (2005). Automated structure solution with autoSHARP.

Wang, X. and J. E. Haber (2004). "Role of Saccharomyces single-stranded DNA-binding protein RPA in the strand invasion step of double-strand break repair." PLoS Biol **2**(1): E21.

Wang, X., G. Ira, et al. (2004). "Role of DNA replication proteins in double-strand break-induced recombination in Saccharomyces cerevisiae." Mol Cell Biol **24**(16): 6891-9.

Watt, P. M., I. D. Hickson, et al. (1996). "SGS1, a homologue of the Bloom's and Werner's syndrome genes, is required for maintenance of genome stability in Saccharomyces cerevisiae." Genetics **144**(3): 935-45.

Weinreich, M. and B. Stillman (1999). "Cdc7p-Dbf4p kinase binds to chromatin during S phase and is regulated by both the APC and the RAD53 checkpoint pathway." EMBO J **18**(19): 5334-46.

Weinstein, J. and R. Rothstein (2008). "The genetic consequences of ablating helicase activity and the Top3 interaction domain of Sgs1." DNA Repair (Amst) **7**(4): 558-71.

West, S. C. (2003). "Molecular views of recombination proteins and their control." Nat Rev Mol Cell Biol **4**(6): 435-45.

Williams, R. S., G. E. Dodson, et al. (2009). "Nbs1 flexibly tethers Ctp1 and Mre11-Rad50 to coordinate DNA double-strand break processing and repair." Cell **139**(1): 87-99.

Wohlschlegel, J. A., S. K. Dhar, et al. (2002). "Xenopus Mcm10 binds to origins of DNA replication after Mcm2-7 and stimulates origin binding of Cdc45." Mol Cell **9**(2): 233-40.

Wohlschlegel, J. A., B. T. Dwyer, et al. (2000). "Inhibition of eukaryotic DNA replication by geminin binding to Cdt1." Science **290**(5500): 2309-12.

Wold, M. S. (1997). "Replication protein A: a heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism." Annu Rev Biochem **66**: 61-92.

Woodward, A. M., T. Gohler, et al. (2006). "Excess Mcm2-7 license dormant origins of replication that can be used under conditions of replicative stress." J Cell Biol **173**(5): 673-83.

Wu, J. R. and D. M. Gilbert (1995). "Rapid DNA preparation for 2D gel analysis of replication intermediates." Nucleic Acids Res **23**(19): 3997-8.

Wu, L. (2007). "Role of the BLM helicase in replication fork management." DNA Repair (Amst) **6**(7): 936-44.

Wu, L. and I. D. Hickson (2003). "The Bloom's syndrome helicase suppresses crossing over during homologous recombination." Nature **426**(6968): 870-4.

Xu, D., R. Guo, et al. (2008). "RMI, a new OB-fold complex essential for Bloom syndrome protein to maintain genome stability." Genes Dev **22**(20): 2843-55.

Xu, X., P. J. Rochette, et al. (2009). "MCM10 mediates RECQ4 association with MCM2-7 helicase complex during DNA replication." EMBO J **28**(19): 3005-14.

Xu, X., S. Vaithiyalingam, et al. (2008). "The basic cleft of RPA70N binds multiple checkpoint proteins, including RAD9, to regulate ATR signaling." Mol Cell Biol **28**(24): 7345-53.

Yamagata, K., J. Kato, et al. (1998). "Bloom's and Werner's syndrome genes suppress hyperrecombination in yeast sgs1 mutant: implication for genomic instability in human diseases." Proc Natl Acad Sci U S A **95**(15): 8733-8.

Yao, J. X., Ed. (1981). On the Application of Phase-Relationships to Complex Structures. Acta Crystallographica Section A, Rantan-Random Multan.

Yodh, J. G., B. C. Stevens, et al. (2009). "BLM helicase measures DNA unwound before switching strands and hRPA promotes unwinding reinitiation." EMBO J **28**(4): 405-16.

Zegerman, P. and J. F. Diffley (2007). "Phosphorylation of Sld2 and Sld3 by cyclin-dependent kinases promotes DNA replication in budding yeast." Nature **445**(7125): 281-5.

Zha, S., C. Boboila, et al. (2009). "Mre11: roles in DNA repair beyond homologous recombination." Nat Struct Mol Biol **16**(8): 798-800.

Zhao, X., E. G. Muller, et al. (1998). "A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools." Mol Cell **2**(3): 329-40.

Zhao, X. and R. Rothstein (2002). "The Dun1 checkpoint kinase phosphorylates and regulates the ribonucleotide reductase inhibitor Sml1." Proc Natl Acad Sci U S A **99**(6): 3746-51.

Zhou, Y. and T. S. Wang (2004). "A coordinated temporal interplay of nucleosome reorganization factor, sister chromatin cohesion factor, and

DNA polymerase alpha facilitates DNA replication." Mol Cell Biol **24**(21): 9568-79.

Zhu, W., C. Ukomadu, et al. (2007). "Mcm10 and And-1/CTF4 recruit DNA polymerase alpha to chromatin for initiation of DNA replication." Genes Dev **21**(18): 2288-99.

Zhu, Z., W. H. Chung, et al. (2008). "Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends." Cell **134**(6): 981-94.

Zou, L. and S. J. Elledge (2003). "Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes." Science **300**(5625): 1542-8.

Zou, Y., Y. Liu, et al. (2006). "Functions of human replication protein A (RPA): from DNA replication to DNA damage and stress responses." J Cell Physiol **208**(2): 267-73.

Appendix

List of Abbreviations

2D	Two dimensional
5-FOA	5-Fluoroorotic acid
9-1-1	Rad9-Rad1-Hus1
A	Alanine
Aa	Amino acid
Ala	Alanine
ACS	ARS consensus sequence
ARS	Autonomous replicating sequence
ATM	Ataxia telangiectasia mutated
ATP	Adenosinetriphosphate
ATR	Ataxia telangiectasia and Rad3 related
Bp	base pairs
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CDK	Cyclin-dependent kinase
ChIP	Chromatin immunoprecipitation
CIP	Calf intestinal phophatase
CK2	Casein kinase 2
CPT	Camptothecin
DAPI	4',6-Diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
DSB	double strand break
dsDNA	double-stranded DNA
DTT	dithiothreitol
E	glutamic acid
FACS	Fluorescence activated cell sorting
FHA	Forkhead associated
GCR	Gross chromosomal re-arrangements

GINS	Go, Ichi, Nii, and San (five, one, two, and three in Japanese),
Glu	Glutamic acid
GST	Glutathione-S-transferase
H2A	Histone 2a
HA	Hemagglutinin
HPLC	High performance liquid chromatography
HR	Homologous recombination
HRP	Horseradish peroxidase
HU	Hydroxyurea
IF	Immunofluorescence
IP	Immunoprecipitation
IPTG	Isopropyl-ß-D-thiogalaktopyranosid
K	Lysine
Kb	Kilobase
M phase	Mitotic phase
Mab	Monoclonal antibody
MAT	Mating type (locus)
MCM	Minichromosome maintenance
Mec1	Mitosis entry checkpoint mutant 1
MMS	Methylmethanesulfonate
MRN	Mre11, Rad50, Nbs1
MRX	Mre11, Rad50, Xrs2
MS	Mass spectroscopy
NHEJ	Non-homologous end joining
Noc	Nocodazole
Nt	Nucleotide
ORC	Origin recognition complex
ORF	Open reading frame
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
Pfu	*Pyrococcus furiosus*
PI3K	Phosphoinositide-3 kinase
PMSF	Phenylmethylsulfonylfluorid
Pol	Polymerase

PP2C	Protein phosphatase 2c
Pre-IC	Pre-initiation complex
Pre-RC	Pre-replication complex
Q	Glutamine
qPCR	Quantitative PCR
Rad53	Radiation sensitive mutant 53
rDNA	Ribosomal DNA
RPA	Replication protein A
RFB	Replication fork barrier
RFC	Replication factor C
RNAi	RNA interference
ssDNA	single-stranded DNA

Acknowledgements

First of all, I would like to thank my supervisor Susan Gasser for giving me the chance to work during my PhD studies on ‘maintenance of genome integrity’, a topic which had always intrigued me. I would like to thank her for giving me the freedom to work as independently as possible and her support, when following own ideas. I’m also grateful for her guidance and the opportunity to present my data on various conferences.

I would like to thank all my PhD committee members: Ellen Fanning, Jennifer Cobb, Nico Thomä and Susan Gasser, for their continuous support and very interesting scientific discussions throughout my whole PhD. Special thanks go to Primo Schär, who accompanied me during all those years with helpful ideas in our joint group meetings and various conferences and who kindly agreed to act as the co-referee for my thesis. Additionally, I would like to thank Nancy Hynes for being an always good-humored room-neighbour and for chairing the final defense.

Next, I would like to thank those people, without their collaboration this PhD work would not have been possible. Many thanks go to Nico Thomä and his lab, especially to Markus Vogel and Philipp Amsler, who worked on the biochemical site of the project. They over-expressed an enormous amount of RPA and Sgs1 fragments and crystallized the N-terminus of yeast RPA70. Thanks also to Seth Rubin, a friend and collaborator from Nico Thomä, for his ITC work on the binding of RPA and Sgs1.

Warm thanks go to Philippe Pasero for his continuous support and for giving me the opportunity to learn and apply DNA combing. I also thank him and his lab members, especially Julian Bianco, Armelle Lengronne and Ana María Poveda Gabaldón, for the friendly atmosphere and for scientific and personal support during my stay.

I acknowledge the whole Gasser lab for the super atmosphere and scientific discussions. Kenji Shimada is an expert on genomic stability and replication. I'm very grateful for his pleasant attitude, scientific ideas and his help in technical matters. I thank the other members of the DNA damage minigroup, especially Thomas Schleker and Haico van Attikum. This work was greatly eased by the lab organization and daily work of Monika Tsai, Veronique Kalck and Razel Arpagaus. Special thanks go to Brietta Pike. She assisted me all those years with scientific questions and has also become in personal matters a close friend of mine.

At FMI, I would like to thank the imaging facility, especially Laurent Gelman, for their help, when I was struggling with MetaMorph. From the bioinformatics facility, I'm grateful to Michael Stadler for statistical analysis of the DNA combing data and Michael Rebhan for his help in analyzing and mapping the RPA-Sgs1 interaction site. Of course, I acknowledge also Alan Naylor, Sjoerd Van Eeden and Thomas Nyffenegger for their help concerning my cranky laptop or other IT questions. Special thanks go also to Gabi Gruber and Dorothy Searles for always being in a good mood and very helpful, when I had organizational or financial questions.

For funding, I would like to thank EMBO short term fellowship, Marie Curie actions and the Novartis Research foundation.

Additionally, I truly thank Helder Ferreira, Brietta Pike, Kenji Shimada, and Mareike Albert for carefully reading my thesis and giving me a lot of useful comments. Mareike and Peter Albert have encouraged me a lot and Peter helped me to tackle my microsoft problems. Sabine Rohner and Astrid Cornils have backed me during the tough end of the thesis.

Needless to say, my biggest thanks go to Sam, my family and to my friends. They always encourage and support me in any way possible.