Chromatin assembly by CAF-1 during homologous recombination : a novel step of regulation

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Assemblage de la chromatine par CAF-1 au cours de la recombinaison homologue : un nouveau degré de régulation chez la levure *S. pombe.*

Jury

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SUMMARY

ABBREVIATIONS

5-FOA: 5 fluorootic acid

Asf1: anti silencing function 1

BER: base excision repair

BIR: break-induced replication model

CAF-1: chromatin assembly factor1

CO: crossing-over

CPT: camptothecin

DDR: DNA damage response

D-loop: displacement loop

dNTP: deoxyribonucleotides

DSB: double strand break

DSBR: double-strand break repair

dsDNA:double strand DNA

FoSTeS: fork stalling and template switching

FRET: fluorescence resonance energy transfer

GCR: global genome repair (NER)

GCRs: gross chromosomal rearrangements

H3K56Ac: histone H3 acetylated on lysine 56

HJ: holliday junction

HR: homologous recombination

HU: hydroxyurea

Hyg: hygromycine

IDLs: insertion-deletion loops

JM: joint molecules

Kan: geneticine

LOH: loss of heterozygosity

MEPS: minimal efficient processing segment

MMBIR: micro-homology mediated BIR

MMC: mitomycin C

MMR: mismatch repair

MMS: methyl methanesulfonate

MRX: Mre11-Rad50-Xrs2

NAHR: non-allelic homologous recombination

Nat: nourseothricin

NER: nucleotide excision repair

NHEJ: non-homologous end joining

PCNA: proliferating cell nuclear antigen

PCR: polymerase chain reaction

PIP: PCNA Interacting Protein

PTMs: post-translational modifications

RFB: replication fork barrier

RFC: replication factor C

RMPs: recombination mediator proteins

RPA: replication protein A

SCE: sister chromatid exchange

SCID: severe combined immune deficiency

SDSA: synthesis-dependent strand annealing

SSA: single strand annealing

ssDNA: single strand DNA

TBZ: tetrabenazine

TCR: transcription-coupled repair (NER)

UV: Ultraviolet radiations

XP: Xeroderma Pigmentosum

PREAMBLE

DNA replication and subsequent segregation of chromosomes requires the coordinated action of a complex network of pathways, which ensure the accurate transmission of the genetic material. DNA replication is a fundamental process for every life form that needs to be orchestrated with chromatin disassembly/assembly, maintenance of epigenetic marks, and DNA repair pathway in order to maintain genome stability and inheritance of epigenetic information (for example to label different chromatin structure heterochromatin versus euchromatin). Defects in the coordination of DNA metabolism pathways during chromosomes replication can result in genomic and epi-genomic instability and ultimately trigger cancer development. Indeed, genome instability is not only a prominent common feature for most, if not all, types of cancer but importantly, it is strongly associated with cancer predisposition in many human syndromes.

Chromosomes replication can be challenged by various endogenous and environmental factors, interfering with the progression of replication fork. Therefore, cells have also to coordinate DNA synthesis with mechanisms ensuring the stability and the recovery of halted forks. Among these mechanisms are included the “checkpoints” and the “homologous recombination” pathways. The DNA damage response promotes cell survival by activating cell cycle checkpoints, allowing time for DNA repair prior to resumption of cell cycle progression, or DNA synthesis resumption before entering into mitosis. Homologous recombination is a universal mechanism that supports DNA repair and robust DNA replication. However, due to its ability to promote genetic exchanges between repeated sequences dispersed through the genome, recombination also contributes to chromosomal rearrangements (mainly by Non Allelic Homologous Recombination, NAHR). Therefore, homologous recombination needs to be accurately regulated to promote efficient DNA repair and robust DNA replication while limiting aberrant recombination outcomes, contributing to genome instability. Nevertheless, mechanisms regulating allelic versus non allelic recombination remain poorly understood.

Replication-coupled chromatin assembly is a vital process that ensures correct wrapping of newly replicated DNA around nucleosomes and proper inheritance of chromatin marks. The Chromatin Assembly Factor 1 (CAF-1) is a histone H3/H4 chaperone playing a major role in replication-coupled nucleosomal deposition. Homologous recombination includes a DNA synthesis step and little is known on the associated chromatin assembly during homologous

recombination. During my PhD, I investigated the role of CAF-1 during the mechanisms of homologous recombination. The data obtained support a model according to which CAF-1 promotes histones deposition during homologous recombination. By doing so, CAF-1 allows the stabilization of early recombination intermediates (D-loop), by preventing their dissociations by DNA helicases. Thus, CAF-1 appears to be part of an equilibrium that regulates stability/dissociation of early recombination events. Importantly, I found that the role of CAF-1 in this equilibrium is of particular importance during non-allelic recombination, revealing a novel regulation level of homologous recombination mechanisms and outcomes by chromatin assembly.

Note to the readers:

The introduction covers several aspects of the maintenance of genome stability, including DNA repair, Homologous Recombination, and chromatin assembly. Our knowledge on these concepts came from studies undertaken in several organisms such as yeast models (*Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) and metazoans. Because our main understanding of homologous recombination results from studies using the yeast *S. cerevisiae*, the mechanisms of recombination are mainly presented in the context of budding yeast. In contrast, our main understanding of chromatin assembly during DNA replication came from studies undertaken in metazoans, especially in mammals; thus the mechanism of chromatin assembly is mainly presented in the context of human cells. Tables provided correspondences between yeast and human homologues are included.

INTRODUCTION

1. DNA REPAIR PATHWAYS

DNA lesions arise from three main causes: environmental agents, products of cellular metabolism and the natural instability of some chemical bonds in DNA. DNA repair mechanisms are evolutionary conserved from prokaryotic to eukaryotic cells, thus revealing the importance of maintaining genomic integrity. Mutations in genes involved in DNA repair are often associated with human diseases with a broad range of clinical features, included cancer susceptibility. The association between cancer predisposition and mutations in DNA repair genes illustrates the importance of DNA repair pathways in maintaining genome stability. During the evolution, cells have developed multiple DNA repair pathways according to the nature of the DNA lesion (Figure n.1).

In this chapter, I will present a brief description of the DNA repair pathways and then I will focus on homologous recombination, which is the predominant repair pathway that was investigated during my PhD.

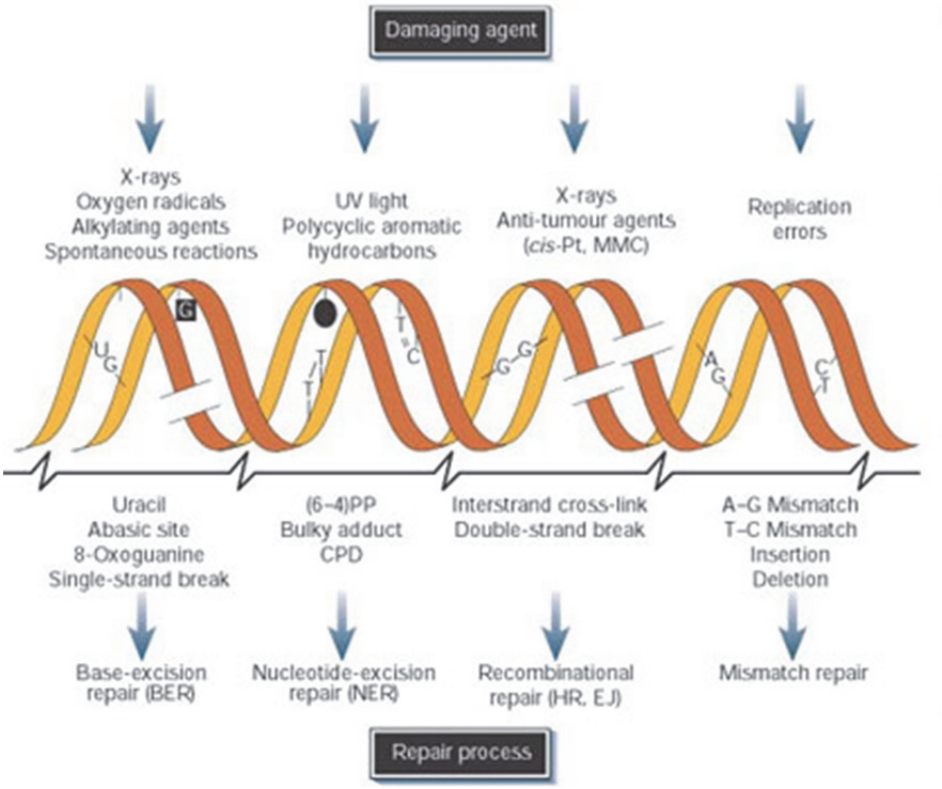


Figure n.1: DNA repair pathways according to the nature of DNA lesions (Hoeijmakers 2001).

1.1. Base Excision Repair (BER)

DNA lesions that do not significantly distort the DNA backbone are corrected by the base excision repair (BER) pathway (Figure 1). A wide variety of damaged DNA bases are recognized by BER: DNA bases chemically modified by oxidation or alkylation, abasic sites resulting from base loss, uracil resulting from deamination of cytosine or from misincorporation during DNA replication, fragmented pyrimidines (Beranek 1990; Christmann, Tomicic et al. 2003; Barnes and Lindahl 2004; Dizdaroglu, Kirkali et al. 2008; Hegde, Hazra et al. 2008; Svilar, Goellner et al. 2011). Reactive oxygen species cause DNA oxidative lesions and among them, the most mutagenic one is the 7,8-dihydro-8-oxoguanine (8-oxogunanine). Indeed, if not repaired, 8-oxoguanine can form a base pair with adenine during DNA synthesis ultimately leading to GC to TA transversions (Kasai, Crain et al. 1986; Hazra, Hill et al. 2001; David, O'Shea et al. 2007).

This pathway is initiated by a specific DNA N-glycosylase that recognizes the damaged base and cleaves it, thus producing an apurinic/apyrimidinic site. Other enzymes are then recruited to excise the remaining sugar fragment. A DNA polymerase inserts the correct base and a DNA ligase seals the nick, thereby repairing the damage (Lindahl 1974; Haring, Rudiger et al. 1994; Kubota, Nash et al. 1996; Aburatani, Hippo et al. 1997; Morland, Rolseth et al. 2002). There are two distinct repair pathways: the short-patch pathway involves a single nucleotide replacement followed by ligation (implication of polymerase β and ligase III) and the long-patch pathway involves DNA synthesis of 2-6 nucleotides (by polymerase λ), removal of displaced nucleotide overhang by a specific Flap endonuclease (FEN-1) and sealing of the nick (ligase I) (Frosina, Fortini et al. 1996; Klungland and Lindahl 1997).

1.2. Nucleotide Excision Repair (NER)

Lesions that significantly distort the DNA helix are excised by the nucleotide excision repair (NER) pathway. NER is a highly conserved DNA repair pathway which removes lesions such as those caused by UV light (6-4 photoproducts and cyclobutane pyrimidine dimers) (Setlow and Carrier 1964; Setlow 1966; Ford and Hanawalt 1997; Dip, Camenisch et al. 2004). NER covers two pathways: the transcription-coupled repair (TCR) and the global genome repair (GGR) pathways. TCR occurs when the lesion is located in the transcribed strand of active genes and thus disturbs transcription elongation, while GCR occurs when the damage is in the non-transcribed strand of active genes or untranscribed regions of the genome (Tornaletti,

Reines et al. 1999; Li and Smerdon 2004). Whereas the same core set of enzymes is used by TCR and GGR, these two pathways rely on different proteins for the initial recognition of the DNA lesion. An intriguing hypothesis is that detection of lesions during TCR (stalling of the transcription machinery) could trigger global relaxation of chromatin structure throughout the genome, thus enhancing access of repair factors for GGR (Rubbi and Milner 2003). The excision of the DNA lesion involves four key events: the recognition of the lesion, the excision of the damaged nucleotide and of flanking regions, the filling of the gap by DNA synthesis and finally ligation of the nick (Huang, Svoboda et al. 1992; Lin and Sancar 1992; Mu, Park et al. 1995; Araujo, Tirode et al. 2000; Hoeijmakers 2001; Guo, Tang et al. 2010). The most notorious disease, resulting from defects in NER pathway, is the Xeroderma Pigmentosum (XP) disease. XP patients present a high risk of skin cancer and eye damage predisposition due to an extreme sun sensitivity (Setlow, Regan et al. 1969; Kraemer, Sander et al. 2007).

1.3. Mismatch Repair (MMR)

The DNA mismatch repair (MMR) pathway is responsible for the removal of mispaired bases or small DNA loop (insertion-deletion loops or IDLs). These errors are caused by DNA polymerases during DNA replication (Iyer, Pluciennik et al. 2006). Wagner and Meselson postulated that mismatch repair contributes to replication fidelity in *Escherichia coli*. This hypothesis was based on the fact that MMR is preferentially directed to the newly synthesized DNA strand (Wagner and Meselson 1976). In prokaryotes, transient hemimethylation during DNA replication distinguishes the two strands: the parental but not the newly synthetized is methylated (Pukkila, Peterson et al. 1983; Lyons and Schendel 1984). In eukaryotes, the MMR pathway is temporally coupled to DNA replication, and MMR components have been recently identified as associated with replication centres (Hombauer, Campbell et al. 2011; Hombauer, Srivatsan et al. 2011). However, the exact mechanism allowing discriminating the parental strand from the newly synthesized one by MMR remains unclear. The replication factor PCNA, which associates with DNA polymerases, might provide a physical link between repair and replication by acting as a strand signal at the fork (Umar, Buermeyer et al. 1996). Another hypothesis, involving incorporation of rNTPs into newly synthetized strands, has recently emerged. Indeed, during normal DNA replication, DNA polymerases have the ability to incorporate rNTPs, instead of dNTPs, at a frequency of one rNTP every 1.250 nucleotides (Nick McElhinny, Kissling et al. 2010). The surprising high level of rNTPs

incorporation into DNA molecules during DNA replication might serve as a signal to recognize the parental strand from newly synthetized one by eukaryotic MMR (Lazzaro, Novarina et al. 2012).

Subsequent steps to the recognition of base-base mismatches are: a) recognition of the nascent strand and generation of a nick, b) unwinding of the 3' end of the nicked strand, c) exonucleolytic degradation of error-containing section of the newly synthesized strand, d) filling of the resulting gap by DNA synthesis and sealing of the repair nick by DNA ligase (Augusto-Pinto, Teixeira et al. 2003; Guo, Presnell et al. 2004; Constantin, Dzantiev et al. 2005; Jiricny 2006; Geng, Du et al. 2011; Pena-Diaz, Bregenhorn et al. 2012).

In humans, the mutator phenotype conferred by defects in MMR contributes to the initiation and promotion of multistage carcinogenesis (Loeb, Loeb et al. 2003). Loss of MMR efficiency leads to cancer predisposition syndromes such as the hereditary non-polyposis colon cancer syndrome (HNPCC) (Liu, Parsons et al. 1996). Loss of MMR efficiency has also been implicated in the development of a subset of sporadic tumors that occur in a variety of tissues (Eshleman and Markowitz 1995; Peltomaki, Gao et al. 2001; Jiricny 2006; Jun, Kim et al. 2006).

1.4. Non Homologous End-Joining (NHEJ)

Among all DNA lesions, double-strand breaks (DSBs) are considered as the most toxic lesions, if not repaired. They are caused by genotoxic agents such as ionizing radiation, but they are also programmed to orchestrate physiological processes, like in V(D)J recombination (somatic recombination). V(D)J recombination is necessary for the development of the vertebrate immune system (Leder, Max et al. 1981; Yaneva, Kowalewski et al. 1997; Coster, Gold et al. 2012). Another example is the programmed DSB introduced by the HO endonuclease to promote mating type switching in the yeast *Saccharomyces cerevisiae* (Haber 2006).

DSBs can also jeopardize genome stability by generating genome rearrangements such as translocations, and thus trigger carcinogenesis by inactivation of tumor suppressor genes or activation of oncogenes. Homologous Recombination (HR) and Non Homologous End Joining (NHEJ) are the two pathways involved in DSB repair. The predominance of one pathway over the other varies according to the organism, but also according to the stage of the cell cycle and the nature of DNA ends at the DSB site (Takata, Sasaki et al. 1998). For

example, in human cells, in which DSBs are mainly repaired by NHEJ, the maximal use of HR occurs during DNA replication (Karanam, Kafri et al. 2012). In contrast, DSBs are mainly repaired by HR in yeast models, but NHEJ occurs also in G1 cells (Roth and Wilson 1985; Godwin, Bollag et al. 1994; Kanaar, Hoeijmakers et al. 1998). Moreover, there is also a possible competition between two key enzymes of NHEJ and HR (Ku 70/80 and Rad52, respectively) for binding of DNA ends (Van Dyck, Stasiak et al. 1999; Pierce, Hu et al. 2001).

Non-Homologous End Joining pathway is highly conserved among organisms. NHEJ was firstly discovered in mammalian cells, but NHEJ is present in all eukaryotes and bacteria. NHEJ consists of the direct joining of two DNA ends and requires at least four steps: a) detection of the DSB b) formation of a molecular bridge that holds the DNA ends together c) a processing mechanism that modifies non-matching and/or damaged nucleotide at DNA ends into compatible ends and d) final ligation step. In vertebrates, these steps require an intimate interplay among the core NHEJ machinery, composed of XRCC4 (for *X-ray repair Cross Complementing* group 4), DNA ligase IV, the Ku70/80 heterodimer and the DNA-PKcs (DNA-dependent protein kinase catalytic subunit) (Roth, Porter et al. 1985; Yaneva, Kowalewski et al. 1997; Walker, Corpina et al. 2001; Mahajan, Nick McElhinny et al. 2002; Mari, Florea et al. 2006; Spagnolo, Rivera-Calzada et al. 2006; Weterings and Chen 2008)

When DNA ends are blunt, NHEJ is a an error-free mechanism (van Heemst, Brugmans et al. 2004) while in presence of complex lesions due to multiple damages, the ligation process leads often to deletions (Roth, Porter et al. 1985; Moore and Haber 1996; Wilson, Grawunder et al. 1997; Pardo, Ma et al. 2006). Defects in NHEJ components lead to a severe immunodeficiency disease termed SCID (Severe Combined Immune Deficiency). In this pathology, B cells and T cells of the adaptive immune system are impaired leading to an increase susceptibility to infectious diseases (Bosma, Custer et al. 1983; Perryman 2004; O'Driscoll and Jeggo 2006; van der Burg, van Dongen et al. 2009). Furthermore, cells derived from patients with a defect in NHEJ are highly radiosensitive (Riballo, Critchlow et al. 1999; Buck, Moshous et al. 2006).

2. HOMOLOGOUS RECOMBINATION

Homologous recombination (HR) is a universally conserved mechanism that promotes DNA repair and supports DNA replication. The fundamental function of HR is to promote strand exchange reactions, leading to genetic exchanges between homologous DNA sequences (Paques and Haber 1999; Mimitou and Symington 2009). The basic substrate of HR is a single stranded DNA (ssDNA) on which the recombinase Rad51 forms a nucleoprotein filament. This Rad51-coated ssDNA is then competent for the search of homologous sequences in the genome. Beyond its function in the repair of DSBs and single stranded gaps (ssDNA gaps), HR has multiple functions in eukaryotes such as support of DNA replication, alternative mechanism for telomere maintenance, diversification of immunoglobulin chains, programmed genetic diversity and chromosomes segregation in meiosis.

Alongside its potential benefits, HR is also considered as a source of genome instability. Indeed, the ability of HR to recombine repeated sequences dispersed throughout the genome can lead to genome rearrangements. Knowing that the human genome contains up to 10 % of repeated sequences, HR has to be accurately regulated to promote efficient DNA repair and to support DNA replication, while limiting aberrant outcomes contributing to cancer development or to genomic disorders. So, HR can be considered a "double-edged sword" for genome stability maintenance (Lambert and Carr 2005; Stankiewicz and Lupski 2006; Lee, Carvalho et al. 2007; Aguilera and Gomez-Gonzalez 2008; Lambert, Mizuno et al. 2010; Simmons, Carvalho et al. 2012).

Thanks to genetic, biochemical and molecular studies, the different steps of HR are well characterized during the repair of DSBs. Therefore, the molecular mechanism of HR will be first described in the context of DSB repair, and then in the context of DNA replication. Moreover, a particular attention will be given to the process of early joint-molecules (D-loop).

2.1. Early steps of homologous recombination

In response to a DSB, DNA ends are resected in 5' to 3' orientation to create a 3' single-stranded DNA. The Mre11-Rad50-Xrs2 (MRX) complex participates in early steps of end resection, via its DNA end-processing activities (Nelms, Maser et al. 1998; Lisby, Barlow et al. 2004; Mimitou and Symington 2008). The exonuclease activity of Mre11 *in vitro* operates in the opposite polarity to that required for DSB resection *in vivo*, so there are additional factors that cooperate with MRX to facilitate DSB processing, such as Sae2 (Rattray, McGill

et al. 2001; Clerici, Mantiero et al. 2005; Limbo, Chahwan et al. 2007; Sartori, Lukas et al. 2007). Sae2 in collaboration with MRX creates a minimally resected DNA intermediate. This intermediate is further processed by the 5'-3' exonuclease Exo1 or the exonuclease Dna2 in conjunction with the helicase Sgs1 (Figure n.2). The resection can result into kilobase-sized 3' ssDNA overhangs (Lee, Bressan et al. 2002; Tran, Erdeniz et al. 2004; Limbo, Chahwan et al. 2007; Sartori, Lukas et al. 2007; Gravel, Chapman et al. 2008; Mimitou and Symington 2008; Zhu, Chung et al. 2008; Bolderson, Tomimatsu et al. 2010; Cejka, Cannavo et al. 2010; Niu, Chung et al. 2010). The resulting ssDNA is initially stabilized through the binding of RPA (Replication protein A), the eukaryotic DNA single stranded binding protein (Figure n.2) (Alani, Thresher et al. 1992).

With the assistance of recombination mediator proteins (RMPs), the recombinase Rad51 nucleates onto ssDNA to form a nucleoprotein filament (pre-synaptic step). This step is mediated by Rad52 the main RMP, which displaces RPA from ssDNA to facilitate Rad51 nucleation onto ssDNA. The Rad51 nucleoprotein filament is then the competent intermediate to initiate the search of homology and the genetic exchange between homologous DNA molecules (Figure n.2). The nucleoprotein filament is able to invade a homologous DNA duplex, to pair the invading ssDNA with the complementary strand of the DNA duplex and to displace the non-complementary strand (synaptic step). The resulted three-stranded intermediate referred to as a joint-molecule, is termed displacement loop (D-loop) (New, Sugiyama et al. 1998; Shinohara, Shinohara et al. 1998). Once the D-loop has been formed, DNA polymerases extend the 3' single stranded DNA invading end, using the donor duplex as a template to restore the lost information (post-synaptic step) (Figure n.2).

Different models have been proposed to explain the step of homology search. The first model proposes that Rad51-filament binds nonspecifically to a dsDNA and then linearly diffuses or slides along the dsDNA, searching for homology (Gonda and Radding 1983). A second model proposes random three-dimensional collisions between the invading strand and the genomic DNA (Adzuma 1998; Gupta, Folta-Stogniew et al. 1999; Folta-Stogniew, O'Malley et al. 2004). The search for homology takes place in a chromatin environment and it is logical to speculate about the effect of nucleosomes on the search of homology or on the DNA invasion step. Nonetheless, nucleosomes do not impose a barrier for successful search of homology and strand invasion. Indeed Rad51-filaments are sufficient to locate the homologous sequence and to form initial joints molecules, even on the surface of a nucleosome *in vitro* (Sinha and Peterson 2008).

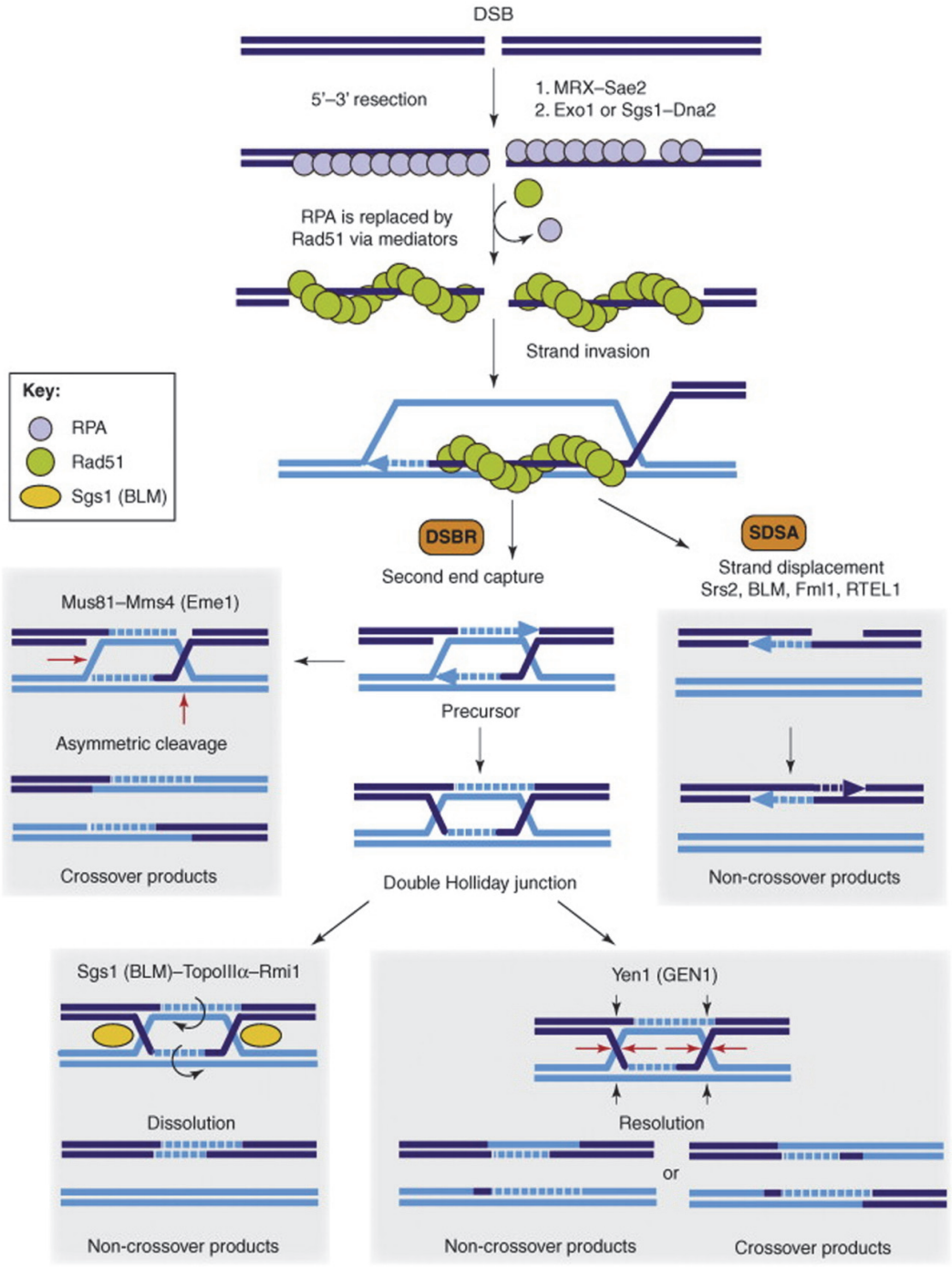


Figure n.2: Mechanism of DSB repair by homologous recombination The initial step of HR consists in the 5'-3' end resection, generating a 3' overhang ssDNA coated by RPA. Then Rad51 nucleates on RPA-coated ssDNA to form a nucleofilament which triggers strand invasion to form a D-loop. In the classical DSBR model, the second end is captured, leading to the formation of double Holliday junctions (HJs) that are resolved by cleavage, giving rise to crossing-over (CO) or non CO products, or resolved by dissolution, giving rise only to non CO products. In the SDSA pathway, the invaded strand is displaced after elongation and result only in non CO products (Mimitou and Symington 2009).

2.2. Models of homologous recombination

Over the last 30 years, several models of recombination have been proposed to report the type of genetic exchanges observed during mitotic or meiotic recombination. The main models are described in details below.

2.2.1. Double Strand Break Repair (DSBR)

The Double Strand Break Repair model was proposed by Szostak and collaborators in 1983 (Szostak, Orr-Weaver et al. 1983) (Figure n.2). In this model, both ends of the DSB are resected to generate 3' ssDNA overhang ends. One of the two ends invades a homologue duplex to form a D-loop structure. This process is catalyzed by the recombinase Rad51 and the main recombination mediator protein Rad52. The invading 3' end primes DNA synthesis using the complementary strand as a donor template. The 3' overhang of the second end is captured by the displaced strand and then elongated by DNA synthesis. The second end-capture is thought to be facilitated by the single strand annealing activity of Rad52 (see below) (Sugiyama, Imamura et al. 2006; McIlwraith and West 2008; Nimonkar, Sica et al. 2009). A double Holliday Junction (HJs) is then formed, which is resolved either by cleavage through the activities of HJ resolvases (Mus81/Mms4, Slx4, Yen1), or by dissolution through the helicase activity of the Sgs1/Rmi1/Top3 complex (Holliday 1964; Boddy, Gaillard et al. 2001; Ira, Malkova et al. 2003; Osman, Dixon et al. 2003; Wu and Hickson 2003; Hollingsworth and Brill 2004; Ip, Rass et al. 2008; Singh, Ahn et al. 2009) (Figure n.2). According to the strands cleaved by HJs resolvases, the genetic exchange results in either gene conversion associated with a cross-over (CO, *i.e.* the reciprocal exchange of flanking markers), or to gene conversion not associated with CO (*i.e.* no reciprocal exchange of flanking markers, see bottom panel of Figure n.2). In contrast, resolution of HJs by dissolution gives rise only to non CO products (Elborough and West 1990; Hyde, Davies et al. 1994; Paques and Haber 1999; Taylor and McGowan 2008). Mitotic cross-overs can result in chromosomal rearrangement such as translocations and thus can be potential threats for genome stability (see below).

Initially, the model of Szostak hypothesized that either the 3'- or the 5'-end of the DSB could be degraded (Szostak, Orr-Weaver et al. 1983). Nonetheless, the results obtained by Sun *et al*. have demonstrated that resection occurs only in the 5'-3' direction (Sun, Dawson et al. 1991). The model of Szostak well describes meiotic recombination events that are mainly associated with COs. However, mitotic recombination is less often associated with COs, thus leading to

the emergence of an alternative recombination model for the repair of DSB: the synthesis-dependent strand annealing (SDSA) pathway.

2.2.2. Synthesis Dependent Strand Annealing (SDSA)

In the Synthesis Dependent strand Annealing model, the invaded strand is elongated, displaced from the D-loop, and anneals back with the 3'ssDNA overhang of the second end (Figure n.2). The remaining gap is then filled by DNA synthesis and the nicks are ligated. The SDSA model forms only non COs products with no alteration of the genetic information of the donor duplex (Figure n.2, right panel) (Nassif, Penney et al. 1994; Paques and Haber 1999; McMahill, Sham et al. 2007; Mimitou and Symington 2009). It appears that the SDSA is the predominant pathway of DSB repair in vegetative cells (Elliott and Jasin 2002; Maloisel, Bhargava et al. 2004).

2.2.3. Break Induced Replication (BIR)

Break Induced Replication is an alternative model of DSB repair and it occurs when only one end of a double-strand break shares homology with a template, thus preventing the capture of the second DSB-end (Figure n.3). BIR has the particularity to promote efficient DNA synthesis over hundreds of kilobases, through the assembly of a single replisome. Therefore, BIR is thought to play a key role in the repair of broken replication forks and in the maintenance of eroded telomeres in cells lacking telomerase, as DNA synthesis can progress until the rebuilt-fork meets a converging fork, or progress from the break to the template chromosomes end (Bosco and Haber 1998; Cullen, Hussey et al. 2007; Lydeard, Jain et al. 2007; Lydeard, Lipkin-Moore et al. 2010; Moriel-Carretero and Aguilera 2010).

The first steps of BIR are similar to the ones of the DSBR model, resulting in a D-loop structure and the 3' end of the invading strand is used to prime DNA synthesis (Davis and Symington 2004; Malkova, Naylor et al. 2005). BIR requires all essential replication proteins, with the exception of those needed for pre-RC assembly. Thus, both leading and lagging strand DNA synthesis are involved in BIR, in contrast to SDSA that requires only leading strand DNA synthesis (Lydeard, Jain et al. 2007; Lydeard, Lipkin-Moore et al. 2010). In *S. cerevisiae*, the requirement of a non-essential polymerase δ subunit (Pol32) distinguishes the DNA synthesis associated to BIR from DNA synthesis associated with canonical replication (Smith, Llorente et al. 2007). Moreover, BIR is prone to template exchange and is hyper-

mutagenic, displaying increased template switching plus frameshift mutagenesis (Gerik, Li et al. 1998; Smith, Llorente et al. 2007; Deem, Keszthelyi et al. 2011).

How BIR allows extensive DNA replication and rebuilding of a replication fork remains unclear. One possible scenario is that the invading strand in the D-loop is unstable and undergoes multiple dissociation/invasion cycles. In this instance, the DNA would be conservatively replicated because all newly synthesized DNA would be associated with the sequences extending from the repair broken end (Figure n.3A) (Formosa and Alberts 1986). Alternatively, the D-loop might be converted into a complete unidirectional replication fork, progressing along the donor chromosome. The fact that all replication factors necessary for the elongation step of canonical replication forks are also required for BIR supports this model (Lydeard, Lipkin-Moore et al. 2010). This results in two semi-conservatively replicated molecules and a single Holliday Junction that needs to be resolved (Figure n.3B and C). BIR is known to drive chromosome rearrangements, such as long tracts of gene conversion, non-reciprocal translocations and multiple templates switching (Bosco and Haber 1998; Ruiz, Gomez-Gonzalez et al. 2009). This takes place when multiple rounds of invasion/DNA synthesis/dissociation occur within dispersed repeated sequences (McEachern and Haber 2006; Smith, Llorente et al. 2007).

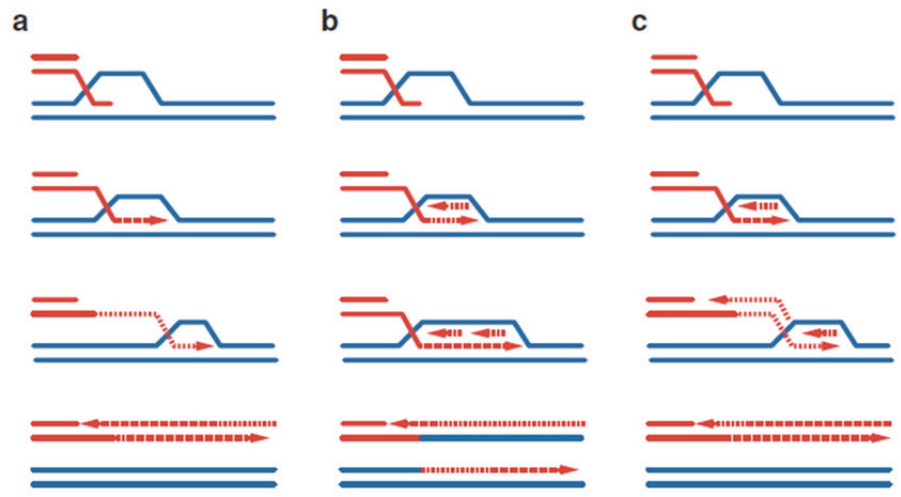


Figure n.3: Mechanism of BIR. **A)** Extension of the invading strand results in the synthesis of one strand that is displaced from the D-loop, thus converted into a DSB. The newly synthetized strand is then used as a template for the synthesis of the second strand. **B)** Extension of the invading strand results a unidirectional replication fork that progresses to the end of the donor chromosome. Resolution of the HJ results in two semiconservative replicated products. **C)** Branch migration of the HJ displaces the two newly synthetized strand. (Mc Eachern and Haber 2006).

2.2.4. Single Strand Annealing (SSA)

When DSB occurs between two direct repeats, the repair of the broken chromosome can result in the deletion of the region between the two direct repeats, leaving a single copy of the repeat. This repair event refers to the Single Strand Annealing (SSA) pathway that was discovered in mammals, and then largely studied in the yeast *S. cerevisiae* (Lin, Sperle et al. 1984; Ivanov and Haber 1995; Paques and Haber 1999).

The first step of SSA is an extensive resection of the 5'-3' DNA end until the homology between direct repeat is exposed as ssDNA (Figure n.4A). The long 3' end is bound by RMPs such as Rad52 and Rad59 that promote the annealing of complementary ssDNA strands (Smith and Rothstein 1995; Smith and Rothstein 1999; Sugawara, Ira et al. 2000) (Figure n.4B). SSA does not require a strand invasion step, therefore SSA is genetically independent of the recombinase Rad51, and RMPs that promote D-loop formation such as Rad54 and Rad55/Rad57 complex (McDonald and Rothstein 1994). After the annealing is completed, non-homologous 3' flap are cleaved by the Rad1/Rad10 nuclease complex (Figure n.4C) (Ivanov and Haber 1995). The final step of SSA is the filling of ss-gaps by DNA synthesis and ligation (Thacker, Chalk et al. 1992). As SSA results in deletion of genetic material, it is considered as a non-conservative mechanism (Sugawara, Ira et al. 2000).

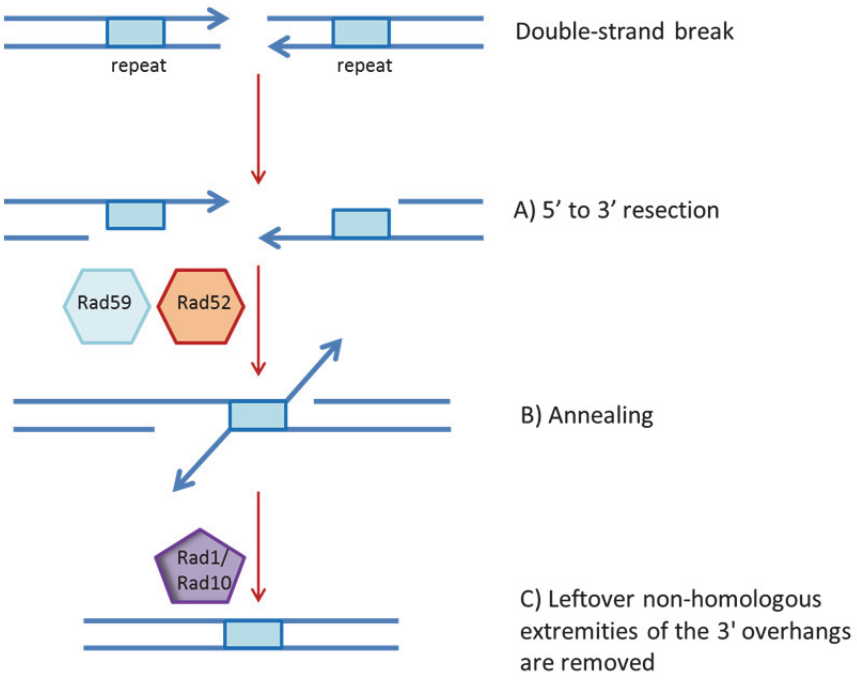


Figure n.4:
Model of Single Strand Annealing.
A) End-resection in the 5'-3' direction until the homology is revealed as ssDNA. **B)** The long 3' ssDNA tail is bound by the RMPs Rad52 and Rad59 that promote the annealing of complementary strands. **C)** Non-homologous 3'flap are cleaved by the Rad1/Rad10 nuclease complex.

2.3. Positive and negative regulators of homologous recombination

The different steps of HR (pre-synaptic, synaptic and post-synaptic) are tightly regulated by positive and negative regulators. Positive regulators are mainly Recombination Mediators Proteins (RMPs) that facilitate Rad51 nucleation onto ssDNA, stabilize the Rad51-filament or favor the strand invasion step. In contrast, negative regulators destabilize the nucleofilament and favor D-loop dissociation. Both negative and positive regulators of HR influence the mode of DSB repair and thus recombination outcome (CO versus non CO products).

2.3.1. Recombination Mediators Proteins (RMPs)

Rad51 nucleates onto a RPA-coated ssDNA. RPA plays a dual role in the formation of Rad51 filament. In one hand, RPA has a positive action by preventing the formation of secondary structures in ssDNA (Kowalczykowski and Krupp 1987). But, on the other hand, Rad51 nucleation is prevented when ssDNA is covered by RPA, as RPA as a greater affinity for ssDNA than Rad51. Therefore, proteins that can either overwhelm the inhibitory effect of RPA on Rad51-binding to ssDNA or promote Rad51 nucleofilament formation are defined as Recombination Mediators Proteins (RMPs) (Table n.1). RMPs can facilitate Rad51 loading onto resected ssDNA, increasing the stability of the nucleofilament. One way to stabilize the nucleofilament is to regulate the ATP-hydrolysis activity (ATPase) of Rad51. The recombinase Rad51 binds ssDNA in an ATP-dependent manner. ATP-binding by Rad51 promotes a structural alteration of the protein that is necessary for efficient and cooperative nucleation of Rad51 onto ssDNA (Namsaraev and Berg 1998). The ssDNA within the filament is then stretched (Ogawa, Shinohara et al. 1993) and this stretching is essential for fast and efficient homology search (Klapstein, Chou et al. 2004; Chen and Ferec 2008). ATP hydrolysis leads to a loss of Rad51 affinity for ssDNA and a subsequent dissociation of the nucleofilament. Therefore, RMPs regulate the stability of the filament by regulating the ATPase activity of Rad51. Alternatively, RMPs can protect the Rad51 filament from dissociation by negative regulators.

Table n.1: Recombination mediator proteins

	Human	*S. cerevisiae*	*S. pombe*
Recombinase	RAD51	Rad51	Rhp51
Recombination mediators	RAD52 BRCA2 RAD51B-RAD51C RAD51D-XRCC2 RAD51C-XRCC3	Rad52 - Rad55-Rad57	Rad22 - Rhp55-Rhp57
	RAD51D SWS1 XRCC2 -	Psy3 Shu2 Shu1 Csm2	Rlp1 Rdlp1 Sws1 -
		Rad59	
	RAD54 RAD54B - RAD51AP1	Rad54 Rdh54/Tid1 Tid4/Uls1 -	Rhp54 -

As mentioned above, the main positive regulator of HR is the **Rad52** protein that interacts with RPA and ssDNA, to form a ring-like structure. Rad52 facilitates the pre-synaptic step: it binds to ssDNA, displaces RPA from ssDNA, and thus facilitates the binding of Rad51 onto ssDNA (Resnick 1975; Ogawa, Shinohara et al. 1995; Sung 1997; Benson, Baumann et al. 1998; New, Sugiyama et al. 1998; Shinohara and Ogawa 1998) (Figure n.5). Genetic and physical interactions have been reported between the N-terminus of Rad52 and RPA (Hays, Firmenich et al. 1995; Hays, Firmenich et al. 1998) while the C-terminus of Rad52 interacts with Rad51 protein (Shinohara and Ogawa 1998; Song and Sung 2000; Sugawara, Wang et al. 2003). Rad52 displays also ssDNA annealing activity which functions independently of Rad51, for example to capture the second DNA end during the repair of DSB or to promote SSA (Mortensen, Bendixen et al. 1996; Van Dyck, Stasiak et al. 1999; McIlwraith and West 2008; Nimonkar, Sica et al. 2009).

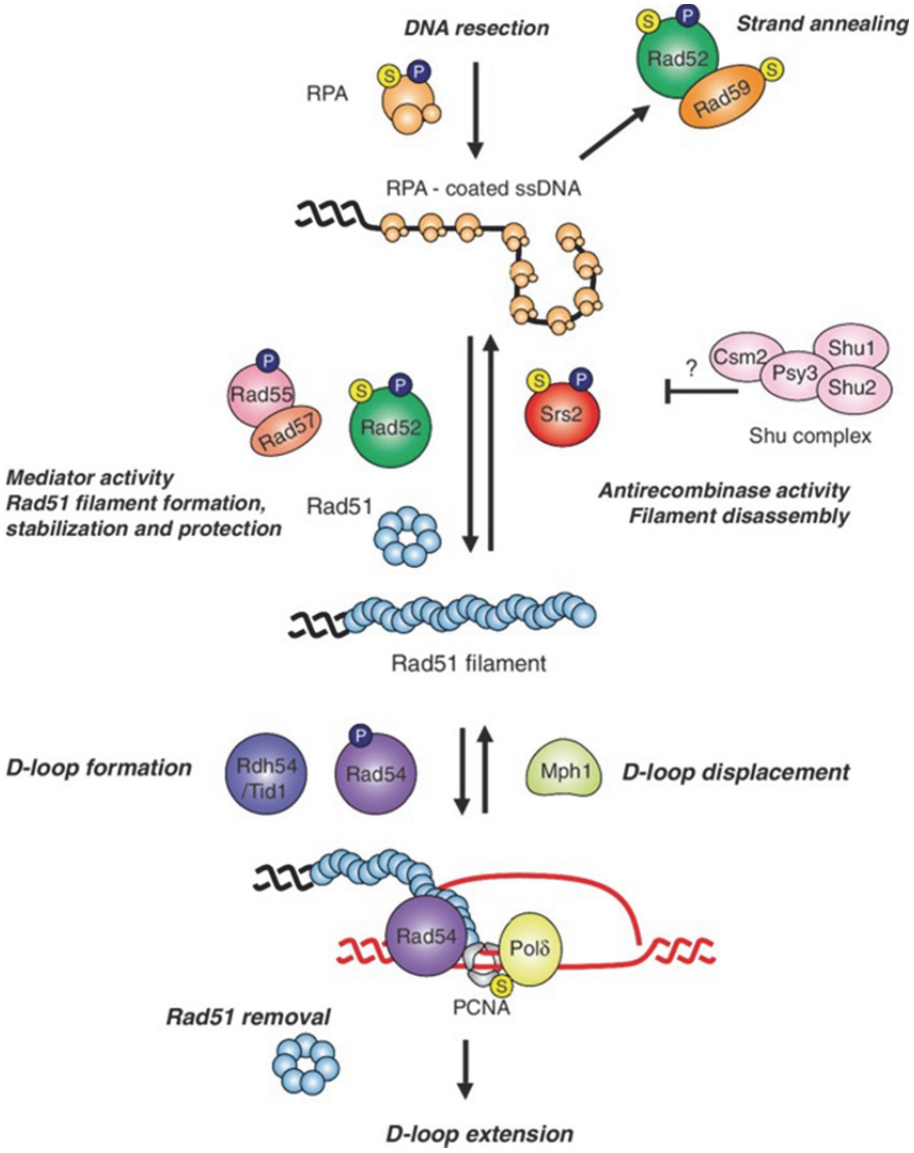


Figure n.5:
Positive and negative regulators during early steps of HR. Rad52 displaces RPA from ssDNA and helps Rad51 nucleation onto ssDNA, with the assistance of additional RMPs as Rad55/Rad57 that also protects Rad51 filament from dissociation by the anti-recombinase Srs2. After strand invasion and D-loop formation, stimulated by Rad54, the D-loop can be dissociated by helicases such as Mph1 or Sgs1 (Krejci, Altmannova et al. 2012)

Rad59 is considered as a Rad52 paralog, sharing significant sequence homology with the N-terminal domain of *S. cerevisiae* Rad52 but lacking the C-terminal domain involved in Rad51 interaction (Davis and Symington 2001; Wu, Sugiyama et al. 2006). Rad52 via its N-terminal region interacts with Rad59 *in vivo* and *in vitro,* thus forming heteromeric rings (Davis and Symington 2003). Rad59 is involved in Rad51-independent recombination, suggesting that Rad59 could stimulate Rad52 single strand annealing activities to promote SSA and/or the second end capture during DSBR. The cooperation between Rad59 and Rad52 is particularly important when the length of direct repeats is reduced (Bai and Symington 1996; Sugawara, Ira et al. 2000; Davis and Symington 2001; Wu, Sugiyama et al. 2006).

Rad55 and Rad57 proteins are Rad51 paralogs, forming a heterodimer to promote the formation and stabilization of Rad51 nucleoprotein filament. Firstly, Rad55-Rad57 heterodimer mediates Rad51 binding to the ssDNA (Heyer 1994; Sung 1997; Sugawara, Wang et al. 2003). Secondly, the Rad55-Rad57 heterodimer forms a co-filament with Rad51 which is more resistant to dissociation by the DNA helicase Srs2, than the Rad51-filament alone. Moreover, the heterodimer stabilizes the Rad51-nucleofilament, increasing the possibility of pairing of Rad51-nucleoprotein filament with a homologue sequence (Sung 1997; Fortin and Symington 2002; Liu, Renault et al. 2011).

The **Shu complex** (Shu1-Shu2-Psy3-Csm2) has been shown genetically to promote DNA repair through HR (Huang, Rio et al. 2003; Shor, Weinstein et al. 2005; Mankouri, Ngo et al. 2007). The Shu complex contains Rad51 paralogs (Shu1 and Psy3) that stabilize the Rad51 nucleofilament by inhibiting its disassembly by the DNA helicase Srs2, notably through Shu1 that modulates Srs2 recruitment, (Shor, Weinstein et al. 2005; Mankouri, Ngo et al. 2007; Ball, Zhang et al. 2009; Choi, Szakal et al. 2010; Bernstein, Reid et al. 2011).
Both RMPs Rad55/Rad57 and the Shu complex illustrate how the assembly and disassembly of Rad51 presynaptic filament is governed by the balance between positive and negative regulators of HR (Liu, Renault et al. 2011).

Rad54 is another positive regulator of recombination acting at the presynaptic, synaptic and post-synaptic steps of HR. Rad54 is a member of the Snf2-family of SF2 helicases, whose components are involved in chromatin remodeling during transcription and DNA repair (Flaus, Martin et al. 2006; Heyer, Li et al. 2006). Rather than operating as DNA helicases, the Snf2-related proteins are viewed as motor proteins using the energy of ATP hydrolysis to translocate on dsDNA, and thus inducing topological changes (Pazin and Kadonaga 1997; Tan, Essers et al. 1999; Mazin, Bornarth et al. 2000; Van Komen, Petukhova et al. 2000). Moving along the DNA filament, Rad54 creates positive and negative supercoils. This negative supercoil could favor the unwinding of the donor duplex and thus facilitates the strand invasion step (Van Komen, Petukhova et al. 2000).
Rad54 stabilizes Rad51 nucleoprotein filaments *in vitro* (Mazin, Alexeev et al. 2003) but the main function of Rad54 *in vivo* is post-synaptic to promote the extension of the invading 3'end by DNA synthesis, and thus stabilizing the D-loop structure (Sugawara, Wang et al. 2003; Li, Zhang et al. 2007). First, Rad54 increases the rate of branch migration in an ATP-dependent manner (Solinger and Heyer 2001). Second, Rad54 promotes Rad51 dissociation from the DNA duplex in the D-loop structure (Figure n.5). Indeed, even upon ATP hydrolysis

Rad51 has a slow turnover and releases slowly from the DNA duplex. Rad54 facilitates Rad51 dissociation from the DNA duplex, in an ATP-dependent manner, thus allowing the extension of the 3'invading end (Sung 1994; Zaitseva, Zaitsev et al. 1999; Kiianitsa, Solinger et al. 2002; Solinger, Kiianitsa et al. 2002).

2.3.2. Regulation of early steps of homologous recombination

Investigations of the dynamics of the early steps of HR during the repair of a site-specific DSB have provided further knowledge on the regulation of HR (Sugawara, Wang et al. 2003; Wolner, van Komen et al. 2003; Wang, Ira et al. 2004; Hicks, Yamaguchi et al. 2011). The 5'-3' end-resection appears to be a fast process as the recruitment of RPA at the site of DSB coincides with DSB induction. In contrast, Rad51 nucleation onto ssDNA occurs 30 minutes after DSB induction, suggesting that the formation of Rad51 nucleoprotein filament is a slow process. The search of homology and the strand invasion step are rather fast processes as Rad51 associates with the donor template only 10 minutes after Rad51 filament formation. Finally, the initiation of DNA synthesis to elongate the 3' invading end occurs 30-40 minutes after the initial association of Rad51 with the donor template. This delay may reflect the reversible nature of the initial Rad51-filament in association with the donor DNA molecule (subsequent cycles of pairing of the ssDNA with the homologue sequence). In support of this, molecular studies have revealed that stable and complete strand exchange is a complex mechanism.

Fluorescence resonance energy transfer (FRET) analyses indicate a rapid annealing exchange of A:T base pairs between the invading nucleoprotein filament and the duplex donor. This A:T exchange is a key step to allow the search of homology (Gupta, Folta-Stogniew et al. 1999). The initial interactions between nucleoprotein filament and homologue sequence are promoted by the extended structure of ssDNA within the Rad51 filament (Klapstein, Chou et al. 2004) and stabilized by Rad51 itself, therefore implicating a protein-protein interaction (Bianco, Tracy et al. 1998; Sung, Krejci et al. 2003; Sinha and Peterson 2008). These early interactions lead to a metastable DNA joint (paranemic joints), in which the 3' end of the Rad51-filament is not engaged in extended base-pairing interactions. The paranemic joint is then converted in a plectonemic joint in which the ssDNA bases pairs with its complementary strand in the D-loop structure (Riddles and Lehman 1985). The ATPase activity of Rad54 plays a crucial role in the conversion of the paranemic joint into a protein-independent joint. Recent data demonstrated that within 10 seconds of Rad54 addition, 20% of the maximum

number of stable joints was formed, *in vitro*. In contrast, this value is reached in about 2 minutes in the absence of Rad54 (Sinha and Peterson 2008). Therefore Rad54 converts initial metastable products into a stable joint molecule termed the D-loop.

The recombinase Rad51 itself plays a critical role in the further stabilization of the nascent D-loop. Indeed, Rad51 not only promotes the formation of branched structure (D-loop) but also promotes branch migration in the 3'-5' direction relative to the invading single stranded DNA (Figure n.6) (Murayama, Kurokawa et al. 2008). I*n vitro,* Rad51-dependent branch migration occurs probably by stabilizing base pairing between heteroduplexes and allows a complete 4-strand exchange reaction (Hall and Kolodner 1994; Namsaraev and Berg 2000). Branch migration by Rad51 results in the migration of the first HJ behind the initial point of strand invasion and in the increase of the length of the heteroduplex, thus stabilizing the D-loop.

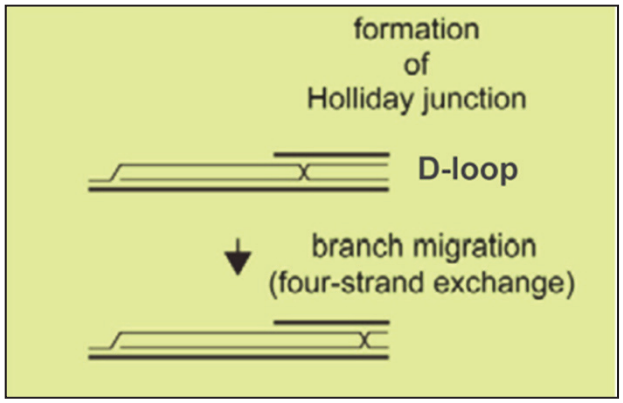


Figure n.6: Four strand exchange reaction by Rad51. After strand invasion, the ability of Rad51 to promote branch migration of the HJ results in the extension of the heteroduplex and complete exchange between duplex-duplex DNA (Murayama, Kurokawa et al. 2008).

In the context of allelic recombination (between homologous chromosomes and sister chromatids), branch migration by Rad51 can occur as the donor and the acceptor DNA molecules share extensive sequence homology. In contrast, during ectopic recombination (between dispersed repeated sequences), branch migration by Rad51 might be restrained due to the limited length of homology between the donor and the acceptor. Therefore, alternative mechanisms of stabilization of the D-loop might be crucial for the success of the genetic exchange.

As mentioned above, a delay of 40 minutes has been detected between initial recognition of Rad51-mediated strand exchange and observable primer extension initiation in budding yeast (Hicks, Yamaguchi et al. 2011). A similar delay of 30 minutes to initiate 3' end polymerization has been observed in mammalian cells (Si, Mundia et al. 2010). This might also suggest that the assembly of a DNA synthesis machinery takes time. Extension of the invading 3'end has been proposed to be mediated by the DNA polymerase eta (pol η). Indeed,

in vitro studies have shown that only pol η is able to extend 3'end within a D-loop structure, but no other polymerases such as pol delta (pol δ), pol iota (pol ι), polymerases T4 and T7 (McIlwraith, Vaisman et al. 2005). In support of this, genetic data in DT40 cells showed that defect in pol η results in impaired DSB repair by HR (Kawamoto, Araki et al. 2005). Moreover, pol η was found to be recruited during the repair of broken replication forks in *xenopus* (Hashimoto, Puddu et al. 2012). Nonetheless, further biochemical studies have established conditions in which pol δ is able to prime DNA synthesis on a D-loop structure (Sebesta, Burkovics et al. 2011). The interaction between pol δ and the replication factor PCNA is crucial for D-loop extension by pol δ. Several genetic evidences support a role for pol δ in mitotic gene conversion and extension of DNA heteroduplex. Indeed, DNA synthesis during gene conversion is mutagenic and pol δ contributes to it; pol δ and pol ε are also required to support extensive DNA synthesis during BIR (Lydeard, Jain et al. 2007; Maloisel, Fabre et al. 2008; Hicks, Chute et al. 2010). Therefore, the exact nature of the DNA synthesis machinery involved in the extension of the heteroduplex within the D-loop remains unclear (Lydeard, Lipkin-Moore et al. 2010; Hashimoto, Puddu et al. 2012).

In competition with the D-loop extension by DNA polymerases, phenomena of D-loop dissociation by several DNA helicases also influence the mode of DSB repair and recombination outcomes. These DNA helicases acting during the early steps of recombination are considered as negative regulators of HR (Table n.2 and Figure n.5).

The DNA helicase/translocase Srs2 is a 3'-5' DNA helicase that suppresses crossover during mitotic recombination by dissociating joint molecules (Fabre, Chan et al. 2002; Aylon, Liefshitz et al. 2003; Ira, Malkova et al. 2003; Robert, Dervins et al. 2006). Srs2 is defined as an anti-recombinase because the deletion of Srs2 leads to a hyper-recombination phenotype, especially an increase in CO-products, which is rescued by deleting Rad51 (Chanet, Heude et al. 1996; Gangloff, Soustelle et al. 2000). *In vitro* studies have established a role for Srs2 in the disassembly of Rad51 nucleoprotein filament (Krejci, Van Komen et al. 2003; Veaute, Jeusset et al. 2003). This activity requires the interaction between Srs2 and Rad51 with subsequent ATP hydrolysis to weaken the Rad51-DNA interaction (Antony, Tomko et al. 2009; Colavito, Macris-Kiss et al. 2009). Moreover, the helicase activity of Srs2 is stimulated by the presence of Rad51 filament, resulting in the ability of Srs2 to unwind D-loop structure (Dupaigne, Le Breton et al. 2008). Therefore, it is thought that Srs2 promotes SDSA pathway, thus preventing mitotic COs.

Table n.2: Negative regulators of homologous recombination

	Human	*S. cerevisiae*	*S. pombe*
Recombinase	RAD51	Rad51	Rhp51
Negative regulators	- FANCM BLM RTEL1 PARI	Srs2 Mph1 Sgs1 - -	Srs2 Fml1 Rqh1 - -

The DNA helicase Sgs1 belongs to the 3'-5' RecQ helicase family. Sgs1 acts at different steps during the repair of DSB by HR. During late steps, the complex Sgs1/Rmi1/Topoisomerase III promotes the dissolution of double HJs, thus preventing mitotic Cos (Ira, Malkova et al. 2003; Wu and Hickson 2003; Ahmad and Stewart 2005; Chang, Bellaoui et al. 2005; Cejka, Cannavo et al. 2010). Sgs1 also promotes end-resection to generate long 3'ssDNA end. In addition, Sgs1 regulates the Rad51-nucleoprotein filament by promoting the disassembly of Rad51 from ssDNA. It is proposed that acting at the post-synaptic step, Sgs1 prevents aberrant multiple strand invasion events (Oh, Lao et al. 2007; Oh, Lao et al. 2008). The helicase BLM, the human homologue of Sgs1, is able to disrupt the binding of Rad51 to ssDNA and thus to promote DSB repair by the SDSA pathway (Bugreev, Yu et al. 2007). Finally, experimental evidences in *Drosophila melanogaster* show that BLM can unwind ssDNA tail from D-loops thus providing a mechanism of channeling the D-loop towards the SDSA pathway (Adams, McVey et al. 2003; McVey, Larocque et al. 2004).
In budding yeast, cells depleted for Sgs1 and Srs2 are either unviable or present a very slow growth phenotype. These phenotypes are suppressed by inactivation of recombination genes involved in the presynaptic step, such as *RAD51*, *RAD55* and *RAD57* (Gangloff, Soustelle et al. 2000). These data suggest that accumulation of unresolved Joint-Molecules, or Rad51 bound to ssDNA are lethal recombination intermediates. Similar genetic interactions were uncovered in fission yeast (Doe and Whitby 2004).

The helicase Mph1 is an ATP-dependent helicase with 3'-5' polarity that works independently of Srs2 and Sgs1 (Prakash, Krejci et al. 2005). Deletion of Mph1 results in an increased rate of mitotic COs. Purified Mph1 binds and unwinds D-loops, by dissociating the invading DNA strand from the template donor. Thus, Mph1 might promote repair of DSB by

promoting the SDSA pathway (Entian, Schuster et al. 1999; Schurer, Rudolph et al. 2004; Gari, Decaillet et al. 2008; Sun, Nandi et al. 2008; Prakash, Satory et al. 2009; Zheng, Prakash et al. 2011). Similarly, Fml1, the *S. pombe* homologue of Mph1, also prevents mitotic COs by disrupting D-loop intermediates (Sun, Nandi et al. 2008).

2.4. Homologous Recombination: a support for DNA replication

HR plays an important role during DNA replication, notably by ensuring the repair or restart of halted replication forks. In eukaryotes, DNA replication is initiated at multiple replication origins that are activated according to a spatio-temporal program (Huberman and Riggs 1966; Wu and Gilbert 1996; Kelly and Brown 2000; Raghuraman, Winzeler et al. 2001; Mechali 2010). Once activated, a replication origin gives birth to two replication forks that progress bi-directionally until they merge with a converging fork. Once established, a single replication fork will replicate several tens of thousands of bases before meeting a converging fork. During DNA synthesis, fork progression can be impeded by many obstacles that refer to Replication Fork Barriers (RFBs), including DNA damages, structure-forming sequences (inverted repeats, G-quadruplexes, hairpins), collisions between advancing forks and transcription machineries, and non-histone proteins bound to DNA (Lambert and Carr 2005; Mirkin and Mirkin 2007) (Figure n.7). In many organisms, natural RFBs results in replication forks pausing during each S-phase at specific loci such as centromeres, telomeres, replication slow zones, tRNA, ribosomal DNA and repeated sequences. Moreover, impediments to fork progression can destabilize the replication machinery and RFBs are known hot spots of recombination, chromosome breakages and rearrangements in yeast models. Thus, replication fork progression requires a well-orchestrated machinery to ensure forks movement, stability, reactivation and fusion with a converging fork.

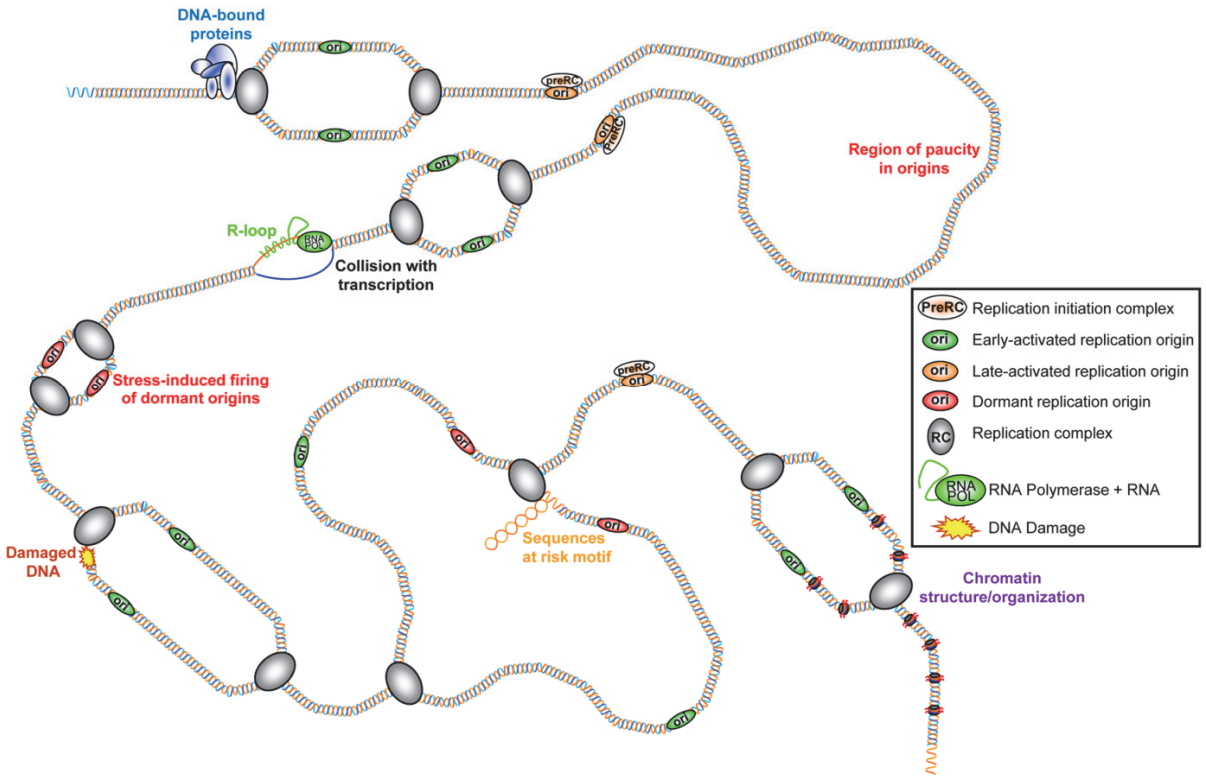


Figure n.7: Overview of obstacles causing RFB. Typical obstacles causing RFBs (DNA-bound proteins, chromatin organization, at risk sequences motif, DNA-damage) and interference between replication and transcription all have the potential to interfere with the progression of replication forks and its associated DNA synthesis. Global replication-stress and impediments to fork progression result in the activation of dormant origins that helps to complete DNA replication. In region of paucity in replication origins, completion of replication relies on long-traveling forks which are thus particularly sensitive to fork arrest and replication inhibition.

A single halted fork does not necessarily prevent the completion of DNA replication because the incoming of an opposite fork initiated from an adjacent origin will replicate up to the site of the arrested fork. Thus, in contrast to the bacterial chromosome replication, the rescue of impeded forks does not rely exclusively on fork-restart mechanisms, but also on activation of dormant origins (Figure n.7). Indeed, in response to replication stress, activation of dormant origins is recognized as an efficient mechanism ensuring complete chromosomal replication (Woodward, Gohler et al. 2006; Ge, Jackson et al. 2007; Petermann, Orta et al. 2010; Kawabata, Luebben et al. 2011). However, at unidirectional replication regions (as the ribosomal DNA locus), or when two converging forks are impeded (Figure n.7) or in regions of paucity in origins as human fragile sites, long travelling forks might have also more risk of accident and fork-restart mechanisms might then become essential to complete DNA replication (Le Tallec, Dutrillaux et al. 2011; Letessier, Millot et al. 2011).

Among fork-restart pathways, both the DNA replication checkpoint and HR are pivotal mechanisms in escorting the progression of replication forks. The integrity of replication forks is guaranteed by the DNA replication checkpoint that maintains the replisome in a replication-competent state to keep DNA polymerases associated with the site of nucleotide incorporation (Katou, Kanoh et al. 2003; Branzei and Foiani 2010; De Piccoli, Katou et al. 2012). How the DNA replication checkpoint modulates replisome activities to maintain it functional at the site of halted fork remains a current debate in the field. In addition, the DNA replication checkpoint also regulates nucleases activities (as Exo1 or Mus81) to preserve the integrity of forked structures (Cotta-Ramusino, Fachinetti et al. 2005; Kai, Boddy et al. 2005; Froget, Blaisonneau et al. 2008). In case of replisome malfunctioning (that refers to as collapsed forks in the literature) or loss of components of the replisome at broken forks, the resumption of DNA synthesis needs the replisome to be rebuilt. In eukaryotes, several evidences indicate that HR is able to promote resumption of DNA synthesis and to rebuild replication machinery at halted replication forks. In mammals, defects in HR result in a decreased fork velocity and, in budding yeast, in the accumulation of unreplicated gaps in response to replication-blocking agents (Daboussi, Courbet et al. 2008; Alabert, Bianco et al. 2009). These data further support the view that HR acts as fork-escort mechanism to support robust DNA replication, thanks to the ability of HR to repair ssDNA gaps left in the rear of moving forks, the ability of HR to restart or repair halted fork, and the ability of HR to protect stalled forks.

2.4.1. Post-replication repair by homologous recombination

Homologous recombination is an efficient mechanism to seal ssDNA gaps in the rear of the moving fork that has encountered a DNA lesion (Figure n.8, middle panel). Several evidences show that HR is required to complete DNA replication when the parental DNA is damaged. For example, analysis of *in vivo* purified damaged replication forks by electronic microscopy have shown that ssDNA gaps accumulate both just at, and behind, the moving fork on both sister-chromatids, especially in the absence of HR (Lopes, Foiani et al. 2006; Hashimoto, Ray Chaudhuri et al. 2010). The formation of ssDNA gaps in the rear of the progressing fork implies that a re-priming event occurs when the synthesis of the leading strand is blocked. While *in vitro* studies pointed out that the bacterial replisome is inherently tolerant to DNA damage, the ability of eukaryotic replisome to re-prime DNA synthesis on the leading strand remains to establish (Yeeles and Marians 2011).

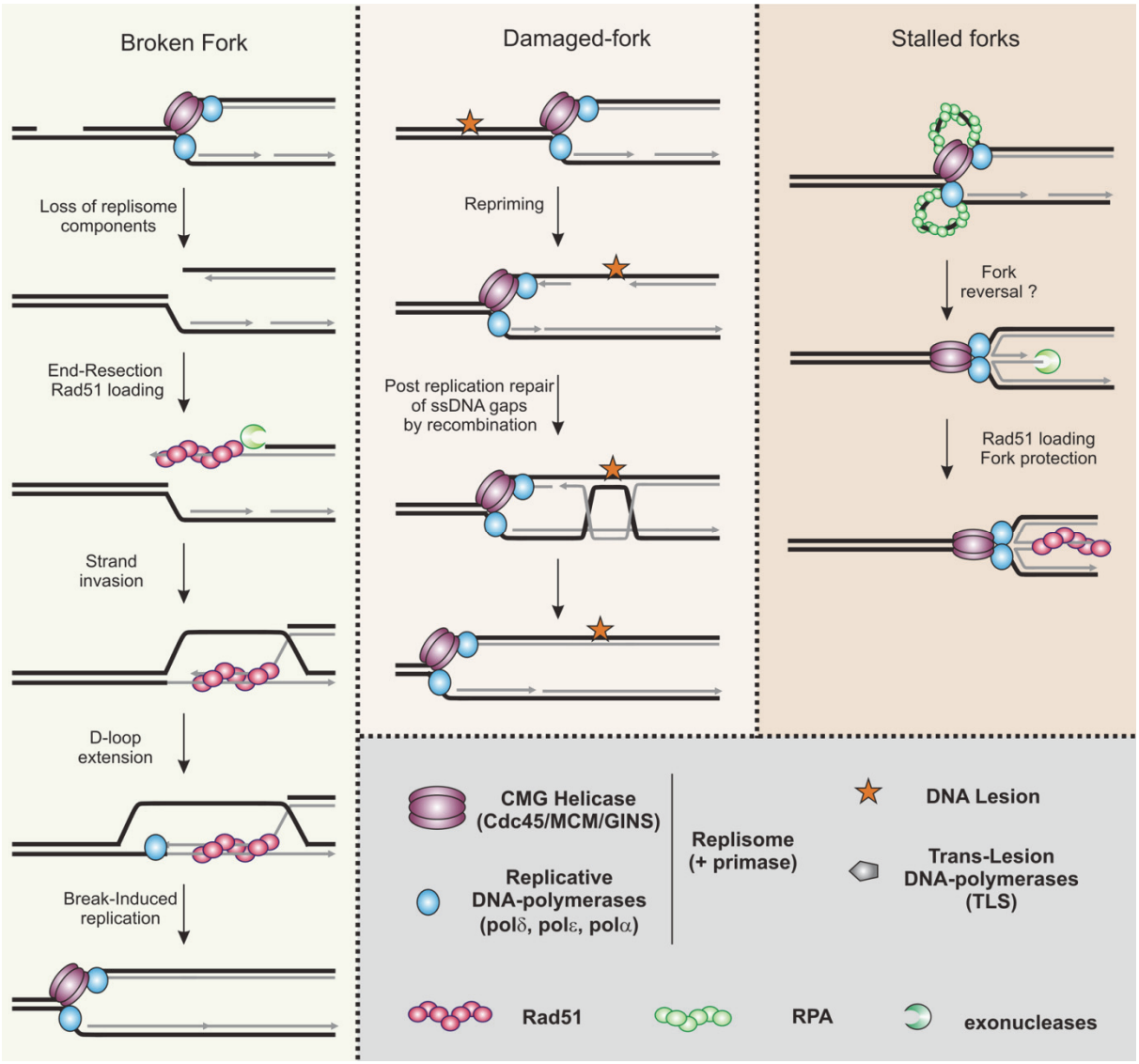


Figure n.8: Three main mechanisms by which HR acts as a fork-escort pathway to ensure faithful genome duplication: repair of broken forks (left panel), repair of ssDNA gaps at damaged-forks (middle panel) and fork protection when fork progression is slowing down (right panel).

To seal ssDNA gaps, homologous recombination copy the information from the undamaged sister-chromatids. This mechanism, which refers as gap filling or template switch in the literature, is expected to result in recombination intermediates between sister-chromatids. By analyzing replication intermediates, joints-molecules formed between sister-chromatids were identified in S-phase cells replicating parental DNA damaged by MMS-treatment (a base-alkylating agent) (Liberi, Maffioletti et al. 2005; Branzei and Foiani 2007). Although the exact nature of these joints-molecules are not clearly established, their genetic dependencies support the view that they correspond to recombination intermediates resulting from a strand exchange reaction by Rad51 between an intact and a gapped sister-chromatid in the rear of replication forks. Sister-chromatid joint-molecules would then be resolved by the helicase

Sgs1 (Branzei, Sollier et al. 2006; Mankouri, Ashton et al. 2011). In budding yeast, RPA, the RMPs Rad52 and Rad55/Rad57, the 5'-3' nuclease Exo1 and the replicative DNA polymerase delta, but not epsilon, are all required to promote sister-chromatid junctions during replication of damaged template (Vanoli, Fumasoni et al. 2010). Thus, it is possible that ssDNA gaps need to be first resected to recruit RMPs and for Rad51 nucleation on ssDNA gaps. Following strand invasion (either by the Rad51-coated ssDNA gap itself or by the 3' end of the newly synthetized strand of the gapped sister-chromatid), the DNA polymerase delta and its accessory subunit Pol32 would then seal the gap by extending the 3'end.

2.4.2. Replication fork restart and repair by homologous recombination

Both in yeast and mammalian cells, HR is able to restart halted replication forks. Fork passage through ssDNA nick or gap in the parental DNA results in a broken fork with a sister-chromatid being physically detached from the fork (Figure n.8 left panel). Such broken forks are efficiently repaired by homologous recombination through BIR mechanism (Roseaulin, Yamada et al. 2008; Clemente-Ruiz and Prado 2009; Moriel-Carretero and Aguilera 2010; Hashimoto, Puddu et al. 2012). In this case, BIR allows the rebuilding of a replisome able to synthetize hundreds of kilobases. All components of DNA replication are required for BIR, except those involved in the assembly of pre-replication complexes (Lydeard, Lipkin-Moore et al. 2010). The DNA polymerase α and δ are necessary for the initial extension step while the DNA pol ε has a later requirement (only after 30 Kb of DNA synthesis). In addition, Pol32, the accessory pol δ subunit, is required for BIR but dispensable for conventional DNA replication (Lydeard, Jain et al. 2007; Moriel-Carretero and Aguilera 2010). Thus, the establishment of a fully functional replication apparatus by HR requires time and maturation of the initial steps. Nonetheless, DNA synthesis associated with BIR is highly inaccurate (Deem, Keszthelyi et al. 2011).

Importantly, DSBs at replication forks are not a pre-requirement for recruitment of recombination factors. Indeed, efficient recruitment of recombination proteins was observed at replication forks impeded by DNA-bound proteins. In this case, fork recovery by HR was proposed to be DSB-independent (Lambert, Watson et al. 2005; Mizuno, Lambert et al. 2009; Hashimoto, Ray Chaudhuri et al. 2010; Lambert, Mizuno et al. 2010). Moreover, similarly to BIR, halted forks recovered by HR are prone to replication slippage (Iraqui, Chekkal et al. 2012 in press). Thus, it appears that recombination-dependent replisomes are error-prone in contrast to origin-dependent replisomes.

2.4.3. A function of "fork-protection" for homologous recombination

Recent investigations have enlightened a novel function for HR as "fork-stabilizer" and how this function serves the robustness of replication forks progression. The first report on the role of BRCA2 (the main RMP in mammals) in stabilizing halted replication forks came from the lab of Venkitaraman (Lomonosov, Anand et al. 2003). In this study, the stability of unidirectional replication forks in the region of the ribosomal DNA (rDNA) was investigated by analysing replication intermediates in murine embryonic fibroblasts. Upon inhibition of DNA replication by HU treatment (an inhibitor of the ribonucleotide reductase leading to depletion of dNTP pool during S-phase), forks were unstable and were liable to breakages in the absence of BRCA2. Recent studies have provided molecular insights in the understanding of the "fork-stabilizer" function of HR. Schlacher and co-authors used DNA fibres techniques to analyse the dynamic of DNA synthesis upon inhibition and resumption of the elongation step. Newly replicated DNA at the fork, synthesized before inhibition of DNA synthesis, was found to be extensively resected in the absence of BRCA2 or Rad51 (Schlacher, Christ et al. 2011). Resection of nascent strands is dependent on Mre11, and it was proposed that BRCA2 ensures fork protection by stabilizing the Rad51 filament at stalled fork, notably by regulating its ATPase activity (Schlacher, Wu et al. 2012). These data establish that HR, in addition to repairing replicative DNA lesions, has a specific function during DNA replication by protecting the integrity of elongated strands at the replication fork.

Interestingly, Rad51 was previously proposed to protect nascent strands in *xenopus* and budding yeast (Hashimoto, Ray Chaudhuri et al. 2010). Direct visualisation of *in vivo* purified forked structures by electronic microscopy has shown that ssDNA gaps accumulate at replication forks upon Rad51 depletion: gaps located behind the moving fork and gaps located at the three-way branched structure of the fork. ssDNA gaps in the rear of the fork result from the activity of the MRN complex, and are likely to reflect defects in post-replication repair by HR. More surprising are ssDNA gaps at the fork that are not dependent on MRN complex, and might reflect a role of HR in preventing uncoupling between leading and lagging strand synthesis. Even in the absence of exogenous DNA damages, 50% of replication forks contain ssDNA of at least 200 nucleotides at the forked structures when Rad51 is depleted. How homologous recombination maintains the coupling between leading and lagging strand synthesis is unclear, but these data support the view that HR escorts the robustness of DNA replication, and provide also molecular basis for a decreased fork speed in the absence of HR (Daboussi, Courbet et al. 2008).

How important is the "fork-stabilizer" function of HR? Replication-associated DSBs have been often observed in cells defective for HR. The initial view was that HR is required to repair spontaneous DSB associated with the process of DNA replication (Sonoda, Sasaki et al. 1998). However, replicative DSBs might also be the consequence of defect in stabilizing halted forks that would then create secondary DNA damages such as breaks or incomplete replication (Venkitaraman 2001). Thus, HR might have a role in preventing breaks by stabilizing replication forks, rather than exclusively by repairing breaks themselves.

2.5. Homologous recombination mediates chromosomal rearrangements

Due to its ability to promote genetic exchange between repeated sequences dispersed throughout the genome, HR can be also a source of chromosomal rearrangements. Eukaryotic genomes contain around 10% of repeated sequences, including Alu sequences, a variety of transposons, pseudo-genes and multigenic families sharing sequence homology. Thus, HR is also viewed as a "double-edged sword" for genome stability maintenance. Genome instability is a hallmark of cancers cells and inherited genome modifications are responsible for human genomic disorders (human diseases that result from genomic rearrangements) (Lupski 1998; Aguilera and Gomez-Gonzalez 2008; Gu, Zhang et al. 2008; Lupski 2009). Nonetheless, genetic variation is also the driving force of evolution and genomes must therefore display adequate plasticity to evolve while remaining sufficiently stable to prevent accumulation of mutations and chromosome rearrangements causing fitness disadvantage.

2.5.1. Non Allelic Homologous Recombination (NAHR)

Homologous recombination between dispersed repeated sequences is defined as Non-Allelic Homologous Recombination (NAHR) or ectopic recombination. NAHR outcomes consist of deletions, duplication, inversions, translocations, acentric and dicentric chromosomes (Figure n.9). NAHR accounts for most of the recurrent rearrangements observed in genomic disorders and some of chromosomal rearrangements occurring in cancer cells.

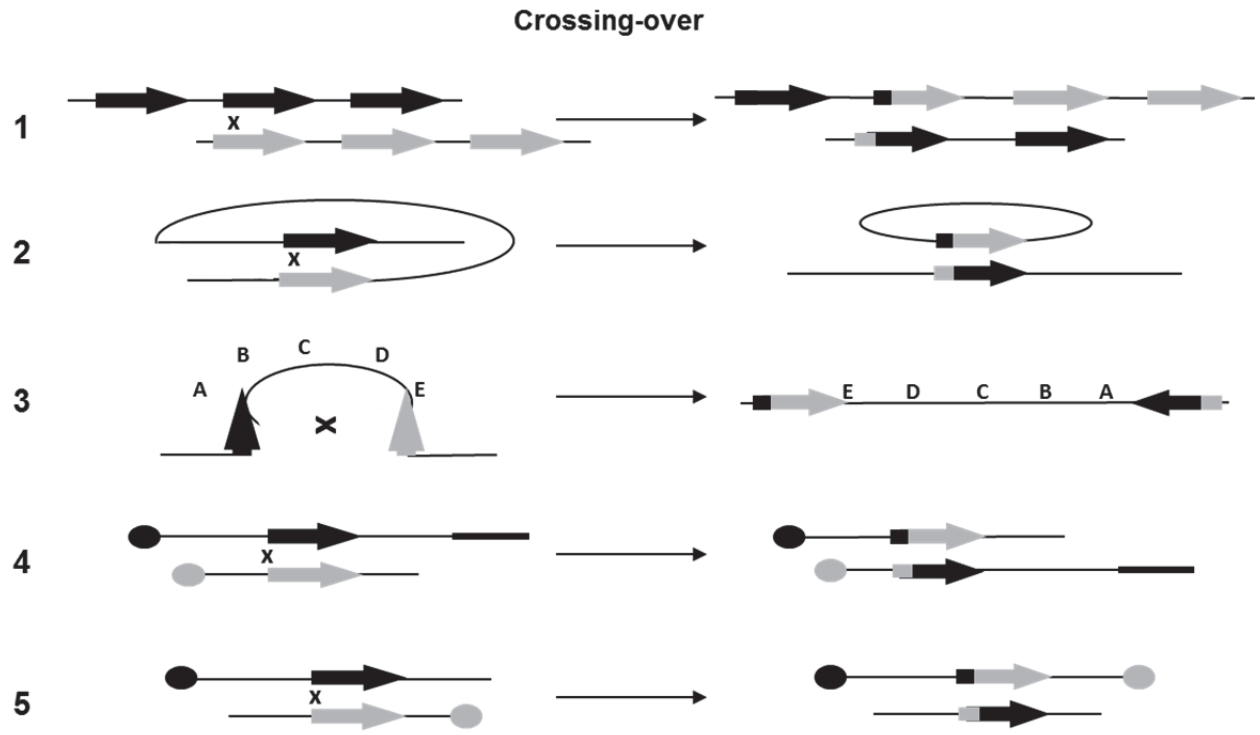


Figure n.9: Recombination outcomes associated to crossing-over. Arrows indicate repeated sequences and their orientations. 1) Copy number variation after inequal CO between sister chromatids (allelic recombination). 2) Segmental deletion after CO between flanking repeated sequences (NAHR). 3) Sequence inversion after CO between inversed repeated sequences (NAHR). 4) Interchromosomal CO between repeated sequences leads to reciprocal translocation (NHAR). 5) Acentric and dicentric chromosomes formation after CO between interchromosomal inverted repeats (Grabarz, Barascu et al. 2012).

NAHR is thought to be induced by DSB, resulting in genetic exchange between repeated sequences, associated or not with CO (Lupski 1998). While COs are responsible for major chromosomal rearrangements, gene conversion mediated by NAHR also results in genome modifications (Grabarz, Barascu et al. 2012). NAHR is also responsible for rearrangements leading to copy number variation of repeated sequences (Figure n. 9), during mitosis and meiosis (Shaw and Lupski 2005; Liu, Carvalho et al. 2012).

Crossing-over between repeated Alu sequences can lead to duplications/deletions, such as in Duchenne or Becker muscular dystrophy and in familiar hypercholesterolemia (Lehrman, Goldstein et al. 1987; Goldmann, Tichy et al. 2010; Takeshima, Yagi et al. 2010; Wang, Yang et al. 2011; Jelassi, Slimani et al. 2012). NAHR between repeated sequences on different chromosomes results in translocations and acentric/dicentric chromosome formation, with subsequent activation of oncogenes or inactivation of oncosuppressors as in the Philadelphia translocation (also known as Philidelphia chromosome) (Onno, Nakamura et al. 1992; Eckardt, Chang et al. 2011; Gahrton 2012). Moreover, crossover events can also lead to

sequence inversions, responsible for mucopolysaccharidose II (Bondeson, Dahl et al. 1995; Rathmann, Bunge et al. 1996; Bunge, Rathmann et al. 1998).

NAHR can also result in Loss of Heterozygosity (LOH) when recombination occurs between a gene and a pseudogene (Figure n.10). The sequence of the functional gene is replaced by a homologous pseudogene, leading to a loss of function. For example, LOH is responsible for tumorigenesis like for restinoblastoma and squamous cell carcinoma (Cavenee, Dryja et al. 1983; de Andrade, da Hora Barbosa et al. 2006; Sang-Hyuk Lee, Lee et al. 2011).

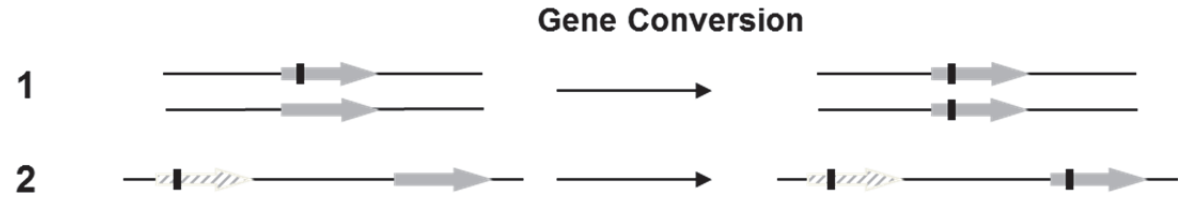


Figure n.10: Loss of Heterozygosity (LOH) by gene conversion. 1) Gene conversion between two alleles can lead to LOH (allelic recombination). 2) A gene conversion event between a pseudo-gene and a functional gene can result in LOH and the subsequent inactivation of the functional gene (Grabarz, Barascu et al. 2012).

NHAR requires a minimal length of homology between repeated sequences, as well as a relatively low level of heterology. The minimal length of homology is defined as the minimal efficient processing segment (MEPS) (Rubnitz and Subramani 1984; Waldman and Liskay 1988). Above a certain number of base pair homology corresponding to the MEPS, a direct correlation between the length of the sequence homology and HR efficiency has been established. When recombinant sequences are under the MEPS, HR efficiency drops dramatically. In mammalian cells, MEPS is found to be about 200-300 bp (Ayares, Chekuri et al. 1986; Liskay, Letsou et al. 1987; Lopez, Bertrand-Mercat et al. 1992) while in S. *cerevisiae* the minimal size is between 26 and 250 bp (Ahn, Dornfeld et al. 1988; Jinks-Robertson, Michelitch et al. 1993; Sugawara, Ira et al. 2000).

2.5.2. Chromosomal rearrangements by Template Exchange/Switch

Alternatively to canonical NAHR, other models responsible for chromosomal rearrangements mediated by micro-homology have been suggested. In these models, the initial event triggering rearrangement is thought to be linked to halted replication forks, and refers to Fork Stalling and Template Switching (FoSTeS). FoSTeS was initially proposed to describe non-recurrent rearrangements observed in human genomic disorders: the replication forks stall,

nascent strands switch template on the basis of micro-homology, initiate DNA synthesis on a non-contiguous template, and then anneal back to the initial template (Figure n.11) (Slack, Thornton et al. 2006; Lee, Carvalho et al. 2007). Because similar template switches of nascent strands were reported during BIR, it was then proposed that FoSTeS results from a BIR-related mechanism mediated by micro-homology and in which HR would not be involved (MMBIR; for Micro-homology Mediated BIR, (Lee, Carvalho et al. 2007; Zhang, Khajavi et al. 2009). Genetic and molecular studies have reported the existence of a similar mechanism in budding yeast: rearrangements resulting in segmental duplication are either mediated by homology and are then dependent on HR, or mediated by micro-homology and are independent of all known DNA repair pathways (Payen, Koszul et al. 2008). Similarly, the group of Ted Weinert reported chromosomal rearrangements induced by replication defects and independent of HR and canonical DSB repair pathways (Paek, Kaochar et al. 2009). Thus, the FoSTeS or MMBIR model, initially proposed in human cells, was further supported by genetic data in yeast models.

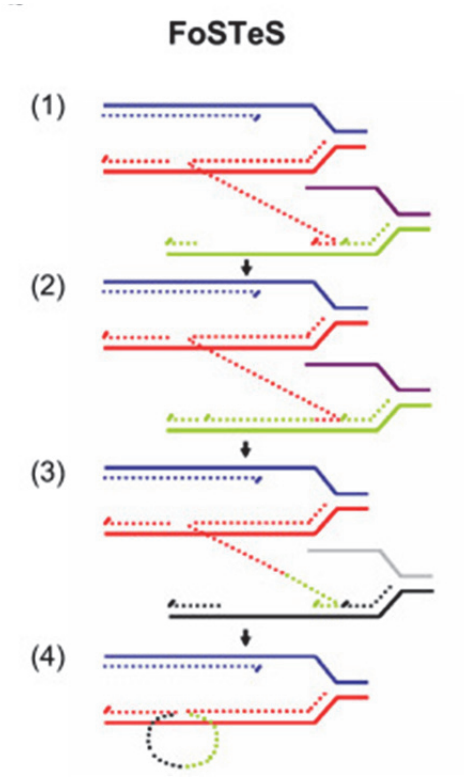


Figure n.11: Model of FoSTes (Fork Stall and Template Switch. 1) after replication fork stalling, the lagging strand (red lines) disengages from the replicon and anneals to a second fork (green lines) on the basis of micro-homology. 2) DNA synthesis occurs on a non-contiguous template. 3) Several template exchange reactions can occur between multiple stalled forks. 4) then the resumption of replication occurs on the initial template (Gu, Zhang et al. 2008)

The use of programmed Replication Fork Barrier (RFB) in fission yeast has been informative regarding the molecular mechanisms by which HR induces genome rearrangements during DNA replication. Ectopically placed RFBs behave as hot spot of recombination and genome rearrangements (Ahn, Osman et al. 2005; Lambert, Watson et al. 2005). Replication forks

halted by the RFB are restarted by HR via a DSB-independent mechanism. During the restart event, nascent strands are proposed to be unwound and can exchange template with a nearby homologous sequence. This homology-driven template exchange of stalled nascent strands occurs as the fork restart and drives NAHR-type. Moreover, our lab recently established that inactivated forks rescued by HR are error-prone and liable to generate small deletions or duplications with micro-homology at the junctions (Iraqui, Chekkal et al. 2012 in press). Altogether, these data establish that inactivated forks can result in both homology and micro-homology-driven rearrangements in a recombination-dependent manner, pointing out a potential role for HR in FoSTeS in human cells (Figure n.12).

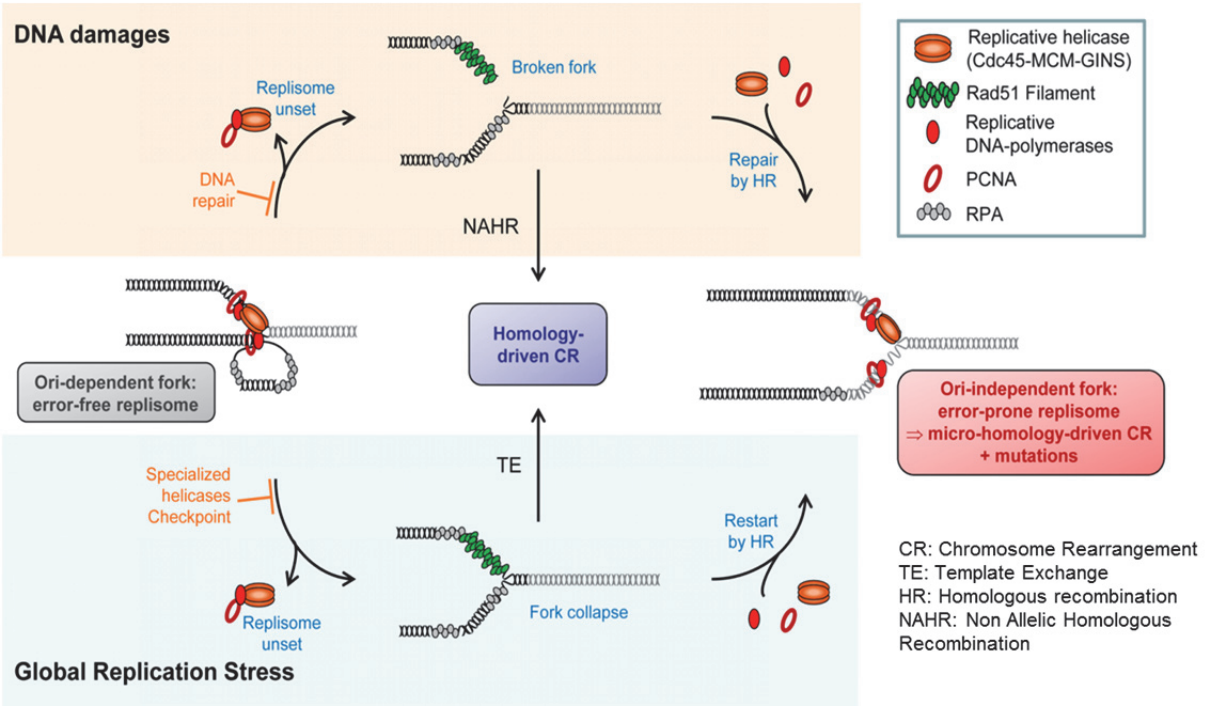


Figure n.12: Model of replication-induced genome instability by homologous recombination, either homology or micro-homology-mediated.

3. CHROMATIN ASSEMBLY DURING DNA REPLICATION

The structural organization of DNA into chromatin is of key importance to regulate genome expression, organization and stability. The basic unit of the chromatin is conserved in all eukaryotes and it is termed the "nucleosome" (Kornberg 1974; Bradbury, Moss et al. 1978). The first level of chromatin organization is the wrapping of the DNA around the nucleosomes, these latter being successively enveloped in a 30 nm chromatin fiber (Kornberg and Lorch 1999; Hayes and Hansen 2001). During meiosis and mitosis, the 30 nm nucleosome array is further packed in metaphase chromosomes (Figure n.13). The chromatin fiber is organized in either euchromatic or heterochromatic domains. Heterochromatin is defined as regions of chromatin that remain cytologically condensed and densely stained throughout the cell cycle, whereas euchromatin is de-condensed during interphase (Heitz, 1928).

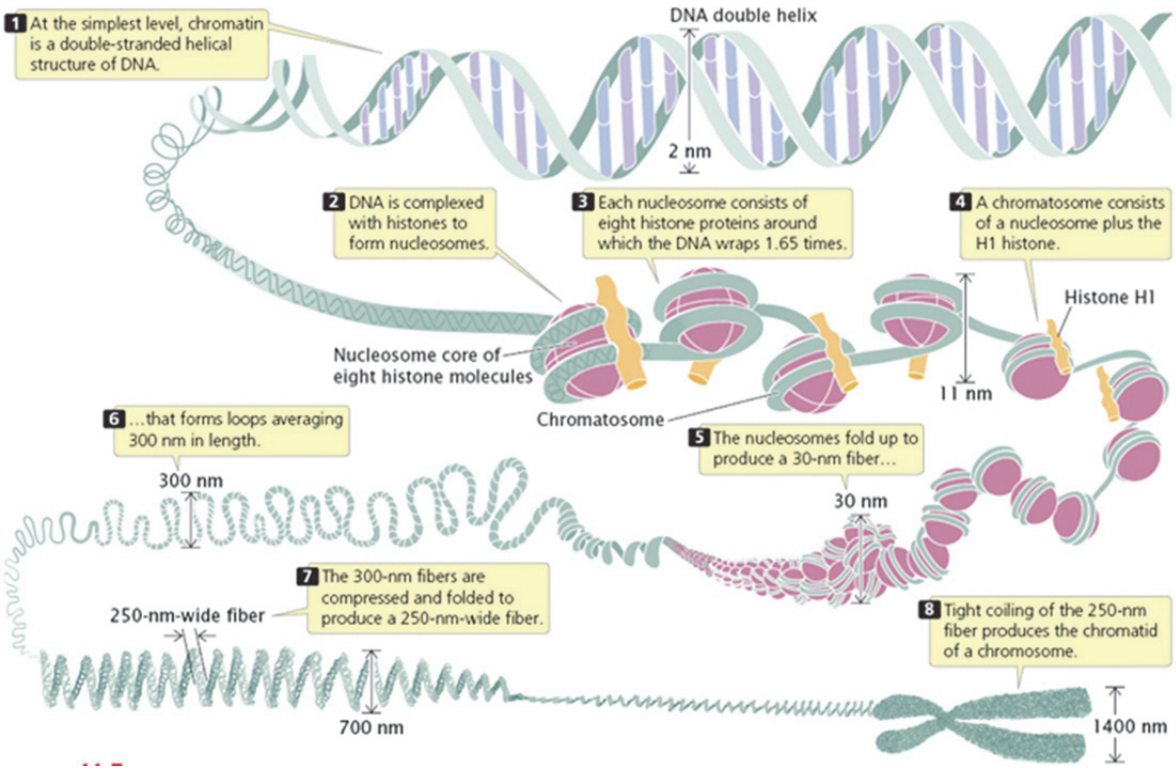


Figure n. 13: Different levels of chromatin organization: from the DNA to the metaphase chromosome (Freeman et al., 2005).

DNA replication occurs in a chromatin context, therefore major changes in chromatin structure are necessary for the replication fork to progress (Almouzni and Mechali 1988; Almouzni, Clark et al. 1990). The nucleosome flow from ahead to behind the replication fork

has to be orchestrated with the maintenance of epigenetic information, through the introduction of specific epigenetic marks (Sogo, Stahl et al. 1986; Jackson 1988; Ransom, Dennehey et al. 2010). Moreover, DNA replication of heterochromatin domains requires additional replication factors, due to the tightly compaction of the chromatin fiber (Taddei, Roche et al. 1999; Ridgway and Almouzni 2001; Nakatani, Ray-Gallet et al. 2004). In this chapter, the mechanisms of nucleosome assembly and disassembly during DNA replication are presented with a focus on the histone chaperone CAF-1.

3.1. Nucleosome structures

The name "chromatin" was given by Flemming in 1882, based on the property to retain basic dyes, and it derives from the Greek *khroma* (coloured). The nucleosome contains about 146 DNA base pairs, wrapped around an octamer of positively charged proteins termed "histones" (Luger, Mader et al. 1997). Eukaryotes present five conserved families of histones: H1/H5, H2A, H2B, H3, and H4. Histones H2A, H2B, H3 and H4 have been identified as "core histones", while histones H1 and H5 as "linker histones".

The nucleosome is constituted of a core of tetrameric $(H3\text{-}H4)_2$, associated with two dimers H2A-H2B (Figure n.14). The molecular weight of each core histone is comprised between 11 and 16 kDa, with more than 20% of their amino acid composition being lysines and arginines (Kornberg 1974; Luger, Mader et al. 1997). H3 and H4 histones are among the most conserved proteins in all organisms, while H2B and H2A display an appreciable sequence variation among different species. In most cells, a further level of chromatin compaction is present due to the association of nucleosome cores with linker histone H1/H5. In addition to binding to nucleosomes, the H1 or H5 histones bind to the "linker DNA" (DNA region between nucleosomes), facilitating the stabilization of the 30 nm chromatin fiber (Crane-Robinson, Dancy et al. 1976; Aviles, Chapman et al. 1978).

In mammalian cells, different histone variants are present (Zweidler 1978). These minor forms of "replacement histones" are non-allelic isoforms of major type histones. Histones variants affect the structure and stability of nucleosome and chromatin, and regulate the access of DNA metabolism enzymes to DNA (transcription machinery, DNA damage repair proteins, etc.) (Cohen, Newrock et al. 1975; Franklin and Zweidler 1977). A classic example is the histone variant H3.1, which is loaded onto replicating DNA, while H3.3 replaces H3.1 in transcribed regions of the genome (Ahmad and Henikoff 2002; Tagami, Ray-Gallet et al. 2004; Orsi, Couble et al. 2009; Ray-Gallet, Woolfe et al. 2011). Another example is the

histone H3-like CENP-A, which appears restricted to centromeric chromatin (Black, Foltz et al. 2004; Dunleavy, Almouzni et al. 2011) and which could be responsible for the maintenance of centromere identity in eukaryotes (Sullivan, Hechenberger et al. 1994). The histone variant H2A.Z is associated with promoters of actively transcribed genes and is involved in heterochromatin metabolism (Guillemette, Bataille et al. 2005; Billon and Cote 2012), while the histone variant H2A.X, which is a mark of DNA double strand breaks, is phosphorylated by checkpoint kinases in response to replication stress and DNA damages, leading to γH2AX (Rogakou, Pilch et al. 1998; Rogakou, Boon et al. 1999; Paull, Rogakou et al. 2000; van Attikum and Gasser 2005). However, in fission yeast such discrimination among different variants does not exist. Histone H3 and H4 are encoded by three identical genes and the only variant is CenpA, a centromere specific histone H3 variant (Lando, Endesfelder et al. 2012).

The histone fold domain is involved in histone/histone and histone/DNA interactions and is formed by three α-helixes, connected by two loops. The N-terminal domain is composed of about 15-30 highly positively charged amino acids, which protrude out of the nucleosome surface (Luger, Mader et al. 1997). The N-terminal tails are the main sites of several covalent post-translational modifications (PTMs), including acetylation, methylation and phosphorylation, which are involved in the regulation of chromatin dynamics, structure and expression (Brownell, Zhou et al. 1996; Hassig, Tong et al. 1998; Loyola and Almouzni 2004; Park and Luger 2008). These modifications could participate in a sort of "histone code", in which histone modifications define different chromatin state through the recruitment of specific proteins (Strahl and Allis 2000).

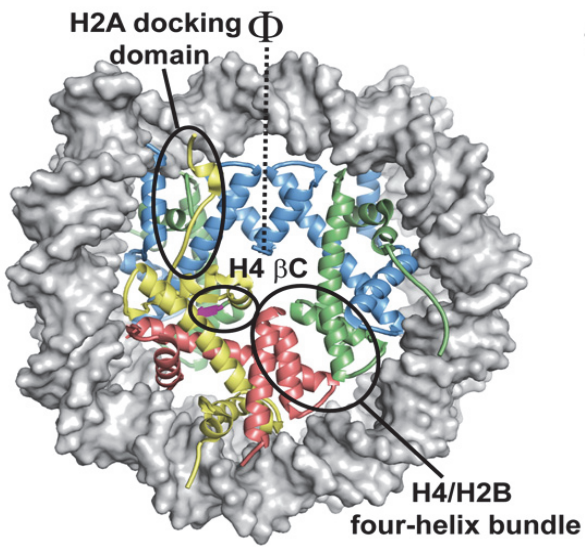


Figure n. 14*:* $(H3\text{-}H4)_2$ tetramer is held together via a tight network of hydrogen bonds and salt bridges that are confined to a small region defined by a four-helix bundle structure. DNA is shown in gray, H2A in yellow, H2B in red, H3 in blue and H4 in green. Solid ovals represent the regions of interaction between H2A-H2B and $(H3\text{-}H4)_2$. The dashed line represents the molecular two-fold axis dissecting the H3 four-helix bundle. H2A-H2B dimer makes intimate contacts with both arms of the W-shaped $(H3\text{-}H4)_2$ tetramer. These contacts involve the docking domain of H2A that interacts with the C-terminal β strand in H4 and parts of H3 on one side, and a four helix bundle between the histone fold regions of H4 and H2B. A minor contact made by the H2A L1 loops exists between the two H2A-H2B dimers. The interactions linking H2A-H2B dimers to $(H3\text{-}H4)_2$ tetramer cannot persist in the absence of DNA and thus the histone octamer is not stable under physiological conditions (Park and Luger, 2008).

3.2. Chromatin assembly and disassembly at replication forks

During DNA synthesis, a transient disruption of nucleosomes occurs ahead of the replication fork. Nucleosomes are then successively transferred onto the nascent strands, behind the moving fork. At the same time, the deposition of newly synthesized histones is essential to restore the correct nucleosome density along the daughter strands (*de novo* assembly) (Figure n.15) (Sogo, Stahl et al. 1986; Jackson 1988; Nakatani, Ray-Gallet et al. 2004; Ransom, Dennehey et al. 2010).

Different models have been proposed to explain the transfer of parental nucleosomes onto newly replicated DNA. A first model suggests that parental nucleosomes are disrupted into two H2A-H2B dimers and one $(H3\text{-}H4)_2$ tetramer. The entire $(H3\text{-}H4)_2$ tetramer is transferred onto the nascent DNA strand to form a subnucleosome structure. Thereafter, either old or newly synthesized H2A-H2B dimers are added to the subnucleosome structure (Jackson 1988). However, available data do not exclude that a different segregation mechanism leads to a further dissociation of parental $(H3\text{-}H4)_2$ tetramers into two H3-H4 dimers. Therefore, both parental and newly synthesized H3-H4 could be concomitantly present in the same nucleosome deposited onto newly replicated DNA. This could facilitate the incorporation of pre-existing modified histones to newly synthesized histones (Tagami, Ray-Gallet et al.

2004). These two mechanisms of parental histone segregation could also be differently used during DNA replication or during chromatin remodeling after DNA repair (Groth, Corpet et al. 2007; Groth 2009).

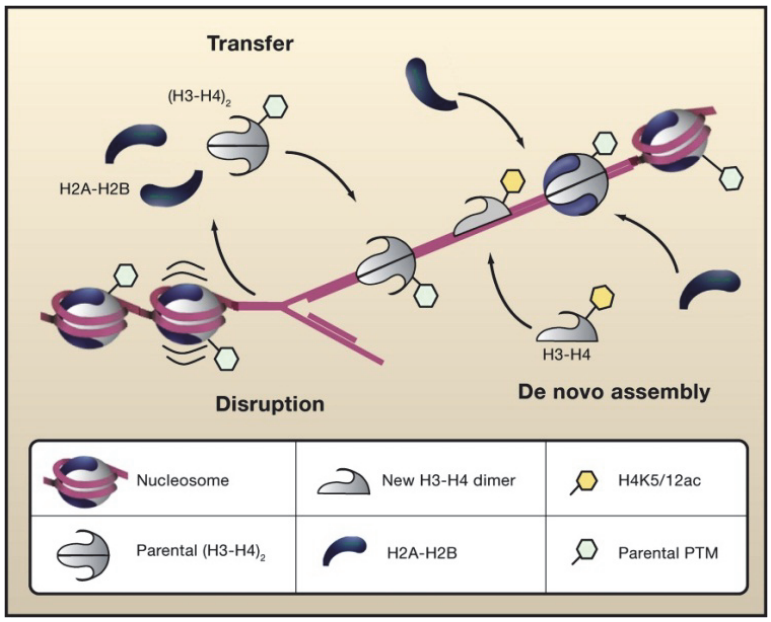


Figure n.15:
Nucleosomes metabolism during DNA replication. Parental histones are evicted ahead of the moving fork, and then transferred onto the daughter strands, in a manner that allows histone variants and histone modifications to be maintained. This segregation mechanism operates together with *de novo* nucleosome assembly to duplicate nucleosomal density and epigenetic information on both daughter strands (Groth, Corpet et al., 2007).

Histones are positively charged proteins and have a general affinity for the negatively charged DNA. Therefore, during DNA replication, histones chaperones are necessary to avoid an indiscriminate bound of histones to DNA. Histone chaperones represent a class of highly conserved eukaryotic proteins that bind single or complexes histones, and release them in a controlled way onto DNA, without being part of the final product (Laskey, Honda et al. 1978; Ray-Gallet, Quivy et al. 2002; Polo and Almouzni 2006). Histone chaperones count many members (Table n.3) that play a key role in the maintenance of genome stability by ensuring proper chromatin structure throughout the cell cycle. Histone chaperones have different relative affinities for histone and histone variants and this might trigger the efficiency by which histones are incorporated into or removed from chromatin (Polo and Almouzni 2006; Eitoku, Sato et al. 2008; Park and Luger 2008). Among the H3-H4 histone chaperones, the Chromatin Assembly Factor-1 (CAF-1) appears to have a major role, together with Anti Silencing Factor 1 (Asf1), in assembling nucleosomes onto newly synthesized DNA during replication and repair (Krude 1999).

Chaperone classification	Histone chaperone	Binding partners	Functions
H3–H4 family	Asf1	H3–H4 dimer HIRA CAF-1 RFC MCMs (via histones) Bdf1	Transcriptional regulation Replication Repair Transcriptional silencing Promotes histone acetylation Assembly of senescence associated heterochromatin foci (SAHF)
	Vps75	H3–H4 Rtt109	Transcriptional regulation Repair Telomere length maintenance Promotes histone acetylation
	Rtt106	H3–H4 CAF-1	Replication Transcriptional silencing Transcription repression
	CAF-1	H3–H4 Rtt106 Asf1 HP1 PCNA MBD1	Replication Repair Transcriptional silencing
	HIRA	H3–H4 Asf1 Pax 3 Swi/Snf	DNA synthesis independent nucleosome assembly Transcriptional repression Transcriptional silencing Assembly of SAHF Sperm chromatin decondensation
	RbAp46 RbAp48 dp55	Component of HAT1 CAF-1 PRC2 NURF NuRD HDAC1 2, 3 complexes	Many aspects of chromatin metabolism and histone modification
	FKBP (Fpr 3, 4)	H3–H4	rDNA silencing Regulation of histone methylation Cell cycle regulation

Table n.3: Classification of H3-H4 histone chaperones in mammalian cells (adapted from Das, Tyler et al., 2010).

3.2.1. The role of histone chaperones during DNA replication

During eukaryotic DNA replication, nucleosomes are transiently disrupted ahead of the fork, allowing the replication machinery to progress along the parental DNA. The replicative helicase MCM2-7, together with Cdc45 and the GINS complex (responsible for binding to both chromatin and DNA replication-associated proteins), mediate DNA unwinding, while the FACT (Facilitates Chromatin Transcription complex) complex removes H2A-H2B dimers (Figure n. 16 left). Asf1 interacts with MCM2-7 and triggers the disruption of the remaining parental $(H3\text{-}H4)_2$ tetramer (Groth, Corpet et al. 2007). Released histones held in close proximity to the fork, and then segregate onto daughter strands. In this way, parental histones act as a fingerprint for specific post-translational modifications for the newly synthesized histones (Natsume, Eitoku et al. 2007; Groth 2009; Clemente-Ruiz, Gonzalez-Prieto et al. 2011; Tanae, Horiuchi et al. 2012).

To maintain correct nucleosome density along the DNA fiber, newly synthesized histones have to be deposited together with parental histones. An important conceptual point is that both evicted and newly synthesized H3-H4 dimers do not associate as tetramers prior to their deposition onto DNA (Polo and Almouzni 2006). Indeed, H3 and H4 are firstly loaded onto the daughter strands and then, H2A and H2B are added by the histone chaperone NAP1, to form a mature nucleosome (Figure n. 16 top). Nucleosome assembly onto newly replicated DNA is achieved by the concerted action of several histones H3-H4 chaperones such as CAF-1, Asf1 and Rtt106. However, it is not clear how the actions of these three histone chaperones are coordinated to achieve rapid deposition of histones onto nascent DNA. Asf1 and CAF-1 are known to interact with components of the replication machinery but how Rtt106 is recruited at the replication fork is unknown (Li, Zhou et al. 2008). Asf1 itself does not deposit histones onto replicating DNA, at least *in vitro*, but synergizes with CAF-1 in replication-coupled nucleosome assembly (Tyler, Collins et al. 2001; Mello, Sillje et al. 2002). The interaction between CAF-1 and Asf1 allows the transfer of H3-H4 dimer from Asf1 to CAF-1. CAF-1 assembles preferentially newly synthesized H3-H4 dimers during DNA replication both on leading and lagging strands (Figure n. 16 right). Rtt106 might physically interact with CAF-1 but it can also function independently of CAF-1 during DNA replication, RNA transcription and heterochromatin assembly (Huang, Zhou et al. 2005; Zunder, Antczak et al. 2012).

Importantly, newly synthetized histones harbors specific post-translational modifications including the acetylation of the lysine K56 on histone H3 (H3K56Ac), at least in yeast models. The histone mark H3K56Ac is promoted by the acetyl transferase Rtt109 that exhibits closed interactions with Asf1 and CAF-1 (Jackson, Shires et al. 1976; Sobel, Cook et al. 1995; Verreault, Kaufman et al. 1996; Verreault, Kaufman et al. 1998; Imhof and Wolffe 1999; Kolonko, Albaugh et al. 2010; Clemente-Ruiz, Gonzalez-Prieto et al. 2011; Su, Hu et al. 2012).

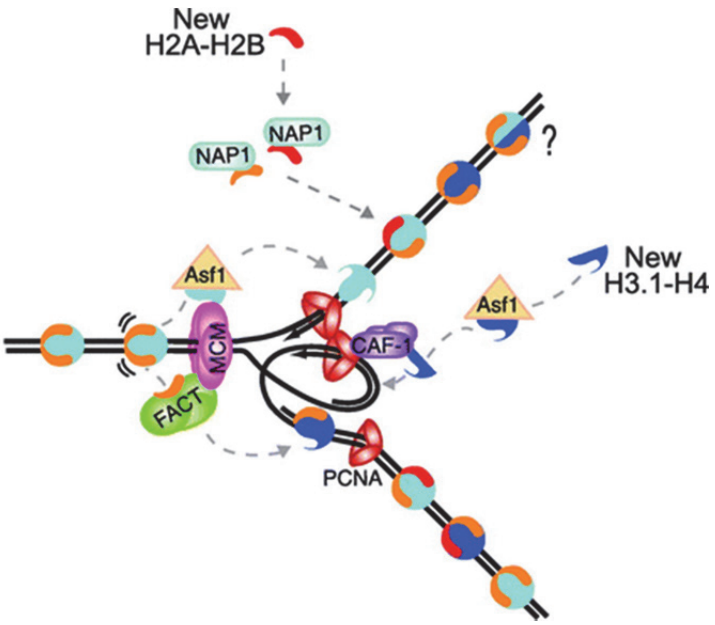


Figure n. 16: Scheme of a replication fork and histones turnover. Nucleosomes are evicted ahead of the fork by Asf1 and FACT, both interacting with the MCM complex. Asf1 transfer H3-H4 dimers to CAF-1. CAF-1 deposits H3-H4 dimers on leading and lagging strand, via its interaction with PCNA. Histone chaperone NAP1 loads H2A-H2B dimers on the $(H3\text{-}H4)_2$ tetramer, to form a complete nucleosome (Groth 2009).

3.2.2. Chromatin Assembly Factor 1

In 1986, Stillman identified an activity that preferentially assembles nucleosomes onto replicating DNA (Stillman 1986). In 1989, Smith and Stillman purified and characterized a replication-dependent chromatin assembly factor: "Chromatin Assembly Factor 1" (CAF-1) (Smith and Stillman 1989). This study was performed in a cell-free system, in which purified proteins can assemble chromatin during DNA replication and this provided an experimental system to investigate chromatin assembly *in vitro*. This system was based on the papovavirus SV40, which replicates in the nucleus of the host cell as a circular chromosome. Moreover, SV40 nucleosomes structure and histones composition are identical to those of the host (Smith and Stillman 1989).

Human purified CAF-1 consists of three subunits: p150, p60 and p48 which copurify in a trimeric protein complex (Stillman 1986; Smith and Stillman 1989; Kaufman, Kobayashi et al. 1995; Verreault, Kaufman et al. 1996; Kaufman, Kobayashi et al. 1997). Since 1989, functional homologues of CAF-1 have been identified in many other organisms such as mouse, *Xenopus, Drosophila, Arabidopsis,* chicken and yeasts. In fission yeast, CAF-1 complex is composed of three subunits, called Pcf1 (p150 homologue), Pcf2 (p60 homologue) and Pcf3 (p48 homologue). The function of CAF-1 in promoting DNA replication-dependent *de novo* nucleosome assembly is evolutionary conserved, thus enabling studies in model organisms to characterize CAF-1 features.

The largest subunit of CAF-1: p150

Human p150, Drospohila p180, budding yeast Cac1 and fission yeast Pcf1 share primary structure homology (Kaufman, Kobayashi et al. 1995; Enomoto, McCune-Zierath et al. 1997; Kaufman, Kobayashi et al. 1997; Tyler, Collins et al. 2001). The largest subunit of human CAF-1, p150, interacts preferentially with newly synthesized histone through its KER and ED domains, also defined as "acidic domain" (Figure n.17A). These domains contain an internal cluster of negatively charged amino acids implicated in histone binding. Deletion of the acidic domain abrogates the binding of p150/p60 to H3 and H4, without affecting the interaction between p150 and p60 (Kaufman, Kobayashi et al. 1995).

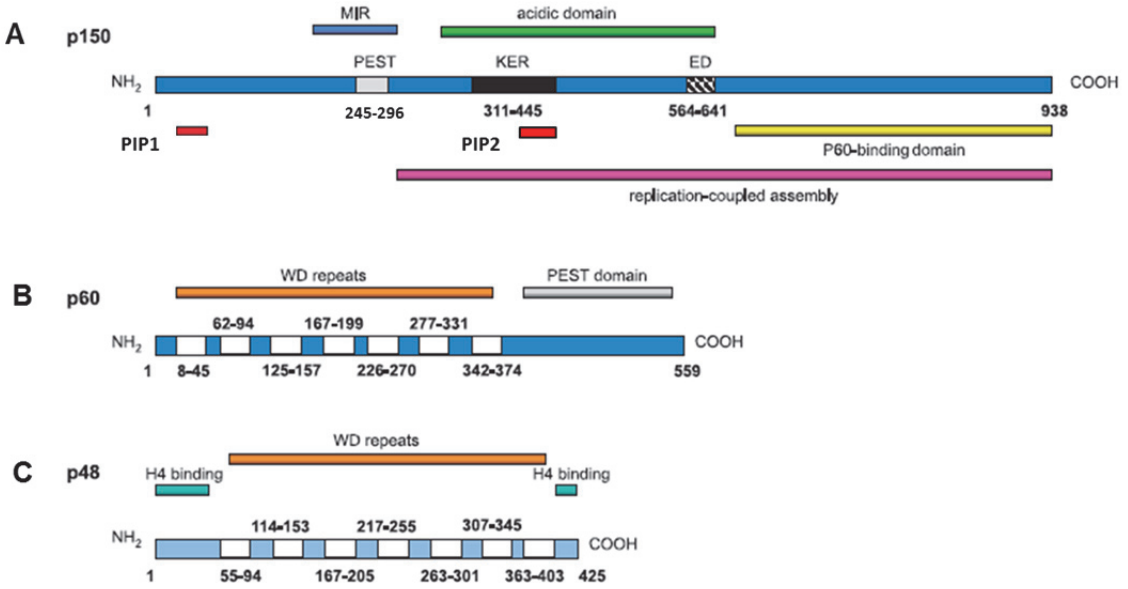


Figure n.17: Features of p150, p60 and p48 proteins, the three subunits of human CAF-1. Amino acids are numbered in bold**. A)** Structure of human p150 protein. The colored lines represent the different domain of the protein. The p150 presents a MIR domain that interacts with heterochromatin proteins, two PCNA binding motifs (PIP1 and PIP2), an acidic domain that interacts with histones and a p60 binding domain. The pink line represents the fragment of p150 necessary to ensure chromatin assembly coupled to DNA synthesis. **B)** Structure of human p60 protein with 7WD repeats (white squares) and a PEST domain. **C)** Structure of human p48 protein with 7WD repeats and the H4 interacting domains (Ridgway and Almouzni 2000; Dohke, Miyazaki et al. 2008).

The C-terminal domain of p150 is involved in the dimerization of p150 and in the interaction with p60 (Kaufman, Kobayashi et al. 1995; Quivy, Grandi et al. 2001). The C-terminal domain contains a high affinity binding site for the p60 protein, which cannot be deleted without abolishing the chromatin assembly activity. The presence of an acidic cluster in this region could be involved in the interaction with other proteins or for the correct three-

dimensional folding of the protein (Kaufman, Kobayashi et al. 1995). Human p150 oscillates between a dimeric and a monomeric state *in vivo* and its phosphorylation by the Cdc7-Dbf4 kinase (DDK) stabilizes p150 as a monomer. The phosphorylation of p150 favors its interaction with the homotrimeric sliding clamp PCNA (proliferating cell nuclear antigen), a component of the replication machinery (Quivy, Grandi et al. 2001; Gerard, Koundrioukoff et al. 2006). Thus, the phosphorylation of p150 and its dimerization state is thought to be a control point regulating the chromatin assembly function of CAF-1.

The N-terminal of p150 is composed of the first 296 amino acids and is characterized by large clusters of acidic residues together with a region enriched in proline, glutamic acid, serine, threonine and aspartic acid residues (PEST region) (Rogers, Wells et al. 1986; Kaufman, Kobayashi et al. 1995). The PEST region can be a signal for the rapid degradation of the protein (Rogers, Wells et al. 1986) but is dispensable for the chromatin assembly activity. The N-terminal of the p150 presents also a MIR domain containing three hydrophobic residues (consensus sequence: PxVxL) that are essential for its interaction with the Heterochromatin Protein 1 (HP1). The interaction between CAF-1 and HP1 is crucial for the maintenance of heterochromatin. However, *in vitro*, the MIR domain is dispensable for the replication-coupled assembly activity of CAF-1 (Murzina, Verreault et al. 1999).

The recruitment of CAF-1 at the replication fork involves a physical interaction between p150 subunit and PCNA (Shibahara and Stillman 1999; Moggs, Grandi et al. 2000; Krawitz, Kama et al. 2002; Rolef Ben-Shahar, Castillo et al. 2009). The interaction between the largest subunit of CAF-1 and PCNA is crucial for the recruitment of CAF-1 at the replication fork and for its chromatin assembly function. PCNA is an essential processivity factor that encircles double-stranded DNA and acts as a sliding clamp to tether DNA polymerases to their substrate, during DNA synthesis. PCNA binds several replication enzymes, such as DNA ligase 1, RFC (replication factor C), DNA polymerase δ, FEN1 (Flap structure-specific endonuclease 1) (Montecucco, Rossi et al. 1998; Gomes and Burgers 2000; Johansson, Garg et al. 2004). Furthermore, PCNA also directly binds to enzymes involved in epigenetic inheritance, such as the DNA methyltransferase DNMT1 in human cells (Chuang, Ian et al. 1997; Warbrick 1998). As mentioned above, PCNA interacts also with CAF-1 to recruit this histone chaperone at DNA replication forks The first 31 residues of human p150 binds to PCNA *in vitro* and *in vivo*, through a particular motif termed PIP-box (for PCNA Interacting Protein) (Figure n.18) (Shibahara and Stillman 1999; Moggs, Grandi et al. 2000; Krawitz, Kama et al. 2002).

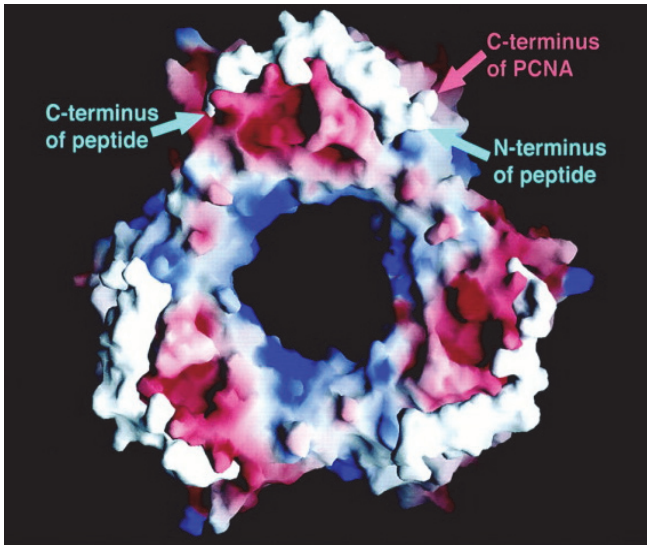


Figure n.18: View of a PCNA bound by three p21 PIPs. Molecular surface of the PCNA-PIP complex, with PCNA colored according to electrostatic potential and the PIP peptide showed in white. Regions of intense positive charge appear blue, and electronegative regions of the surface are red (Gulbis, Kelman et al. 1996).

Mammalian p150 subunit presents two PIP-box motifs: PIP1 exhibits a canonical sequence (Qxx(I/L/V)xx(F/Y)(F/Y); while PIP2 exhibits a degenerated sequence. In human cells, mutations in the canonical PIP-box (PIP1) decrease the ability of p150 to interact with PCNA but have a modest contribution on the targeting of p150 to DNA replication foci *in vivo*. Moreover, the mutation of the consensus sequence of the PIP-box has no impact on nucleosome assembly *in vitro*. A degenerated PCNA-interacting region within the KER domain of human p150 has been identified (PIP2) (Moggs, Grandi et al. 2000; Rolef Ben-Shahar, Castillo et al. 2009). The PIP2 motif lacks a key conserved residue (Figure n.19). Despite its weaker interaction with PCNA, the PIP2 motif is of crucial importance for replication-dependent nucleosome assembly *in vitro* and for targeting CAF-1 to DNA replication foci *in vivo* (Rolef Ben-Shahar, Castillo et al. 2009).

The weak binding of p150 with PCNA is explained on the basis of the available crystal structures of canonical PIPs in complex with PCNA. In these structures, the carbonyl and the amino groups of the lateral chain of the highly conserved glutamine within the canonical PIP motif are involved in several interactions with PCNA. In addition, two positively charged residues of PCNA are in close proximity to the glutamine side chain. In the PIP2 motif of human p150, the conserved glutamine residue of canonical PIP boxes is replaced by a lysine. Due to the loss of the multiple interactions mediated by the glutamine side chain and the proximity of positively charged PCNA residues, a lysine at that position would considerably weaken PCNA binding (Gulbis, Kelman et al. 1996; Bowman, O'Donnell et al. 2004; Bruning and Shamoo 2004; Chapados, Hosfield et al. 2004; Hishiki, Hashimoto et al. 2009; Rolef Ben-Shahar, Castillo et al. 2009).

In budding yeast, the largest subunit of CAF-1, named Cac1, binds PCNA as well (Zhang, Shibahara et al. 2000). This interaction occurs via a canonical PIP-box (PIP1) (Figure n.19). Indeed, yeast Cac1 exhibits only one canonical PIP-box motif, in contrast to human p150. Mutations in the PIP-box of Cac1 abolish its interaction with PCNA, resulting in a severe impairment in nucleosome assembly *in vitro* and mild effects in gene silencing. Importantly, mutations of the PIP-box do not affect the assembly of CAF-1 complex, as Cac1 remains able to interact with Cac3, the third subunit (the budding yeast homologue of human p48) (Krawitz, Kama et al. 2002). A second consensus PIP-box was identified in Cac2 subunit of CAF-1 (the budding yeast homologue of human p60). The function of this PIP-box remains unclear.

Nonetheless, a single mutation in the PIP-box of Cac1 is sufficient to abolish the interaction between CAF-1 and PCNA and to affect the chromatin assembly function *in vitro*. Thus, it appears that potential interactions between Cac2 and PCNA are not sufficient to promote a robust chromatin assembly activity (Krawitz, Kama et al. 2002).

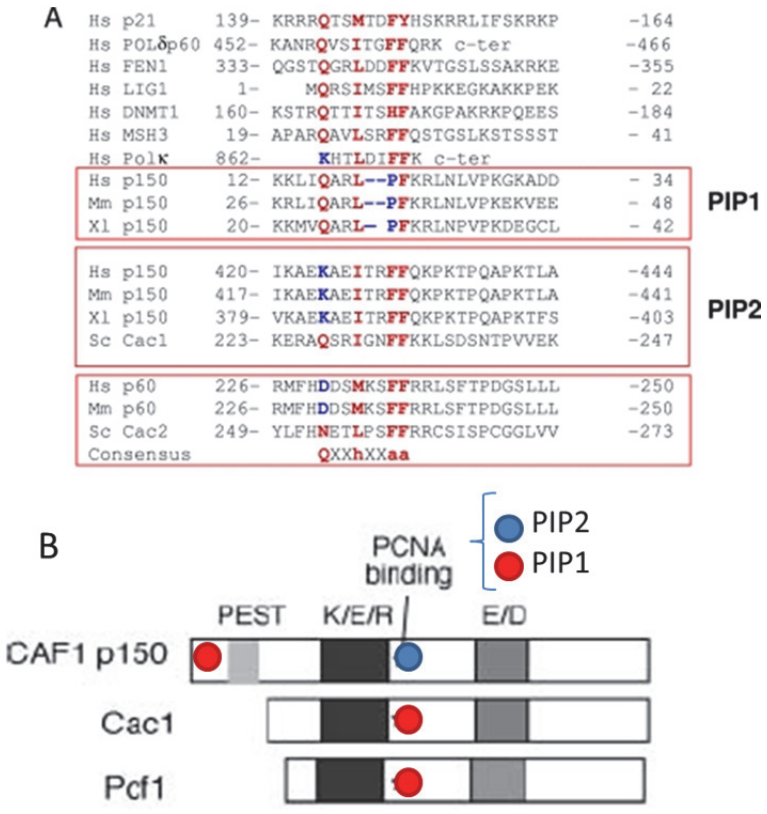


Figure n. 19: the PIP-box motifs of CAF-1: PIP1 and PIP2.
A) Sequence alignment for PIP1 and PIP2 in different model organisms. Hs is *Homo sapiens*, Mm is *Mus musculus*, Xl is *Xenopus laevis*, Sc is *S. cerevisiae*. Red residues are conserved, while blue residues are not.
B) Scheme of human p150, Cac1 and Pcf1 and their relative PIP boxes. PIP1 is a canonical PIP box (red circles) and PIP2 is a degenerated PIP box (blue circle). (Adapted from (Rolef Ben-Shahar, Castillo et al. 2009).

The p60 subunit of CAF-1

The human p60, *Drosophila* p105, the budding yeast Cac2 and the fission yeast Pcf2 are members of protein family of tryptophan-aspartate (WD)-repeat (Kaufman, Kobayashi et al. 1995; Verreault, Kaufman et al. 1996). Such proteins contain four to eight WD repeated motifs; each one is approximately of 40 amino acids in length (Figure 17B). The human p60 has seven WD repeats, although the fifth and sixth repeats show significant divergences from canonical residues (Verreault, Kaufman et al. 1996). WD repeats are proposed to mediate the interaction between p60 and p48 (the third subunit of CAF-1), which is also a member of (WD)-repeat protein family (Komachi, Redd et al. 1994; Kaufman, Kobayashi et al. 1995; Kaufman, Kobayashi et al. 1997; Tyler, Collins et al. 2001).

The p60 subunit physically interacts with the large subunit p150 and the deletion of p60 binding domain in the C-terminal part of p150 subunit impairs the nucleosome assembly activity (Kaufman, Kobayashi et al. 1995). The human p60 subunit also exhibits a C-terminal PEST domain and a B-domain-like motif that bind to Asf1 (Malay, Umehara et al. 2008). The interaction between CAF-1 and Asf1 is crucial during replication-coupled nucleosome assembly to allow the transfer of histones from Asf1 to CAF-1 (Mello, Sillje et al. 2002; Tang, Poustovoitov et al. 2006).

Another interesting aspect is that both p60 and p150 contains consensus target sites for various kinases (Smith and Stillman 1991; Kaufman, Kobayashi et al. 1995; Martini, Roche et al. 1998). The human p60 is phosphorylated by Cyclin/Cdk activities *in vitro* (Keller and Krude 2000) and this could be a way to regulate CAF-1 activity. Indeed p60 phosphorylation correlates with the cell cycle dependent regulation of CAF-1 activity and localization (Marheineke and Krude 1998; Martini, Roche et al. 1998).

The p48 subunit of CAF-1

Human p48, *Drosophila* p55, budding yeast Cac3 and fission yeast Pcf3 present a large domain containing WD repeats (Figure 17C) (Tyler, Bulger et al. 1996; Verreault, Kaufman et al. 1996; Kaufman, Kobayashi et al. 1997). The human p48 subunit, in complex with p150 and p60, binds the H4 histone during DNA replication-coupled chromatin assembly (Verreault, Kaufman et al. 1998).

The p48 and the p46 proteins are homologue histone chaperones that play key roles in establishing and maintaining chromatin structure. Both p48 and p46 can interact with core histones H2A and H4 *in vitro*. The interacting domains between H4 and p48 and p46 were

mapped in *S. cerevisiae* and *Xenopus* and data showed that p48 and p46 bind directly to helix1 of histone H4 (Verreault, Kaufman et al. 1998; Vermaak, Wade et al. 1999; Murzina, Pei et al. 2008). However, despite p46 and p48 present 90% identical at the amino acid sequence level, in both human and yeast cells p46 is found exclusively in complexes with histone acetyltransferase 1 (HAT1), whereas p48 is instead found in the CAF-1 complex (Parthun, Widom et al. 1996; Taunton, Hassig et al. 1996; Martinez-Balbas, Tsukiyama et al. 1998; Verreault, Kaufman et al. 1998).

Although the interaction between p48 and the N-terminal of H4 occurs, it has been demonstrated that deletion of both H3 and H4 N-terminal domains does not affect CAF-1-mediated nucleosome deposition onto newly synthesized DNA *in vitro*. Moreover, each of CAF-1 subunit binds the recombinant $(H3\text{-}H4)_2$ tetramers lacking the N-terminal domains of both H3 and H4 (Shibahara, Verreault et al. 2000). Thus, CAF-1 interacts with histone H3-H4 by multiple interactions. However, it is well known that in human cells and in budding yeast, that deletion of any one of CAF-1 subunit leads to a loss of function of the entire complex *in vivo* (Kaufman, Kobayashi et al. 1997; Linger and Tyler 2005).

The p48 protein was found in CAF-1 complex in stoichiometric amounts, but p48 was also found uncomplexed to p150 and p60 in cytosolic replication extracts (Smith and Stillman 1989; Verreault, Kaufman et al. 1996; Marheineke and Krude 1998). Indeed, in a broad range of species, human p48 is present in several chromatin-related complexes involved in histone metabolism, including the histone deacetylase 1 complex (HDAC1), histone deacetylase 2 complex (HDAC2), the histone methyltransferase complex ESC-E(Z) and the ATP-dependent nucleosome remodeling complex NURF (Parthun, Widom et al. 1996; Taunton, Hassig et al. 1996; Martinez-Balbas, Tsukiyama et al. 1998; Verreault, Kaufman et al. 1998; Ahmad, Takami et al. 1999).

3.2.3. Anti silencing function 1

The histone H3-H4 chaperone CIA/Asf1 (For *CCG1-interacting factor* A or *Anti-silencing Factor 1*) is the most conserved histone chaperone among eukaryotes. Asf1 was first identified from a genetic screening in budding yeast as a gene whose overexpression induces the expression of loci that are normally transcriptionally silent (Le, Davis et al. 1997) Later on, the histone chaperone function of Asf1 was uncovered by Tyler and colleagues in Drosophila. The authors identified Asf1 in a complex with newly synthesized histone H3/H4

and showed that Asf1 acts as a factor with CAF-1 to synergize replication-coupled nucleosome assembly *in vitro* (Tyler, Adams et al. 1999). The most evolutionarily conserved region of Asf1 is the first 155 residues within the N-terminal (Daganzo, Erzberger et al. 2003; Mousson, Lautrette et al. 2005). This domain is characteristic of an immunoglobulin (Ig)-fold domain, in both yeast and human cells. The role of the Ig-fold domain is to bind histone H3/H4 and to stimulate CAF-1 dependent nucleosome assembly (Umehara, Chimura et al. 2002; Daganzo, Erzberger et al. 2003). The C- termini of Asf1 is rather divergent between species or human isoforms and could be a good candidate for the basis of distinct functions (Umehara, Chimura et al. 2002; Daganzo, Erzberger et al. 2003).

During DNA replication, Asf1 regulates the histone turnover at the replication fork (see Introduction 3.2). Three-dimensional structure of Asf1 from *S. cerevisiae*, *S. pombe* and human has been resolved and these studies provide the molecular basis for the role of Asf1 in the assembly/disassembly of histones H3/H4 *in vivo* (Umehara, Chimura et al. 2002; Antczak, Tsubota et al. 2006; English, Adkins et al. 2006; Agez, Chen et al. 2007; Natsume, Eitoku et al. 2007; Malay, Umehara et al. 2008).

Newly synthesized H3/H4 histones are associated with Asf1 in the cytoplasm and are then imported into the nucleus (English, Adkins et al. 2006; Natsume, Eitoku et al. 2007). Current models suggest that the N-terminal of Asf1 binds to the C-terminal of histone H3, thus physically blocking the formation of H3-H4 tetramer (Agez, Chen et al. 2007). Asf1 also contacts and causes a conformational change of the C-terminus of histone H4, which otherwise interacts with H2A (Natsume, Eitoku et al. 2007). Once in the nucleus, Asf1 binds other histone chaperones including CAF-1 and Rtt106 which mediate chromatin assembly following DNA synthesis (Shibahara and Stillman 1999; Tyler, Adams et al. 1999; Mello, Sillje et al. 2002). Structural analysis demonstrated that Asf1 acts as a histone donor for CAF-1 with a molar ratio of approximately 1:1 between Asf1 and H3-H4 dimer (Agez, Chen et al. 2007; Natsume, Eitoku et al. 2007).

In vertebrates, *D. melanogaster*, and *S. pombe,* Asf1 is essential for cell survival, with a critical role in maintaining genomic stability (Groth, Ray-Gallet et al. 2005; Sanematsu, Takami et al. 2006; Schulz and Tyler 2006; Tanae, Horiuchi et al. 2012). For example, the *asf1-3*3 thermosensitive mutant, which lacks the N-terminal part of Asf1, is incapable of binding histone H3 and leads to a mislocalization of the Asf1-33 protein, in *S. pombe*. Cells harboring the *asf1-33* mutation exhibit an alteration of overall chromatin structure, accompanied by degradation of chromosomal DNA, DNA-damage checkpoint activation,

formation of recombination center (Rad22 foci) and impairment of heterochromatin structure (Tanae, Horiuchi et al. 2012).

Asf1 is also involved in gene silencing and heterochromatin maintenance (Sanematsu, Takami et al. 2006; Yamane, Mizuguchi et al. 2011; Tanae, Horiuchi et al. 2012). In budding yeast, strains deleted for Asf1 display deficiencies in gene silencing at telomere and mating-type loci (Le, Davis et al. 1997; Tyler, Adams et al. 1999; Meijsing and Ehrenhofer-Murray 2001; Sharp, Rizki et al. 2005). Asf1 function in gene silencing could be due to its interaction with HIRA (for *HIstone cell cycle Regulation defective homolog A*) another histone chaperone. HIRA mediates chromatin assembly independently of DNA synthesis, prevalently during DNA repair, gene silencing and transcription (Kaufman, Kobayashi et al. 1997; Ray-Gallet, Quivy et al. 2002; Blackwell, Martin et al. 2004; Tagami, Ray-Gallet et al. 2004; Zhang, Poustovoitov et al. 2005; Fillingham, Recht et al. 2008). The interplay between Asf1 and HIRA was shown to be of crucial importance in fission yeast to maintain gene silencing, via the recruitment of the heterochromatin protein Swi6 at heterochromatic loci (Yamane, Mizuguchi et al. 2011). Moreover, it has been structurally demonstrated that the interaction of Asf1 to CAF-1 and HIRA involved the same interface, suggesting a mutually exclusive interaction (Malay, Umehara et al. 2008). This exclusive interaction might represent a point of regulation for histone deposition associated or not with DNA synthesis. In addition, histone deposition appears also regulated by the selective binding of histone chaperone CAF-1 and HIRA to histone H3 variant present in the H3-H4 dimer (Nakatani, Ray-Gallet et al. 2004; Tagami, Ray-Gallet et al. 2004).

3.2.4. The replication-coupled histone mark H3K56

Dynamic variations in the structure of chromatin influence DNA-related processes in eukaryotes, which are controlled in part by the post translational modifications of histones (Krude 1999). Histones H4 deposited during S-phase are acetylated on lysines 5, 8 and 12 (H4K5Ac, H4K8Ac and H4K12Ac), a pattern highly conserved from yeast to humans. Acetylation of newly synthetized histones is promoted by the acetyltransferase HAT1 (Jackson, Shires et al. 1976; Sobel, Cook et al. 1995; Verreault, Kaufman et al. 1998; Imhof and Wolffe 1999). The histone H3 is also acetylated within its amino terminal tail, but its acetylation pattern is more variable among organisms (Sobel, Cook et al. 1995; Kuo, Brownell et al. 1996). The acetylation of H3 on lysine 56 (H3K56Ac) has been implicated in

the regulation of nucleosome assembly during DNA replication and repair, and nucleosome disassembly during gene transcription (Verreault, Kaufman et al. 1996; Chen, Carson et al. 2008; Li, Zhou et al. 2008; Tjeertes, Miller et al. 2009; Yuan, Pu et al. 2009; Clemente-Ruiz, Gonzalez-Prieto et al. 2011; Su, Hu et al. 2012). All the studies performed to demonstrate the importance of the histone mark H3K56Ac in DNA metabolism were undertaken in yeast models because this mark is globally abundant in yeast and flies, but its presence has been uncertain in mammals. However, recent studies demonstrated the presence of this marker also in human cells (Das, Lucia et al. 2009; Tjeertes, Miller et al. 2009; Xie, Song et al. 2009).

In yeast, histone chaperones play a recurrent role in the biology of H3K56 acetylation: Asf1 binds and presents newly synthesized dimers H3-H4 to the histone acetyltransferase Rtt109, that in turns acetylates H3 on lysine 56 (Recht, Tsubota et al. 2006; Driscoll, Hudson et al. 2007; Han, Zhou et al. 2007; Tsubota, Berndsen et al. 2007; Li, Zhou et al. 2008). The histone mark H3K56Ac promotes S-phase specific chromatin assembly by enhancing the ability of histone chaperones CAF-1 and Rtt106 to bind histone H3 and assemble it with histone H4 into nucleosomes (Li, Zhou et al. 2008; Su, Hu et al. 2012). In *S. cerevisiae,* the absence of acetylation on lysine 56 of histone H3 (either by deleted *asf1*, *rtt109* or expressing a non-acetylable form of H3, K56R) prevent co-purification of CAF-1 and Rtt106 with H3, indicating that H3 association with histone chaperones requires acetylation of H3K56 (Huang, Zhou et al. 2005; Li, Zhou et al. 2008; Su, Hu et al. 2012). The specific recognition of the acetylated form of H3 on K56 by CAF-1 and Rtt106 extends the "histone code" hypothesis (Strahl and Allis 2000). Indeed, as H3-H4 dimers harboring the H3K56Ac mark are likely to display concomitantly H4 marks (such as H4K5Ac, H4K12Ac), it is possible that multiple marks are necessary to recognize newly replicated DNA and to promote chromatin assembly (Tyler, Adams et al. 1999; Fillingham and Greenblatt 2008; Li, Zhou et al. 2008). Moreover, in *S. cerevisiae,* cells lacking Rtt109 or Asf1 present defects in MMS-induced HR while the HR induced by HO-induced DSBs does not require any of these genes. Indeed K56 acetylation may promotes sister-chromatid exchanges, considering that cells lacking Rtt109 or Asf1 present a dramatic decrease in SCE during S-phase (Duro, Vaisica et al. 2008).

During DNA replication, after nucleosome assembly in the rear of moving forks, histones have to be deacetylated by deacetylases as marks of "mature" chromatin. The interaction between Asf1 and the deacetylases Hst3/Hst4 in *S. cerevisiae,* promotes the deacetylation of H3K56 (Maas, Miller et al. 2006; Yang and Freudenreich 2010; Hachinohe, Hanaoka et al. 2011). An interesting theory is that the human p48 subunit of CAF-1 complex could play a

role in histones deacetylation. There are many evidences suggesting that p48 is involved in alterations of the chromatin structure, based on deacetylation level of core histones. For example, human p48 associates with the catalytic subunit of the human deacetylase HD1 (Taunton, Hassig et al. 1996). Moreover, chicken p48 interacts with histone deacetylases chHDAC-1 and -2 *in vivo* and *in vitro*, probably through one or two N-terminal and C-terminal WD repeated motifs (Ahmad, Takami et al. 1999). An intriguing hypothesis is that p48 subunit could have a role in chromatin maturation independently of the two other subunits of CAF-1. Indeed, p48 could switch from an interaction with CAF-1 to an interaction with enzymes involved in histone deacetylation and thus signaling the progression of the cell cycle.

Defects in the histone mark H3K56Ac during chromatin assembly lead to genetic instability which is linked to a higher susceptibility of fork stalling, damage or breakage (Han, Zhou et al. 2007; Clemente-Ruiz, Gonzalez-Prieto et al. 2011; Su, Hu et al. 2012). The absence of H3K56Ac or the simultaneous knock-out of CAF-1 and Rtt106 affect the integrity of advancing replication forks, thus increasing homologous recombination (HR). An intriguing hypothesis is that H3K56Ac might recruit additional chromatin factors required for fork stabilization. Alternatively, fork instability may result from defective chromatin assembly and/or transfer of parental histones behind the fork (Clemente-Ruiz, Gonzalez-Prieto et al. 2011). Defective chromatin assembly by partial depletion of H4 is rapidly followed by the collapse of replication forks, which are efficiently rescued by homologous recombination, suggesting that correct nucleosome deposition is required for replication fork stability (Prado and Aguilera 2005). The loss of integrity of advancing replication forks may end up in the collapse of some of them resulting in exposed ssDNA that is susceptible to be process by homologous recombination. HR participates in the rescue of these forks using the sister chromatid and, consistent with this, *asf1-d* strain shows an increase in sister chromatid exchange (SCE) (Prado, Cortes-Ledesma et al. 2004; Endo, Kawashima et al. 2010; Clemente-Ruiz, Gonzalez-Prieto et al. 2011).

Beyond its role in marking newly replicated DNA, the histone mark H3K56Ac plays a crucial function in the DNA damage response, together with Asf1. In *S. cerevisiae,* the absence of Asf1 results in cell sensitivity to hydroxyurea (HU), camptothecin (CPT), cisplatin (CIS) and methyl methansulfonate (MMS). This sensitivity to DNA-damaging agents is shared by cells harboring the non acetylable form of histone H3 on K56 (mutation K56 to R56 leading to H3K56R), suggesting a common role for Asf1 and the histone mark H3K56Ac in genome

stability (Le, Davis et al. 1997; Tyler, Adams et al. 1999; Ramey, Howar et al. 2004; Franco, Lam et al. 2005; Wurtele, Kaiser et al. 2012). The expression of H3 mutant (H3K56Q) that mimics the acetylation modification rescues the cell sensitivity to HU, CPT and MMS observed in the absence of Asf1, showing that the role of Asf1 in the DNA damage response is directly linked to its ability to promote the deposition of H3K56 onto DNA, either after DNA repair or during DNA replication (Agez, Chen et al. 2007). Nonetheless, the links between H3K56Ac, the nascent chromatin structure and the DNA damage response remain unclear. In response to MMS, cells expressing H3K56R (the non-acetylable form) cannot complete DNA replication and accumulate recombination foci, suggesting that H3K56Ac helps to complete the repair of DNA lesions by homologous recombination (Wurtele, Kaiser et al. 2012). One possibility is that both Asf1 and H3K56Ac are necessary to restore chromatin structure following DNA repair (Chen, Carson et al. 2008), an aspect developed in the next chapter.

3.2.5. Chromatin assembly at replication forks is essential to maintain genome stability

Several studies have pointed out an increased level of homologous recombination or general genome instability when replication-coupled chromatin assembly is impaired, as for example in the absence of CAF-1 or Asf1. While these data could be interpreted as a potential role for histones chaperones in repressing HR, alternative explanations such as impaired stability of replication forks and/or accumulation of DNA lesions are also possible.

As mentioned above, the absence of Asf1 results in an increased level of SCE. The hyper-recombinogenic effect of *asf1-d* is epistatic over *cac1-d*, suggesting that Asf1 and CAF-1 participate in the same pathway in the maintenance of genome integrity (Prado, Cortes-Ledesma et al. 2004). This is consistent with the fact that *asf1* and *cac1* mutants present an increased rate of gross chromosomal rearrangements (GCRs) (Myung, Pennaneach et al. 2003). Another evidence of increased level of spontaneous DNA lesions and genome instability in the absence of CAF-1 was provided by a study on post-replication repair by unequal sister chromatid recombination (uSCR), in budding yeast. Cells deficient for *cac1* or *cac2* display a higher level of HR during post-replication repair while the level of H3K56Ac remains unaffected. Others data suggest that the deposition of H3K56Ac onto newly synthesized DNA in CAF-1 deficient cells, even if delayed, is required for the induction of

sister chromatin recombination (Tong, Evangelista et al. 2001; Pan, Ye et al. 2006; Li, Zhou et al. 2008; Endo, Kawashima et al. 2010).

In plants, CAF-1 is also important for maintaining genome stability by suppressing illegitimate homologous recombination. In *Arabidopsis thaliana,* depletion of either subunit of CAF-1 complex increases the frequency of somatic homologous recombination of about 40 fold. This means that CAF-1 complex is responsible to maintain HR at low level in wild-type plants (Endo, Ishikawa et al. 2006). Furthermore, the frequency of transferred DNA (T-DNA) integration was also elevated in CAF-1 mutants, and. T-DNA integration occurs mainly through non-HR mechanisms such as NHEJ. Increased level of illegitimate HR is probably due to an increased level of general genome instability, rather than enhanced expression of NHEJ pathway genes (Endo, Ishikawa et al. 2006).

Several lines of evidences establish that chromatin assembly onto newly replicated DNA, especially the deposition of the histone mark H3K56Ac is crucial to maintain replication fork stability. In budding yeast, Asf1 interact with the replication factor RFC and defects in Asf1 results in loss of component of the replisome, including RFC, PCNA and DNA polymerase alpha (Franco, Lam et al. 2005). Defects in histone deposition appear to destabilize the fork and thus trigger a need for repairing halted forks by homologous recombination. Replicative stress induces a complex DNA damage response in which S-phase checkpoints aim to maintain the stability of stalled replication forks and provide time to repair or tolerate the damage (Lopes, Cotta-Ramusino et al. 2001). In cells defective for replication-coupled chromatin assembly pathway, the integrity of advancing but not stalled replication forks is compromised, which leads to checkpoint activation and accumulation of recombinogenic DNA damage (Clemente-Ruiz, Gonzalez-Prieto et al. 2011). Thus H3K56Ac/CAF1/Rtt106-dependent chromatin assembly provides a mechanism for the stabilization of replication forks, whose deficiency causes a “hyper-rec” phenotype probably associated with the generation of DSBs (Clemente-Ruiz, Gonzalez-Prieto et al. 2011). Furthermore, the partial depletion of histone H4 due to defects in histone chaperone activity, leads to recombinogenic DNA damage, as well. Indeed, the depletion of H4 is followed by the collapse of unperturbed and stalled replication forks, even when the S-phase checkpoint remains functional (Clemente-Ruiz and Prado 2009). This has been demonstrated in budding yeast by a reduction in the amount of replication intermediates and by an increase of recombination intermediates such as HJ-like structures, likely reflecting fork restart by HR (Clemente-Ruiz and Prado 2009).

To summarize data described above, defects in chromatin assembly at replication forks due to deficiencies in histone chaperones activities lead to abnormal recombination phenotypes. Several explanations can be proposed:

a) **The general decrease in nucleosome density** could lead to a novel chromatin context, being more liable to DNA lesions or facilitating DNA access to DNA repair enzymes, including HR. In support of this, recent data showed that a reduction in histone H3 and H4 gene dosage results in cell resistance to all common DNA damaging agents in S. *cerevisiae*. Cell resistance results from an increased efficiency in DNA repair, especially by HR, suggesting that histones might indeed act as a barrier to DNA repair enzymes from accessing DNA lesions (Liang, Burkhart et al. 2012). Liang and colleagues data might support the initial hypothesis that reducing nucleosome density in the absence of chromatin assembly facilitates recombination efficiency.

b) **The collapse of replication forks** occurs more frequently when H3K56Ac deposition is impaired, while a general decrease in nucleosome deposition per se might not increase the instability of advancing replication forks. Restart or repair of collapsed forks requires HR, resulting in a hyper-rec phenotype (Clemente-Ruiz and Prado 2009; Clemente-Ruiz, Gonzalez-Prieto et al. 2011).

c) **Histone chaperones could have a direct role** in repressing HR. However, this explanation is unlikely because recent data that have highlighted the role of CAF-1 during DNA repair by HR rather support a role for CAF-1 in promoting HR.

3.3. Models for heterochromatin replication

During DNA replication, heterochromatin domains constitute a challenge for the replication machinery because of the high level of compaction. To allow the replisome to pass through these domains, there is the need for additional factors to confer flexibility to the heterochromatic DNA (Taddei, Roche et al. 1999; Loyola, He et al. 2004; Maison and Almouzni 2004). In the meantime, a flow of epigenetic marks has to be established to guarantee the correct transfer of the epigenetic information behind the replication fork. Heterochromatin domains such as those corresponding to the centromere are crucial for proper chromosome segregation, maintenance of epigenetic information and genome stability. Hallmarks of heterochromatin domains in mammalian cells are the presence of epigenetic marks, such as DNA methylation, hypoacetylated histones, the trimethylation of histone H3 on lysine 9 (H3K9Me3), and the recruitment of the protein HP1 (Heterochromatin Protein 1),

as well as the prevalence of the H3.1 histone variant in the core nucleosome (James and Elgin 1986; Jeppesen, Mitchell et al. 1992; Tweedie, Charlton et al. 1997; Peters, O'Carroll et al. 2001; Bird 2002).

CAF-1, interacting with proteins involved in DNA replication and heterochromatin maintenance, plays a role in setting up the repressed state of chromatin, demonstrating that propagation of silenced chromatin is intimately linked to histone deposition process (Taddei, Roche et al. 1999; Quivy, Roche et al. 2004; Houlard, Berlivet et al. 2006). This function seems to be conserved from yeast to multicellular organisms, indicating the importance of maintaining a correct epigenetic status, independently on the complexity of organism (Eissenberg and Elgin 2000; Song, He et al. 2007; Dohke, Miyazaki et al. 2008; Quivy, Gerard et al. 2008; Huang, Yu et al. 2010). In eukaryotes, the domains next to centromeres are rich in HP1, which would ensure the self-perpetuation of the epigenetic state of the domain (James and Elgin 1986). Quivy and colleagues proposed a model in which human p150 could be used to facilitate the passage of the replication fork at heterochromatic regions, through its interaction with PCNA. This occurs by displacing chromatin bound HP1 ahead of the replication fork and transferring HP1 to the daughter strand, on H3K9Me3 (Bannister, Zegerman et al. 2001; Lachner, O'Carroll et al. 2001; Quivy, Roche et al. 2004; Quivy, Gerard et al. 2008; Hayashi, Takahashi et al. 2009). Such a mechanism could ensure the maintenance of the heterochromatin structure, and therefore the memory of the repressed state (Quivy, Roche et al. 2004; Quivy, Gerard et al. 2008). The interaction between CAF-1 and HP1 allows the replication machinery to progress efficiently through heterochromatin while the heterochromatic structure is destabilized. The disruption of p150-HP1 interaction prevents S-phase progression, without activation of the DNA damage checkpoint. This can be due to the presence of complexes formed by the interaction of HP1 with the DNA, which act as replication fork barriers (Quivy, Roche et al. 2004).

An emerging theme for replication-coupled memory is the use of PCNA as a hub that couples chromatin reorganization to replication, establishing a connection between genetic and epigenetic inheritance (Zhang, Shibahara et al. 2000). PCNA recruits a large number of chromatin-modulating enzymes to sites of DNA replication such as the methyltransferase DNMT1 (Leonhardt, Page et al. 1992; Chuang, Ian et al. 1997), CAF-1 (Shibahara and Stillman 1999), the histone deacetylase 1 (HDAC) (Milutinovic, Zhuang et al. 2002) and the complex formed by the nucleosome-remodeling factor SNF2H and the Williams syndrome transcription factor WSTF (Poot, Bozhenok et al. 2004). Some of these enzymes can be

considered as general chromatin-maturation factors but others operate in a domain specific manner such as DNMT1 (Leonhardt, Page et al. 1992; Chuang, Ian et al. 1997).

It seems likely that the actions of DNMT1, WSTF-SNF2H and CAF-1 are coordinated to ensure efficient DNA methylation prior to chromatin maturation. DNMT1 and CAF-1 themselves have the ability to recruit a number of enzymes implicated in chromatin maturation. This interplay probably ensures that replication-coupled propagation of DNA methylation coincides with formation of a repressive chromatin state (Leonhardt, Page et al. 1992; Chuang, Ian et al. 1997). In this scenario, the role of CAF-1 will go beyond the mere HP1 mobilization. The interaction between CAF-1 with other chromatin maturation enzymes suggests that CAF-1 could be a direct player in epigenetic inheritance. Another intriguing possibility is that the transient presence of acetylated histones onto newly synthesized DNA could regulate the kinetics of nascent chromatin maturation. Histone-modifying enzymes interacting or not with CAF-1 could provide a window of opportunity to maintain or alter specific chromatin structures, before histone deacetylation (Groth, Corpet et al. 2007).

In the fission yeast *S. pombe*, spCAF-1 and Swi6 (the homologue of HP1) are involved in the maintenance of heterochromatin. The fission yeast centromeres are organized into distinct domains that consist of the central core (cnt) surrounding by large inverted-repeat structures (otr and imr). Therefore, *S. pombe* centromeres bear a close resemblance to higher eukaryotic centromeres. Moreover, centromeric heterochromatin is characterized by H3K9 methylation and by the recruitment of Swi6 heterochromatin binding protein (Dohke, Miyazaki et al. 2008). The depletion of spCAF-1 causes defects in silencing at centromeric and mating loci heterochromatin, accompanied with a decrease in Swi6 recruitment. These defects likely reflect the ability of CAF-1 to deliver HP1 to foci of heterochromatin replication in S-phase. Dohke and colleagues (2008) proposed a model for the implication of CAF-1 in heterochromatin maintenance, similar to Quivy's one, suggesting an interaction among yeast HP1 (Swi6), p150 (Pcf1) and p60 (Pcf2), with consequent Swi6 delivery to H3K9 histones, in the wave of the moving fork (Figure n. 20).

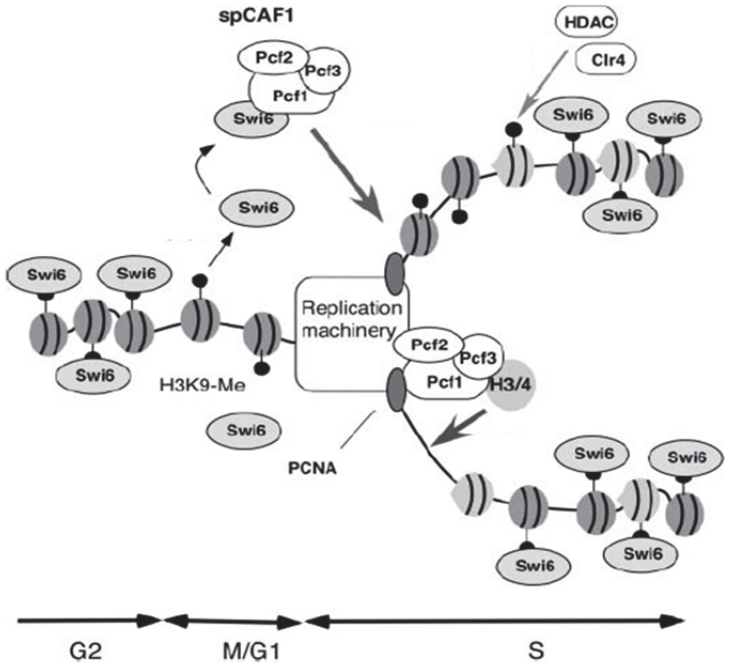


Figure n.20: Swi6 dynamics during heterochromatin replication, in fission yeast. The dissociation of Swi6 from heterochromatin during mitosis is induced by the phosphorylation of the serine 10 on histone H3. Swi6 does not dissociate from subtelomeric heterochromatin in G1/S, suggesting that the regulation of these regions is different from the centromeric heterochromatin. At the G1/S transition, CAF-1 interacts with released Swi6 and when heterochromatin undergoes replication early in S-phase, the complex CAF-1-Swi6 is recruited to replication forks through an interaction with PCNA. At this moment, Swi6 is transferred onto daughter strands that retain K9 methylation. Chromatin bound Swi6 might recruit, directly or indirectly, H3K9 specific histone methyl transferase, Clr4 and HDACs to modify the newly assembled chromatin. The interaction between Swi6 and Clr4 has never been reported but, in mammalian cells, HP1 was shown to interact with the human homologue of Clr4, SUV39H (Dohke, Miyazaki et al. 2008).

Epigenetic inheritance is necessary to ensure the proper genetic information and to maintain genome stability in daughter cells, after DNA replication. While the histone chaperone function of CAF-1 is conserved among all eukaryotes, CAF-1 appears to acquire novel important function in multicellular organisms. The three genes coding for the 3 subunits of CAF-1 are essential in multicellular eukaryotes including plants, mouse, chicken, *Xenopus, Drosophila* and human cells (Hoek and Stillman 2003; Loyola and Almouzni 2004; Houlard, Berlivet et al. 2006). In contrast, CAF-1 is not essential in unicellular eukaryotes (like yeasts). It is possible that CAF-1 acquired novel functions for the necessity of specific developmental programs and for the determination of different tissues. Chromatin-based inheritance of the epigenetic information is instrumental in development, when the genome information must be used selectively to shape a highly complex organism. An evidence to corroborate this hypothesis is that the absence of CAF-1 in *Arabidopsis* leads to defects in plant structures organization, sustaining a critical role in proliferating tissues (Endo, Ishikawa et al. 2006; Ramirez-Parra and Gutierrez 2007). Moreover, the depletion of CAF-1 in mouse embryos leads to developmental arrest concomitant with severe alterations in heterochromatin organization (Houlard, Berlivet et al. 2006). In *Drosophila,* CAF-1 is an essential gene

required to achieve a correct organism development, because homozygous mutants are unviable and heterozygous mutants show defects in repression of gene silencing at heterochromatic loci (Song, He et al. 2007). In human cells, CAF-1 depletion results in a severe loss of viability, defects in S-phase progression, accompanied by spontaneous DNA damage and activation of the checkpoint kinase Chk1. Similar phenotypes were reproduced in human cells by expressing a dominant negative form of p150 (Hoek and Stillman 2003; Loyola and Almouzni 2004). The accumulation of cells in early and mid S-phase could be caused by stalling of replication forks or by the activation of intra-S-phase checkpoint in response to chromatin defects, or because of specific defects in heterochromatin replication (Houlard, Berlivet et al. 2006).

In mammalian cells, CAF-1 loads H3.1 onto replicated DNA, whereas H3.3 replaces H3.1 in particular regions of the genome, such as transcribed genes, telomeric and centromeric heterochromatin (Mito, Henikoff et al. 2005; Jin, Zang et al. 2009; Wong, Ren et al. 2009; Ray-Gallet, Woolfe et al. 2011). In yeast, the discrimination between the two histone variants does not exist because only the histone H3.3 is present. Thus, there is no need for histone-substrate differentiation among the histone chaperones. The lack of histone variants in the yeast could be an evolutionary feature, due probably to the fact that most of the genome is transcribed (Hoek and Stillman 2003). The absence of substrate differentiation among the histone chaperones suggests that, at least in yeast, the deficiency in CAF-1 could be overwhelmed by other histone chaperones, such as HIRA and/or Rtt106. Therefore, yeast cells do not present any proliferation issue in the absence of CAF-1.

Despite differences between multicellular and unicellular eukaryotes, the function of CAF-1 and Asf1 in the maintenance of genome stability in unicellular eukaryotes is undeniable. In yeast, disruption of CAF-1 results in gene silencing defects at telomeres and at the mating type loci and these phenotypes are enhanced when Asf1 is deleted (Game and Kaufman 1999). Moreover, loss of CAF-1 and Asf1 genes, in conjunction with checkpoint genes such as *tel1, chk1* and *dpb11,* leads to an increase in gross chromosomal rearrangements (GCRs) and in mutation rate. These GCRs includes translocations and deletion of chromosome arms with associated *de novo* telomere addition (Myung, Pennaneach et al. 2003).

4. CHROMATIN RESTORATION DURING DNA REPAIR

4.1. The role of CAF-1 during DNA Repair

Beyond their role in replication-coupled nucleosome assembly during S-phase, histones chaperones such as Asf1 and CAF-1 are involved in the restoration of chromatin after DNA repair (Gaillard, Martini et al. 1996; Green and Almouzni 2003; Lewis, Karthikeyan et al. 2005; Schopf, Bregenhorn et al. 2012). During the process of chromatin assembly, CAF-1 deposits histones at sites of repair-associated DNA synthesis, therefore CAF-1 is a key protein for the maintenance of chromatin memory at sites of DNA repair (Green and Almouzni 2003; Ishii, Koshiyama et al. 2008). Moreover, recent evidences suggest that CAF-1 could have additional role during DNA repair and not be merely required to restore chromatin after the completion of DNA repair (Baldeyron, Soria et al. 2011). An interesting hypothesis is that CAF-1 could act firstly in the recruitment or in the regulation of DNA repair enzymes at sites of DNA repair. Subsequently, CAF-1 function could switch toward a histone chaperone mode (Ishii, Koshiyama et al. 2008; Baldeyron, Soria et al. 2011; Schopf, Bregenhorn et al. 2012). In this chapter, the role of histones chaperones in DNA repair and the DNA damage response are presented.

4.1.1. Role of CAF-1 during Nucleotide Excision Repair (NER)

Access to DNA lesions by the NER machinery required major remodeling of the chromatin structure (Smerdon and Lieberman 1978). After completion of DNA repair, the chromatin has to be faithfully reassembled to restore the preexisting epigenetic information. Impairment in chromatin restoration might result in loss of epigenetic information and thus genetic instability (Green and Almouzni 2003; Polo, Roche et al. 2006).

Human CAF-1 promotes nucleosome assembly on damaged templates, both *in vitro* and *in vivo* during NER (Gaillard, Martini et al. 1996; Martini, Roche et al. 1998; Mello, Sillje et al. 2002; Green and Almouzni 2003). *In vivo,* CAF-1 is locally recruited at sites of UV-induced DNA damages in a PCNA-dependent manner. Recruitment of both CAF-1 and PCNA occurs at damaged sites that have undergone the dual incision of the initial damaged lesion (Figure n.21) (Moggs, Grandi et al. 2000; Green and Almouzni 2003). This data suggest that CAF-1 acts at late stages during NER, to restore locally chromatin structure at damaged-sites. Chromatin restoration at repair sites might occur synergistically with Asf1, but Asf1 does not

appear to be recruited at DNA lesion sites *in vitro*, neither it is able to promote nucleosome assembly on its own on a damaged template (Mello, Sillje et al. 2002). Moreover, CAF-1 could also act as a trap for histones evicted during DNA repair, therefore providing a hub to maintain epigenetic information (Gaillard, Martini et al. 1996; Green and Almouzni 2003; Polo and Almouzni 2006). In support of the role of CAF-1 in chromatin restoration following NER, deletion of any of the three genes encoding CAF-1 subunits in *S. cerevisiae* and in *Arabidopsis* moderately increases sensitivity to UV irradiation (Kaufman, Kobayashi et al. 1997; Game and Kaufman 1999; Endo, Ishikawa et al. 2006; Kirik, Pecinka et al. 2006; Kim and Haber 2009).

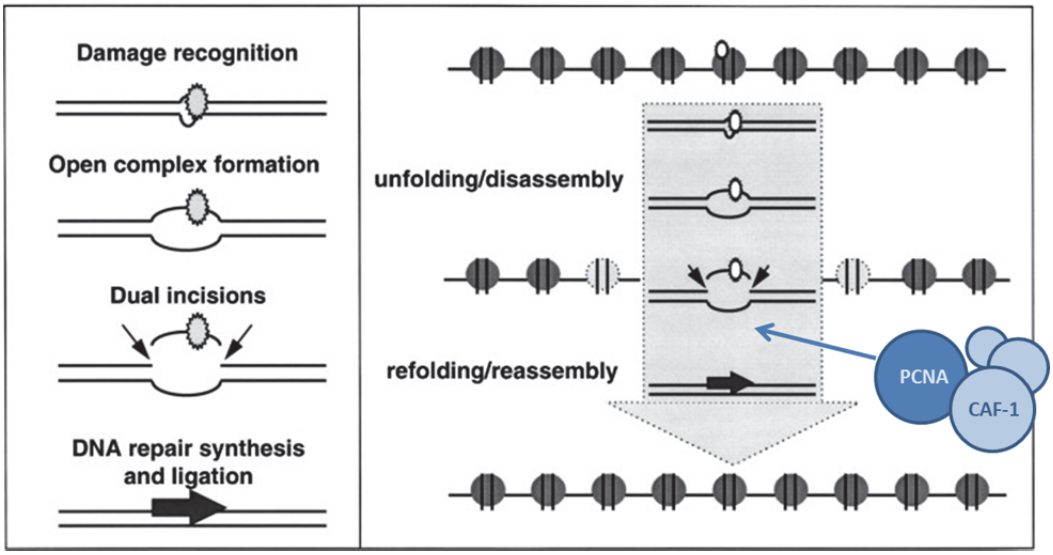


Figure n. 21: Chromatin remodeling during NER. After the DNA damage recognition, major remodeling of chromatin structure occurs, to allow the repair machinery to gain access to the DNA lesion. CAF-1 and PCNA are recruited at damaged sites that have undergone the dual incision stage of repair processing. After completion of DNA repair, CAF-1-dependent chromatin reassembly is essential to restore the preexisting epigenetic information (Adapted from (Moggs and Almouzni 1999).

4.1.2. Role of CAF-1 during Mismatch Repair (MMR)

During DNA replication, DNA polymerases can mis-incorporate dNTP, thus creating a mismatch in the nascent strand. Histone depositions occurs around 250 bp behind the DNA synthesis point, in the rear of the moving fork. Therefore, the time window to correct potential mismatches is reduced and MMR pathway has to be tightly coordinated with chromatin assembly (Sogo, Stahl et al. 1986).

The interplay between MMR and chromatin assembly seems to be regulated by the interaction between MutSα and p150 (Wilson, Carney et al. 1998; Schopf, Bregenhorn et al. 2012). MutSα is a heterodimer composed of MSH2 and MSH6 that act as a sliding clamp to initiate Exo1-dependent degradation of the error-containing DNA strand (Wilson, Carney et al. 1998; Genschel, Bazemore et al. 2002; Wei, Clark et al. 2003). The crosstalk between MMR pathway and nucleosome assembly through CAF-1-MutSα interaction might imply a decreased affinity of CAF-1 to bind histones, or potential interference in the ability of CAF-1 to associate with the fork. Therefore, chromatin assembly would be delayed giving time to MMR to repair mismatches. Once the mismatch is repaired, MutSα no longer binds to DNA and CAF-1 can restore the chromatin organization (Kadyrova, Blanko et al. 2011; Schopf, Bregenhorn et al. 2012). These data support a dual role for CAF-1 during MMR. Indeed, CAF-1 has an early role as MMR regulator, via its interaction with MutSα and a late role as histone chaperone for replication-coupled nucleosome deposition.

4.1.3. Role of CAF-1 during Double Strand Break (DSB) repair

CAF-1 appears to have a role in DSB repair, both in non-homologous end-joining (NHEJ) and homologous recombination. While the impacts of CAF-1 defects on NEHJ are rather indirect, some data point out a direct role for CAF-1 during DSB repair by HR (Lewis, Karthikeyan et al. 2005; Nabatiyan, Szuts et al. 2006; Ramirez-Parra and Gutierrez 2007).

In *S. cerevisiae,* inactivation of both CAF-1 and Asf1 reduces NHEJ efficiency and accuracy. However, Lewis and colleagues demonstrated that the effects on NHEJ, in CAF-1 depleted strains, are an indirect consequence of derepression of silencing at the mating locus (Lewis, Karthikeyan et al. 2005). Inactivation of both CAF-1 and Asf1 results in a 10-fold decrease in DSB-induced plasmid:chromosome recombination, and ionizing radiation-induced loss of heterozygosity was abolished in the mutants (Lewis, Karthikeyan et al. 2005). In *Drosophila*, the deletion of the largest subunit of CAF-1 (p180) affects the efficiency of gap repair by HR *in vivo* (Song, He et al. 2007). Gap repair consists in the repair of a chromosomal DSB, using a non-chromosomal homologous template. Song and colleagues demonstrated that the efficiency of the gap repair is reduced by 2.5 fold in the absence of the largest subunit of CAF-1, though not completely abrogated. Altogether, these data suggest a role for CAF-1 in DNA repair by HR and that this function is conserved. Nonetheless, the exact function of CAF-1 during the different steps of HR remains unclear.

Further evidences involving a direct role of CAF-1 during DSB repair by HR were then highlighted. In human cells, a rapid and transient accumulation of all HP1 isoforms occurs at laser-induced DNA damage, both within euchromatin and heterochromatin, only 5 minutes after damage induction (Ayoub, Jeyasekharan et al. 2009; Luijsterburg, Dinant et al. 2009; Zarebski, Wiernasz et al. 2009; Baldeyron, Soria et al. 2011). Baldeyron and colleagues focused on HP1α and they found that HP1α recruitment at DNA damage is rapid and transient. Importantly, HP1α recruitment depends on its ability to interact with p150 of CAF-1, but independently of heterochromatin marks. They also investigated KAP-1 dynamics at DNA lesions. KAP-1 is the transcriptional co-repressor KRAB-associated protein 1 and was found in complexes with HP1 (Ryan, Schultz et al. 1999). Deletion of p150 impaired both HP1α and KAP-1 accumulation at damage sites while depletion of HP1α or KAP-1 reciprocally impaired their accumulation at laser-induced DNA lesions, without affecting p150 recruitment. Interestingly, HP1α and KAP-1 accumulation was not impaired by p60 deletion, showing that only p150, but not the function of CAF-1 in chromatin assembly, has a role in DNA repair (Baldeyron, Soria et al. 2011).

Depletion of either p150 or HP1α results in impaired recruitment of DSB repair proteins, including 53BP1, BRCA1 and Rad51 at laser-induced DNA lesions. While the depletion of HP1α leads to a clear defect in DSB repair by HR, depletion of p150 has only a modest effect on HR-mediated repair (Baldeyron, Soria et al. 2011). The impact of p150 depletion on HR might be hidden by the fact that inactivation of p150 results in accumulation of cells in S-phase. Importantly, phosphorylation of RPA2, a single strand binding protein, was reduced when HP1α is depleted, as it is also observed in the absence of CtIP, a nuclease involved in end-resection. One possible explanation is that HP1α, together with p150, promote homologous recombination by stimulating the early step of end-resection necessary for Rad51 nucleation onto ssDNA. Considering that the recruitment of BRCA1, a CtIP partner, is affected in the absence of HP1α, the latter might have a role in promoting CtIP recruitment at damaged sites.

The p150 subunit of CAF-1 stays longer than HP1α and KAP-1 at damaged sites and a possible explanation is that the function of p150 switches toward an active histone chaperone mode, as part of the CAF-1 complex. By doing so, CAF-1 might ensure the restoration of chromatin organization after completion of DNA repair (Green and Almouzni 2003; Polo and Almouzni 2006; Polo and Almouzni 2007; Baldeyron, Soria et al. 2011). These results

suggest a dual role for CAF-1 in HR: early function in the process of DNA repair, and a later function as histone chaperone.

Interestingly, direct interactions between CAF-1 and the meiotic recombinase Lim15/Dmc1 have been reported (Ishii, Koshiyama et al. 2008). Programmed DSBs occur in meiosis to promote crossover events, leading to genetic variability among organisms. Chromatin organization following meiotic recombination is critical to ensure the correct segregation of homologous chromosomes into gametes and an involvement of histone chaperones in these processes is conceivable. Meiotic recombination between homologue chromosomes requires a meiosis-specific recombinase, Dmc1, in addition to Rad51. In the basidiomycete *C. cinerea,* after the formation of meiotic DSB, Lim15/Dmc1 coats ssDNA to perform strand invasion (Namekawa, Iwabata et al. 2005; Neale and Keeney 2006). After the strand invasion with the homologue chromosome, PCNA and DNA polymerases are recruited to ensure extension of the invading strand on the homologous template, allowing the pathway to progress toward chromatin assembly (Figure n.22) (Sakaguchi and Lu 1982; Sawado and Sakaguchi 1997; Namekawa, Hamada et al. 2003; Namekawa, Hamada et al. 2003). A physical and direct interaction between the largest subunit of CAF-1 and the meiosis-specific recombinase protein Lim15/Dmc1 has been reported (Ishii, Koshiyama et al. 2008). Despite the lack of evidences for the function of this interaction, it was proposed that CAF-1 is recruited during extension of the D-loop to promote histones deposition (Figure n.22). This late role could allow chromatin assembly following meiotic recombination, contributing toward the establishment of the memory of recombination events (Ishii, Koshiyama et al. 2008).

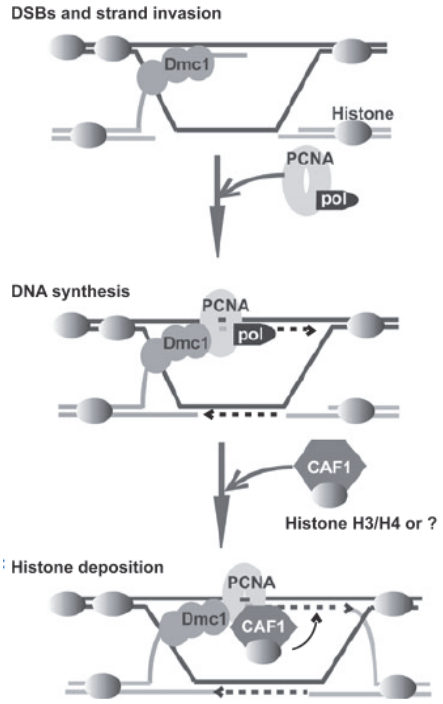


Figure n. 22 – Model of interaction between CAF-1 and the recombinase Lim15/Dmc1. After DSB formation, Lim15/Dmc1 coats single strand end, during strand invasion. PCNA recruits the DNA polymerase to the site of Lim15/Dmc1. CAF-1 forms a complex with Lim15/Dmc1 and PCNA and this is necessary to deposits histones H3 and H4 or other factors, such as histone variants on the newly synthesized DNA (Ishii, Koshiyama et al. 2008).

4.2. The role of histone chaperones in the DNA damage response

Genome integrity is constantly threatened by exogenous and endogenous agents; therefore it is essential for cells to detect DNA lesions and to coordinate DNA damage response with cell cycle progression (Foiani, Ferrari et al. 1998; Lambert and Carr 2005; Lambert, Froget et al. 2007; Branzei and Foiani 2009; Ciccia and Elledge 2010). The signalization cascade that leads to cell cycle arrest is called DNA Damage Response (DDR) and it consists of three main steps: a) sensing the presence of DNA damage by "sensor proteins" b) signaling to "transducer proteins" that amplify the signal c) activation of "effector checkpoint kinases", responsible for regulation of cell cycle progression, transcription, cell death, DNA repair. Checkpoint pathways are essentials to arrest cell cycle progression, providing additional time for DNA repair and DNA replication completion, contributing to genome integrity (Qin and Li 2003; Lambert and Carr 2005; Lambert, Froget et al. 2007).

Once DNA damages are repaired, the checkpoint has to be turned OFF (checkpoint recovery) for cells resuming cell cycle progression. Completion of DNA repair implies not only the successful repair of initial DNA lesions, but also the restoration of the chromatin structure. Importantly, chromatin restoration has been shown to be crucial to turn-off checkpoint signaling (Tyler, Adams et al. 1999). The restoration of chromatin implies: a) nucleosome reassembly involving either parental histones recycling or *de novo* histones deposition and b) restoration of locus-specific chromatin-structure, including epigenetic marks. Defects in epigenetic reinstatement at post-repair sites may contribute to epigenetic instability, leading to loss of epigenetic information and increased genetic instability (Koundrioukoff, Polo et al. 2004).

In *S. cerevisiae,* Asf1 might play a direct role in the inactivation of the DDR via its dynamic physical interaction with Rad53 (Emili, Schieltz et al. 2001; Hu, Alcasabas et al. 2001). The checkpoint kinase Rad53 is part of the DDR but also prevents the accumulation of free histones in the cell, as a surveillance mechanism (Gunjan and Verreault 2003). The inactive form of Rad53 binds to Asf1 but upon phosphorylation of Rad53, Asf1 is released and cell cycle is arrested (Emili, Schieltz et al. 2001; Hu, Alcasabas et al. 2001; Schwartz, Lee et al. 2003). Thus, the binding of Asf1 to Rad53 might be a signal for the checkpoint recovery (Figure n. 23).

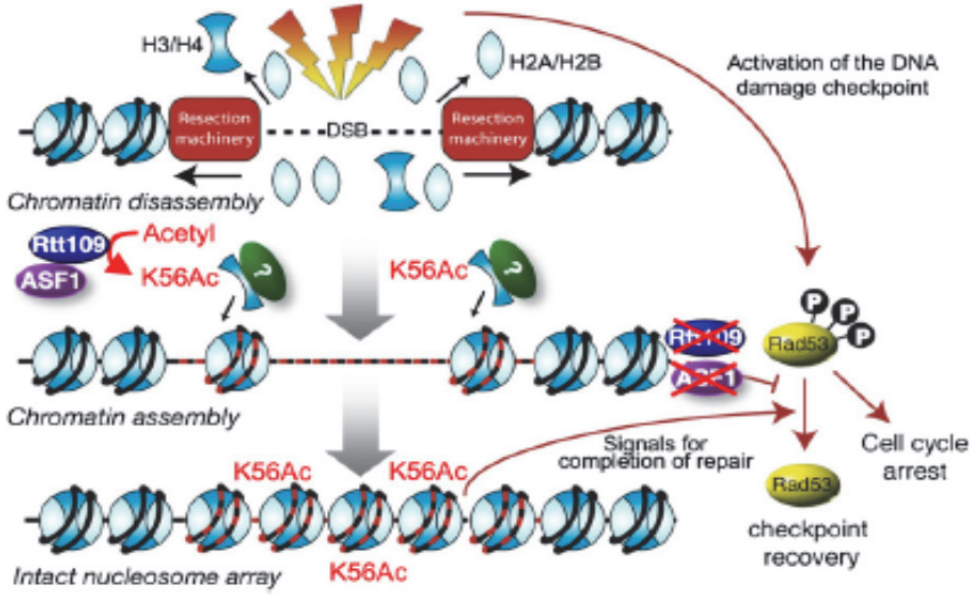


Figure n. 23: Model for the role of chromatin restoration in deactivation of the DNA-damage checkpoint. During DSB response, the DNA damage checkpoint is activated and the chromatin is disassembled. Although chromatin remodeling is required for recruitment of the resection machinery, DNA resection itself is sufficient to disassemble the chromatin in the vicinity of a DSB. Inactive Rad53 is bound to Asf1 and upon checkpoint activation Rad53 becomes hyperphosphorylated, releasing Asf1. Asf1 promotes chromatin assembly indirectly, through the acetylation of H3 on K56, via Rtt109. K56 acetylation drives chromatin restoration after DSB and this is a signal for the completion of the repair. The mechanism is not understood, but it has been demonstrated that H3K56 is sufficient to turn off the DNA damage checkpoint, even in absence of Asf1 (Chen, Carson et al. 2008).

Kim and colleagues demonstrated that CAF-1 and Asf1 cooperatively restore chromatin at DNA repair sites and mediate efficient deactivation of the checkpoint, especially upon robust checkpoint activation (Kim and Haber 2009). The double mutant *asf1-d cac1-d* presents a defect in checkpoint recovery, while neither *asf1-d* nor *cac1-d* single mutant exhibits this phenotype. These data are not in agreement with those presented by Chen and colleagues, who found that the single deletion of *asf1* was sufficient to impair checkpoint recovery (Chen, Carson et al. 2008).

Checkpoint deactivation following chromatin restoration requires the histone mark H3K56Ac. (Masumoto, Hawke et al. 2005; Collins, Miller et al. 2007; Driscoll, Hudson et al. 2007; Han, Zhou et al. 2007; Tsubota, Berndsen et al. 2007; Wurtele, Kaiser et al. 2012). Asf1-dependent H3K56Ac occurs also outside of S-phase, suggesting that this histone mark may function in replication-independent chromatin assembly pathways, for example during DDR (Rufiange, Jacques et al. 2007; Li, Zhou et al. 2008). Although cells deficient for Asf1 are able to efficiently repair DNA lesions caused by DNA damaging agents, they are highly sensitive to

them. This defect in chromatin reassembly is correlated with a defect in the ability of cells to re-enter the cell cycle (Tyler, Adams et al. 1999; Wurtele, Kaiser et al. 2012). Cells lacking Asf1 perform normal resection and DNA repair, but their ability to reassemble chromatin at the site of DNA damage, after DNA repair is completed, is severely impaired (Tyler, Adams et al. 1999; Chen, Carson et al. 2008; Wurtele, Kaiser et al. 2012). Nevertheless, Asf1 is not the histone chaperone that directly deposits histones on repaired DNA but it promotes cell resistance to DNA damaging-agents by promoting H3K56Ac via Rtt109. Indeed, the presence of the histone mark H3K56Ac onto repaired chromatin is proposed to be the signal of repair completion and thus checkpoint recovery (Chen, Carson et al. 2008). Importantly, in the absence of Asf1, no histones were detected at site of DNA repair after repair of the DSB. Furthermore, the deletion of Rtt109 leads to similar phenotype that loss of Asf1, in term of checkpoint recovery. Mimicking the histone mark H3K56Ac (K56Q) bypasses the requirement for Asf1 in turning off the checkpoint, whereas mutations that prevent K56 acetylation (K56R) blocks chromatin reassembly after DSB-repair (Recht, Tsubota et al. 2006; Xhemalce, Miller et al. 2007; Chen, Carson et al. 2008). In support for a role of H3K56Ac in promoting checkpoint recovery, the deacetylase Hst3 is transcriptionally repressed and degraded by the DNA damage checkpoint (Maas, Miller et al. 2006; Thaminy, Newcomb et al. 2007). Consistent with this, H3K56Ac is massively present in the chromatin fractions undergoing DNA repair (Masumoto, Hawke et al. 2005).

The mechanism by which H3K56Ac-dependent chromatin assembly leads to checkpoint deactivation remains unclear. However, it seems likely that DNA integrity or cell cycle progression could be monitored by a mark at the chromatin level (Chen, Carson et al. 2008; Fillingham and Greenblatt 2008).

5. OBJECTIVES: Investigating the role of CAF-1 in replication-induced recombination

Histones chaperones, such as CAF-1 and Asf1, appear to have a significant influence on homologous recombination pathways. In one hand, defects in replication-coupled chromatin assembly drives genome instability, especially at replication forks, thus creating a need for homologous recombination pathway to restart/repair stalled forks. This is likely to result in a hyper-rec phenotype. In contrast, analyzing DNA repair following induction of specific DNA lesions, such as DSBs, reveals a potential direct role, at least for CAF-1, in regulating HR.

The objectives of my PhD project were to investigate the role of CAF-1 in the regulation of replication-induced recombination in the fission yeast *Schizosaccharomyces pombe*. In addition to classical recombination assays available in fission yeast, I made use of conditional replication fork barriers. These genetic assays allow the arrest of a single replication fork at a specific locus to be induced. Fork arrest induces recombination events linked to the restart of the collapsed fork by homologous recombination. Importantly, these conditional fork arrest substrates allow the visualization of joints molecules (recombination intermediates) and the products of their resolution, thus permitting to investigate the role of various factors in the regulation of homologous recombination at a molecular level.

5.1. The fission yeast Schizosaccharomyces pombe as a model organism

The model organism *Schizosaccharomyces pombe* (fission yeast), commonly used in biology, derives from a strain isolated in 1921 in Switzerland, by Osterwalder. The same strain was isolated in 1893 by Lindner from East African millet beer. The fission yeast name "*Schizosaccharomyces"* is derived from the latin "saccharum" which means sugar and from the greek "mukes" which means mushroom. Lindner specified the name of the species which is the Swahili word for beer (*pombe*) (Figure n. 24A).

The *S. pombe* strain isolated by Osterwalder was firstly used as model organism in the 1950s, by Leupold for genetic studies and by Mitchison for cell cycle studies. Fission yeast cells present features that are common to multi-cellular organisms included conserved cellular pathways. Moreover, the rapidity of the cell cycle and the lack of pathogenicity mark the fission yeast as an ideal model organism (Yanagida 2002).

S. pombe is a unicellular eukaryote with rod-shaped cells of 3.5 micrometers in diameter and 7-15 micrometers in length (Figure n. 24B). The name fission yeast indicates that cells divide

by medial fission producing a septum. The cells maintain their shape during cell cycle and they grow only to the tips, dividing by medial fission and producing daughter cells of the same size.

A

Schizosaccharomyces pombe	
Biological classification (taxonomic hierarchy)	
Kingdom:	Fungi
Phylum:	Ascomycota
Subphylum:	Taphrinomycotina
Class:	Schizosaccharomycetes
Order:	Schizosaccharomycetales
Family:	Schizosaccharomycetaceae
Genus:	Schizosaccharomyces
Species:	S. pombe

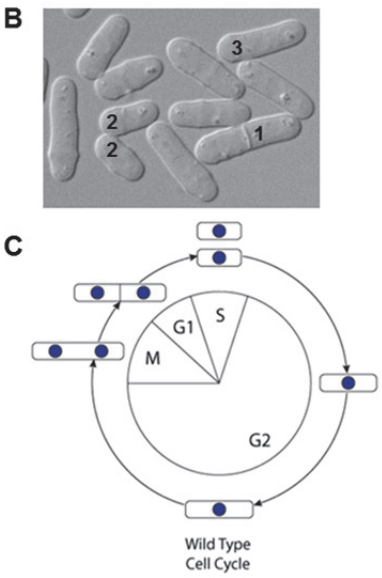


Figure n. 24: Features of the model organism *S. pombe.*
A) Biologic classification of the model organism *S. pombe*
B) Rod- shaped cell. In the picture, it is possible to notice cells in different phases of the cell cycle: 1.S-phase cell 2.late S-phase cell 3.G2-phase.
C) Cell cycle of *S. pombe*: the 80% of the time yeasts cell are in G2 phase while M, G1 and S-phase are rapid.

The cell cycle of the fission yeast is prevalently haploid. Cells of opposite mating type (h+ or h-) undergo conjugation, induced by nitrogen starvation. The conjugation of the two cells leads to unstable diploid zygote that rapidly undergoes into meiosis to generate four haploid spores (tetrad). These four spores, in favorable conditions, can proliferate to form haploid colonies. The mating type of *S. pombe* can be divided in two categories: homothallic strains (h^{90}) that switch mating type and thus undergo conjugation among them, leading to tetrads formation; heterothallic strains (either h+ or h-) that are stab le in their mating type and thus undergo conjugation when h+ cells mate h- cells.

The cell cycle of *S. pombe* is rapid, around 3 hours, and varies according to culture media and temperature. The mitotic phase (M-phase) is constituted of chromosome segregation (nuclear division) and cytokinesis (cell division). A characteristic of yeast models is that nuclear envelope does not break down during mitosis, and proteins responsible for microtubule organization remain associated with the nuclear membrane. The G1 phase follows M-phase and is extremely fast in *S. pombe*, thus mitosis is rapidly followed by the entry into S-phase, while cytokinesis is not yet completed. Because of this, cells in S-phase are easily recognized in an asynchronous population, as cells having a septum (septated cells). Subsequent to S-phase, the G2 phase is very long and it occupies 80% of the cell cycle timing (Figure n. 24C).

The genome of *S. pombe* was sequenced and published in 2002 by a consortium led by the Sanger Institute (http://old.genedb.org/genedb/pombe/) (Wood, Gwilliam et al. 2002). The entire genome size is approximately of 14.1 Mbp divided in three chromosomes: chromosome I of 5.7 Mb, chromosome II of 4.6 Mb and chromosome III of 3.5 Mb. Chromosomes present a classical structure with a large centromere constituted of repeated sequences, and telomeres. Importantly, the extremities of the chromosome III contain repeated transcription units of ribosomal RNA (rDNA) and are thus variable in size from one strain to another one. In addition to the three linear chromosomes, there is also a circular mitochondrial chromosome of 20 Kbp. About 60% of the fission yeast genome codes for proteins (4.970 protein-coding genes). The genome contains also around 450 non-coding RNAs. The gene density decrease in telomeric regions and genes are absent in centromeric regions.

The fission yeast CAF-1 complex is composed of three subunits encoded by the folloing genes:

- *pcf1* (SPBC29A10.03c), on chromosome II, codes for the human homologue p150. It is an intron-free gene of 1.635 bp. The Pcf1 protein is composed of 544 amino acids with a mass of 62.2 kDa and an isoelectric point at pH 4.6.
- *pcf2* (SPAC26H5.03), on chromosome I, codes for the human homologue p60. It is a gene containing 3 introns and the unspliced RNA length is of 1.740 bp, while the mRNA is of 1.539 bp. The Pcf2 protein is constituted of 512 amino acids with a mass of 57.4 kDa and an isoelectric point at pH 6.5.
- *pcf3* (SPAC25H1.06), on chromosome I, codes for the human homologue p48 homologue. It is a intron-free gene of 1.227 bp. The Pcf3 protein is composed of 408 amino acid, with a mass of 46.7 kDa and an isoelectric point at pH 6.5.

5.2. The RTS1-Replication Fork Barrier in S. pombe

The conditional replication fork barrier *RTS1* is a polar RFB mediated by a DNA-bound protein. The binding of the Rtf1 protein to the *RTS1* sequence causes the polar arrest of replication forks. The *RTS1*-RFB is naturally located closed to the *mat* locus on the chromosome II. Mating type switching requires the *mat* locus to be replicated in a unidirectional manner, by fork progressing from the telomere toward the centromere. By blocking replication forks progressing in the opposite direction (from telomere towards the centromere), the *RTS1*-RFB allows the *mat* locus to be replicated in a unidirectional manner.

Thus, the function of the *RTS1*-RFB is to coordinate replication dynamic at the *mat* locus, to ensure efficient mating type switching (Dalgaard and Klar 2001; Codlin and Dalgaard 2003).

The RTS1 sequence if of 859 bp and is constituted of two regions:

- The region B of 450 bp is essential for the activity of the RFB. It contains four repetitions of a 55 bp motif binds by the Rtf1 protein. The current view is that Rtf1 binds to *RTS1* and by interacting with additional proteins, as Rtf2, mediates polar fork arrest
- The region A of about 60 bp, rich in purine, which is thought to reinforce the RFB activity of the region B. The proteins Swi1, Swi3 and Rtf2 can bind the region A (Dalgaard and Klar 2000; Codlin and Dalgaard 2003; Lee, Grewal et al. 2004).

The *RTS1*-RFB causes fork arrest because of a non-histone protein complex binding to the DNA. As proposed for other polar RFBs, the *RTS1*-RFB is thought to block fork progression by directly (contact between proteins and the replisome) or indirectly (topological constraint) affecting the replicative helicase activity and thereby preventing DNA unwinding (Eydmann, Sommariva et al. 2008; Kaplan and Bastia 2009).

Lambert and colleagues exploited the *RTS1*-RFB to design conditional fork arrest substrates in order to decipher the impact of impediments to fork progression on genome stability and molecular mechanisms of fork-restart by homologous recombination (Figure n. 25A). It was initially established that fork arrested by the *RTS1*-barrier are hot spots of recombination, and of chromosomal rearrangements and that the HR pathway, but not the checkpoint pathway, is required to ensure full cell viability upon induction of the *RTS1*-RFB (Lambert, Watson et al. 2005). Importantly, it was then established that HR promotes efficient recovery of fork arrested at the *RTS1*-barrier by a DSB-independent mechanism (Mizuno, Lambert et al. 2009; Lambert, Mizuno et al. 2010). The polar *RTS1* barrier was introduced on the centromere-proximal (*cen*-proximal) side of the *ura4* locus, 5 kb away from the strong replication origin (ori) 3006/7 on chromosome III. This created the *uraR* locus (Figure n. 25A). The activity of the polar *RTS1*-barrier is dependent on its interaction with the protein Rtf1 which the expression is controlled by the presence or the absence of thiamine in the media (Figure n. 25B). Indeed, the Rtf1 promoter was merged with the thiamine-repressible promoter *nmt41*. Thereby, in the presence of thiamine, the *nmt41* promoter is repressed, Rtf1 is not expressed and the *RTS1* barrier is inactive (defined as “OFF” conditions). In contrast, in the absence of thiamine, the *nmt41* promoter is induced, Rtf1 protein is expressed and the barrier is active (defined as “ON” conditions). One *RTS1* barrier is bound by several Rtf1 proteins in association with protein-protein interactions. Efficient induction of Rtf1 expression requires

incubation for 12-16 hours in thiamine-free media. By analyzing replication intermediates by native 2-dimensional gel electrophoresis (2DGE), it was reported that more than 95 % of forks moving from ori 3006/7 toward the telomere (*tel*) are efficiently blocked by the *RTS1*-RFB at the *uraR* locus. Arrested forks were not detected without Rtf1 induction (Figure n. 25B-D).

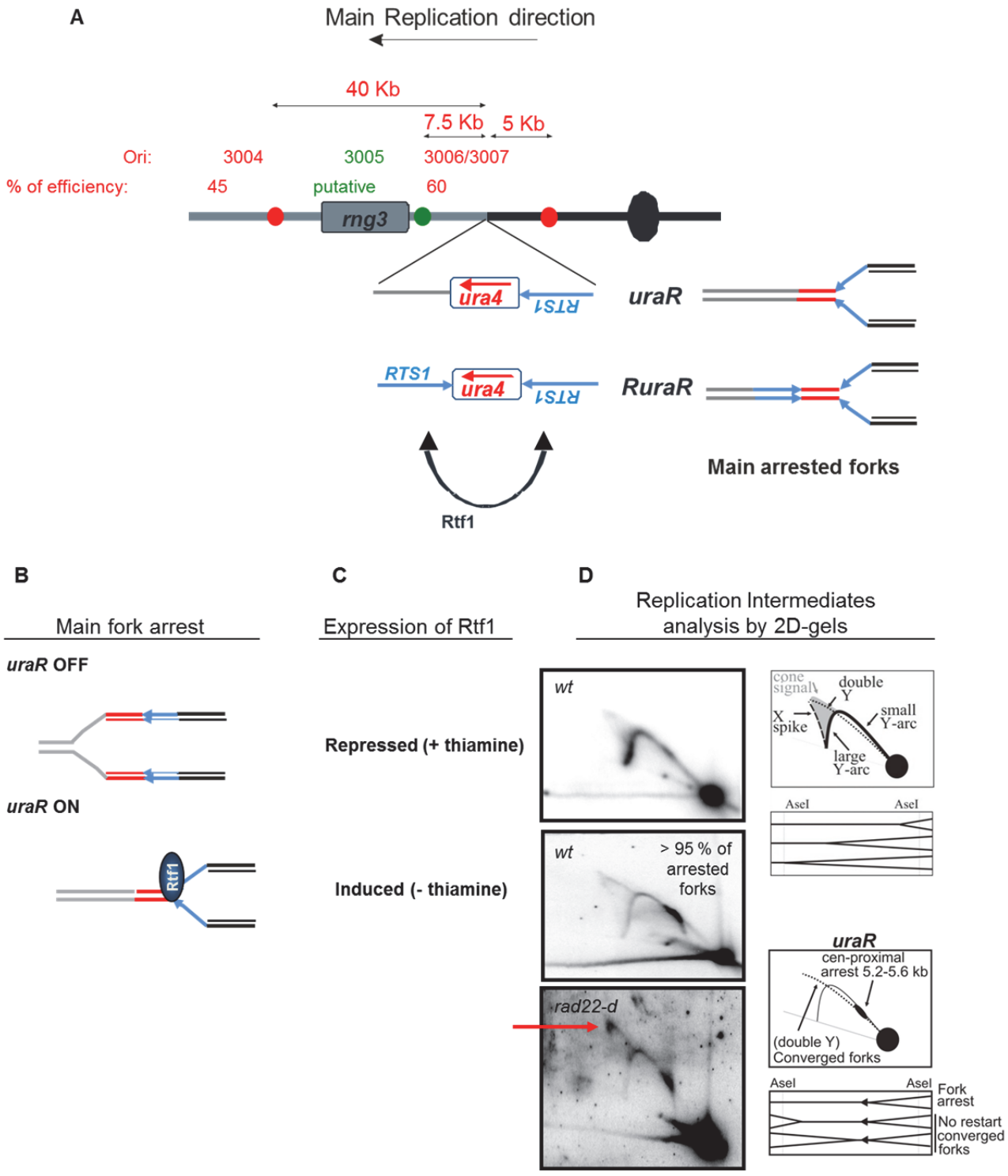


Figure n.25: An inducible site-specific fork arrest assay in *S. pombe*. A) *RTS1*-RFB sequences are integrated at one side or both sides of *ura4* gene, blocking the incoming replication fork. *Centromere*-proximal and *telomere*-proximal regions are represented in black and grey, respectively. Strong or putative replication origins (ori) and the centromere are indicated by red, green and black circles, respectively. Blue arrows indicate the polarity of the *RTS1*-RFB. The transcription direction of *ura4*$^{+}$ gene is indicated by a red arrow. On the bottom, representations of the primary arrested fork structure are given for each construct (Lambert, Watson et al., 2005). B) Scheme of fork arrest substrate. C) Condition of Rtf1 expression. D) left panels: analysis of replication intermediates by 2DGE in *wt* strain in OFF (Rtf1 being repressed) and ON (Rtf1 being expressed) conditions, and in *rad22-d* strain (ON condition). The red arrow indicates accumulation of termination signal, a sign of defective fork-restart in the absence of homologous recombination. Right panels: scheme of replication intermediates observed in *uraR* strains within the AseI restriction fragment (Lambert, Mizuno et al. 2010).

In a second construct, a second *RTS1*-barrier was introduced on the tel-proximal side of *ura4*, such that the two *RTS1* sequences are in inverted repeats. This created the *RuraR* locus in which block of converging forks can virtually occur (Figure n. 25A). However, 2D gel analysis showed that forks arrested on the *cen*-proximal side of *ura4* were efficiently recovered by recombination before forks are arrested on the *tel*-proximal side. Indeed, Joint molecules (JMs) resulting from recombination between *RTS1* repeats were detected by 2D gel (Figure n. 26). Resolution of these JMs gives rise to chromosomal rearrangements.

Recovery of the arrested fork occurs by a DSB-independent mechanism and involves the recruitment of recombination proteins at the *RTS1*-RFB site (Figure n. 26A). The causative protein barrier then has to be removed either by DNA helicase or by the recombination machinery itself, to allow fork-progression to resume. Based on 2D gel and genetic analysis, it was proposed that recombination proteins associate with unwound nascent strands that then anneal with the initial template to allow DNA synthesis to restart (Figure n. 26 A, left panel). Occasionally, the unwound nascent strand can mistakenly anneal with a homologous template in close proximity to the collapsed fork (the second *RTS1* sequence in the *RuraR* construct), resulting in the restart of DNA synthesis on non-contiguous template (Figure n. 26A, right panel).

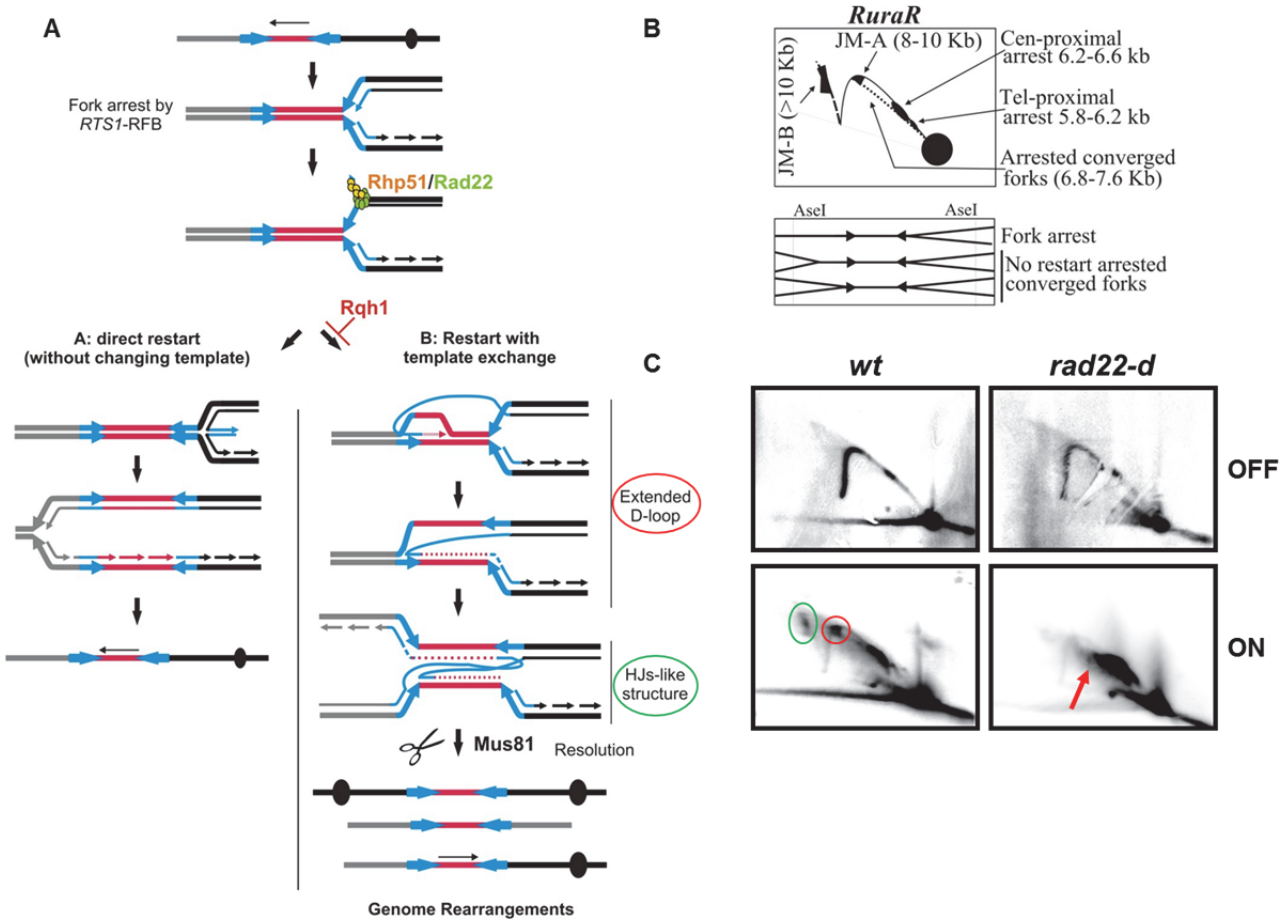


Figure n.26 – Model of fork restart by recombination. A) Blue arrows indicate *RTS1*-RFB and its polarity. The *ura4* gene is indicated in red and black arrow indicates its transcription orientation. *Centromere*-proximal and *telomere*-proximal regions are represented in black and grey, respectively. Centromere is represented by a black circle. The model propose that unwinding of stalled nascent strands allows the recruitment of Rhp51 (yellow circle) and Rad22 (green circle). In the *RuraR* system, two mechanisms have been reported: the restart occurs either on the initial template (A, direct restart, left panel) or by template exchange of stalled nascent strands thus allowing resumption of DNA synthesis on a non-contiguous template (B, right panel). Template exchange between the two *RTS1* repeats result in formation of joint-molecules visualized by 2DGE and whose resolution leads to chromosomal rearrangements (switch of *ura4* orientation, acentric and dicentric iso-chromosome). B) Scheme of replication intermediates and joint-molecules observed by 2DGE in the *RuraR* strain within the AseI restriction fragment. C) 2DGE in indicated strains and conditions. Red circle indicates joint-molecules that are proposed to be extended D-loop and green circle indicates joint-molecules that are proposed to be Holliday-junctions-like structures (HJs-like) (Lambert, Mizuno et al. 2010).

During template exchange reaction, the incorrect template switch of nascent strands results in formation of JMs detected by 2D gel and which resolution results in inversions and iso-acentric and dicentric chromosomes in ~2- 3 % of cells/generation (Figure n. 26B-C, and 27). In the absence of homologous recombination (*i.e.* in a *rad22-d* mutant), JMs were not detected and termination signals accumulated (Figure n. 26C). Similarly, termination signals

accumulated in the *rad22-d uraR strain* (Figure n. 25 D). Therefore, accumulation of termination signal is interpreted as sign of defect in restarting forks at the *RTS1*-barrier.

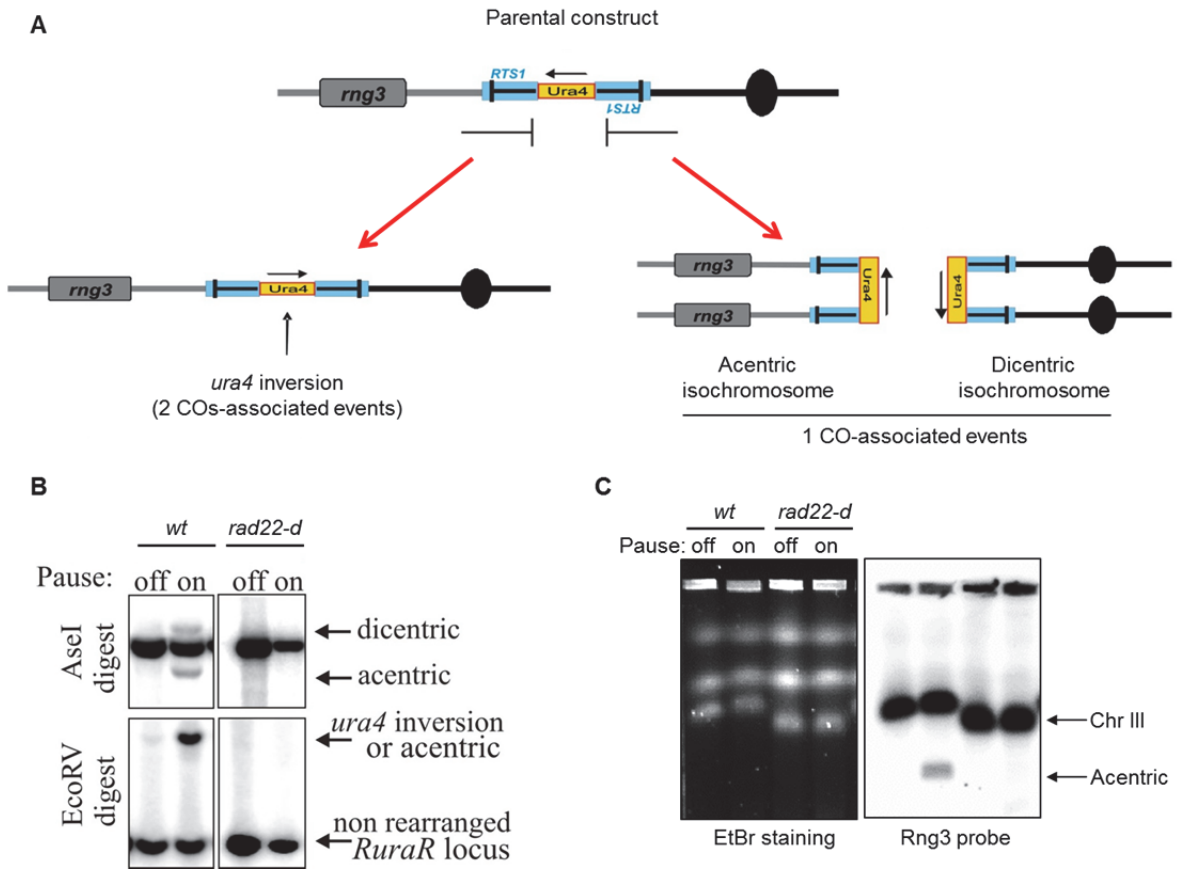


Figure n.27: Chromosomal rearrangements accumulate upon fork arrest within inverted repeats. A) Scheme of the *RuraR* parental construct. Blue squares with black lines inside represent *RTS1* sequences and their polarity. *Centromere*-proximal and *telomere*-proximal regions are represented in black and grey, respectively. The centromere is indicated by a black circle. The transcription direction of *ura4*$^{+}$ gene is indicated by a black arrow. Fork arrest at *RTS1*-RFB within inverted repeats results in chromosomal rearrangements including the switch of *ura4* orientation (resolution of HJ-like structure in one crossing over direction), and acentric and dicentric iso-chromosomes (resolution of HJ-like structure in two crossing over direction) (Lambert, Watson et al. 2005; Lambert, Mizuno et al. 2010). B) Restriction Fragment Length Analysis (RFLA) of the *RuraR* locus in indicated strain and conditions. *wt* and *rad22-d* strains. Note that no chromosomal rearrangements are observed in the *rad22-d* strain (Lambert, Mizuno et al. 2010). C) Pulse Field Gel Analysis (PFGE) followed by Southern-blot of chromosome III in indicated strains harboring the *RuraR* construct and in indicated conditions.

MATERIALS AND METHODS

1. GENETIC AND CELL BIOLOGY TECHNIQUES

1.1. Growth media

Amino acids and bases: adenine hydrochloride (≥ 99 %, Sigma-Aldrich), uracil (≥ 99 %, Sigma-Aldrich), L-leucin (≥ 98 %, Sigma-Aldrich), L-arginin monohydrochloride (≥ 98 %, Sigma-Aldrich) and L-histidin monohydrochloride monohydrate (≥ 98 %, Sigma-Aldrich): stock solution at 1.25 %, used at 0.0125% in media.

Media:

a) EMM glutamate (EMM-Glu): Edinburgh minimal medium (MP Biomedicals), Glutamate 27.14 g/L.

b) Yeast Extract (YE): yeast extract (DIFCO)

Solid media contain bacto agar (BD) 20 g/L.

Culture media, growth conditions and basis protocols of fission yeast manipulation are detailed in the "*Fission yeast Handbook*" written by Paul Nurse (http://biosci.osu.edu/~nile/nurse_lab_manual.pdf). Fission yeast cultures realized in liquid media were performed in *Yeast Extract* (YE) (BD Biosciences) a rich medium containing 2% final concentration of glucose or *Edinburgh Minimum Media L-Glutamate* (EMM-Glu) (MP Biomedicals), a minimal medium in which the nitrogen source is glutamate and not ammonium chloride (as for others common minimal media). Liquid culture media were supplemented in amino acids, necessary for strains growth depending on their genotype (adenine, leucine, histidine, arginine and uracil at 12.5 mg/mL - Sigma).

For fission yeast growth on solid media, 2% of agar (BD Biosciences) was added. Antibiotic selection was realized on solid media by adding:

a) 200mg/L of geneticine (G418) (Invitrogen) for Kan (kanamycin) resistant strains,
b) 200mg/L of nourseothricin (Werner Bioagents) for Nat resistant strains
c) 200mg/L of hygromycin (Invitrogen) for Hyg resistant strains.

Regarding strains harbouring the fork arrest system at *ura4* locus, the activity of the replication fork barrier is regulated by the conditional addition of thiamine. The expression of Rtf1 protein is under the control of the inducible *nmt41* promoter, which was repressed by 60µM of thiamine. Therefore, depending on the necessity of repression (OFF condition) or induction (ON condition) of the replication fork barrier, media were respectively supplemented or not in thiamine at a final concentration of 60 µM (Calbiochem).

1.2. Strains and plasmids

Fission yeast strains were constructed using standard genetic techniques (Moreno, Klar et al. 1991; Bahler, Wu et al. 1998; Hentges, Van Driessche et al. 2005) and are listed in *Table n.4*.

Gene deletion was carried out by gene targeting, using PCR products containing homology with flanking region of the gene of interest. To achieve this purpose, primers were designed with two segments: the first one was 100 bp complementary to flanking region of the gene and the second one is 20 bp complementary to flanking region of antibiotic marker on pFA6a-derived plasmid (Bahle, Wu et al. 1998; Hentges, Van Driessche et al. 2005). By PCR, deletion cassettes were obtained, containing the antibiotic marker and 100 bp corresponding to the complementary flanking region of the target gene. For gene replacement by a kanamycin, nourseothrycin or hygromycin marker, PCR were respectively performed on pFA6a-kanMX6, pFA6a-natMX6 or pFA6a-hphMX6 plasmids. Deletion cassettes were finally used to transform *Schizosaccharomyces pombe* cells.

Primers used for the construction of a strain deleted for *swi6* gene: the first 100 bp (capital letters) are complementary to the flanking region of the gene while the last 20 bp (minuscule letters) are complementary to the flanking region of the marker cassette.

*Forward:*AACGAGCAAACAACCTGTAAAGACCAACGCGAAATTGATGTTTAGTACTTTTTAAAATAT
TCTGAAATCTCGTTTATTTTCATATTAAGACAAGTGAAAAcggatccccgggttaattaa

*Reverse:*TACTACGACACGGGACTTGATAAAAGCAATAACGTCAGAAAAAAGCGAAATCTAATTTAT
AAACAAAAGAAAAAGAATTTTTTAAAGGAACACAAAAAAAgaattcgagctcgtttaaac

Construction of a strain Pcf1-YFP: The Kan marker associated with YECitrine was amplified by PCR (plasmid: pFA6a-link-YECitrine-Kan). The PCR product was used to transform a fission yeast wild-type strain. The primers used had the same features mentioned above.

*Forward:*TATTATCAGCGAAACTCTTGGAAAAGTCGCTGTGCGTAAAGGAAAATCAGTATCTGATGG
TTGGATAATCAAGGAGAATTTTGCATCCCTTTTATCCTCTGGTGACGGTGCTGGTTTA

*Reverse:*TACAGTATTAATGAATGATATTATACATATTTGCTTAATCGTATAAAAAGAGAGAGGTATA
AATAGTCCTTGAGTGTTCAAGATAATTGCACACTGTGAATCGATGAATTCGAGCTCG

Construction of a strain Pcf2-MYC: The Kan marker associated with 13 Myc repeats was amplified by PCR (plasmid: pFA6a-13Myc-KanMX6). The PCR product was used to transform a fission yeast wild-type strain. Two kinds of transformant were obtained: respectively with 13 Myc repeats and with 4.5 Myc repeats. After western blot analysis, the

strain used for the experiments contained 4.5 Myc repeats. The reason was that, in the strain containing the 13 Myc repeats, Pcf2 migrates at the same size of Pcf1-YFP. This could lead to problems in the revelation of the two proteins, by western blot.

*Forward:*CAATACGGCTGGGGGACCAGCTACTACAACACTAATTCCCAGAAAAGTTGAATCTTCAAA
AGTATCAAAGAAGCGTATTGCACCTACCCCCGTTTATCCACGGATCCCCGGGTTAATTAA
*Reverse:*AGGTTCTTTAAAGTTACTAGTACAAAACAAGAAGTACGAGATTTTTACTCCAAAAATCAAA
GCGTATTGTCGAAGTAGAACTTTTTTCATAAACATGAAAGAATTCGAGCTCGTTTAAAC

Construction of a strain Pcf1-PIP*: To obtain the mutation in the PIP-box, two PCR reactions were performed. For each reaction, a primer containing the desired mutations was used *(F-pcf1 PIP** or *R-pcf1 PIP*)* together with a second primer outside *pcf1* ORF *(U-pcf1-check* or *L-pcf1-check)*. The two PCR products obtained were ligated via fusion PCR, using only the two primers outside *pcf1* ORF.

KF31	*U-pcf1-check*	AGTCTATCAACATTCACCCAC
KF32	*L-pcf1-check*	GATATTCACTGTAACAAGAACCTCG
KF42	*F-pcf1 PIP**	GGAAAGACAAGCATTGAAGGCAAACAACGCTGCCACCAAAGGAG
KF43	*R-pcf1 PIP**	CTCCTTTGGTGGCAGCGTTGTTTGCCTTCAATGCTTGTCTTTCC

The final PCR product containing the entire *pcf1* ORF with the desired mutations in the PIP-box, was used to transform a strain in which *pcf1* gene was deleted by *ura4*$^+$. Clones resistant for 5-FOA (1.6 g/L) were selected (loss of *ura4*$^+$ means integration of *pcf1-PIP**)

1.3. S. pombe transformation

This technique consent the integration of a specific DNA fragment containing the marker cassette, into the fission yeast genome at a specific locus. The transformation consists in the permeabilization of the yeast cell wall, allowing the DNA molecule to enter into the cell. Once in the nucleus, the bases complementarity between the DNA fragment and the flanking region of the target gene permits the integration of the exogenous sequence.

2.10^8 cells from an exponential culture of *S. pombe* were pelleted and washed with an equal volume of sterile water. After a second wash in 1 ml of sterile water, cells were resuspended in 1 ml LiAc-TE 1X (Sigma). Then, cells were spinned and resuspended in LiAc-TE 1X at a concentration of 2.10^9 cells/ml, mixed with 2 µl of herring sperm DNA (Invitrogen) at 10mg/ml and with 1 µg of the DNA fragment for the transformation. After 10 minutes of incubation at room temperature, 260µl of 40% PEG/LiAc-TE was added and then cells were incubated for 60 minutes at 30°C. 40µl of DMSO was added and heat shock was given at

42°C, during 5 minutes. Cells were pelleted and washed with 1 ml of sterile water. Then, cells were resuspended in 3 ml of recovery medium (EMM-Glu) for three hours, then spread on media containing antibiotics. Another option was that cells were not resuspendend in EMM-Glu, but in 0.5 ml of water and then plated on YE. The day after YE plates were replica-plated on selective plates containing Kan or Nat or Hyg antibiotics.

1.4. Serial dilution assay to study the response of fission yeast cells to particular growth conditions

The serial dilution assay was used to characterize the growth and the survival of cells experiencing different kinds of DNA replication challenges, such as the induction of the fork blockade or the exposure to a variety of genotoxic agents.

a) The genotoxic agents tested at different doses were hydroxyurea (Sigma), camptothecin (Sigma), mitomycin C (Sigma), cisplatin (Sigma), methyl methanesulfonate (Sigma) and tetrabenazine (Sigma). I also irradiated plates with UV-C Stratalinker (Stratagene).

   To perform a serial dilution assay cells in the culture were counted and diluted in water, at different concentrations (1.10^7 to 1.10^3 cells/ml). Then, 10 µl of each 1/10th serial dilution were dropped onto plates YE and EMM-Glu, implemented in genotoxic agents.

Drug	stock	concentration used on plate (25 ml)
CPT	10 mM in DMSO	1 µM, 5 µM, 10 µM, 15 µM, 25 µM
HU	1.2 M in water	1 mM, 5 mM, 7.5 mM, 10 mM, 15 mM
MMS	100%	0.01%, 0.015%, 0.02%, 0.025%
CIS	100 mM in water	0.1 mM, 0.25 mM, 0.5 mM, 0.65 mM
MMC	3.125 mM in water	150 µM, 200 µM, 250 µM
TBZ	10 mg/ml in DMSO	5 µg/ml, 10 µg/ml, 20 µg/ml, 30 µg/ml, 60 µg/ml
UV-C		50 J/m², 100 J/m², 150 J/m², 200 J/m², 250 J/m²

b) The survival of different strains to the replication fork blockade was tested by a serial dilution assay. A single colony was inoculated in minimal media (EMM-Glu) with thiamine (pause OFF), to obtain a pre-culture. The pre-culture was washed twice in sterile water and an aliquot was inoculated in two liquid cultures, with or without thiamine (60 µM). The cells were grown for 24 hours (the fork arrest system is induced after 16-18 hours without thiamine). From these two cultures (with or without

thiamine), 10 µl of 1/10th serial dilution (1.10^7 to 1.10^3 cells/ml) were dropped on EMM-Glu plates with (pause OFF) or without (pause ON).

1.5. Assay to score recombination events between dispersed repeated sequences

This assay allows the investigation of recombination events between the two *RTS1* sequences integrated on both sides of *ura4* locus and the *RTS1* sequence naturally present on chromosome II. Recombination events lead to *ura4* loss, including deletions or translocations. Colonies growing on EMM-Glu plates without uracil were switched to EMM-Glu plates with uracil and with or without thiamine. Colonies growing on plates with and without thiamine were respectively inoculated in liquid EMM-Glu media containing or not thiamine, until saturation. From the saturated cultures, 1.10^7 cells were plated on YE supplemented with 5-fluoroorotic acid (1.6 g/L) (Euromedex). At the same time, 100µl of an appropriate dilution were spread on complete YE, to determine the number of viable cells. Colonies were counted after 4 days at 30°C. The rate of *ura4* loss was determined with the method of the median, comparing the number of colonies growing on 5-FOA plates and the viable colonies growing on complete YE plates. Statistical significance was detected using the non-parametric Mann-Whitney test. Colony PCR was performed on 5-FOA resistant colonies to score *ura4* deletions and translocations (See 2.1 Material and Methods for colony PCR). The percentage of deletion/translocation events determined by the PCR assay, was used to balance the rate of *ura4* loss.

Primers used (Sigma- 100 µM):

Ura4Up5: AAAACAAACGCAAACAAGGC
Ura4Lo5: GTTTAACTATGCTTCGTCGG
TLII: TTTCCTTTCACGGCTAACCC
TLIII:TGTACCCATGAGCAAACTGC
Rng3Lo:AAGGACTGCGTTCTTCTAGC
Rng3Up:TGAATCCTCCGTTCAGTAGG

1.6. Assay to score recombination between sister chromatids and between homologous chromosomes.

To perform the investigation of allelic recombination in specific genetic backgrounds, we disposed of fission yeast haploid strains harbouring the construct to check spontaneous sister chromatid exchange (Hartsuiker, Vaessen et al. 2001) and of fission yeast diploid strains

harbouring the construct to score spontaneous homologous recombination and chromosome loss (Hartsuiker, Vaessen et al. 2001).

One colony, growing on complete media was disaggregated in 0.25 ml of ultra-pure water and dilutions were plated on EMM-Glu –ade, EMM-Glu –ade-ura and complete EMM-Glu for survival. After 5 days colonies were counted and the median calculated with the 95% confidence interval for deletions and gene conversion (sister chromatid exchange) and for homologous recombination and chromosome loss (diploid strains). The experiment to score sister chromatid exchanges was performed also in presence of CPT, to determine the rate of induced SCE. Therefore, colonies growing on complete media were switched in a liquid culture and the day after, treated with 20 µM of CPT for 4 hours or only with DMSO as control, before being plated on the selective plates, mentioned above.

1.7. Assay to score the repair of a site-specific polar DSB by recombination between sister- chromatids.

The strain *h+ mat1-PΔ17 pcf1-d* (-SSB + donors) was crossed with the h^- *mat1-M2,3*Δ (+SSB, - donors) strain on a YE plate (Roseaulin, Yamada et al. 2008). After two days the patches were streaked onto G-plates (YE plates not implemented in amino acids) to select the white diploids. The selection of the diploid was possible because the two strains presented two *ade6* alleles that can complement (*ade6 M-210* and *ade6 M-216*). Such diploids were patched on SPA plates (specific plate for sporulation) and after two days, tetrads were dissected. Spores were analyzed by mating type colony PCR for the presence of the SSB (h^-), the presence of the donors (h^+) and the deletion of *pcf1*.

Primers (Sigma - 100 µM):

MT1:AGAAGAGAGAGTAGTTGAAG
MM:TACGTTCAGTAGACGTAGTG
MP:ACGGTAGTCATCGGTCTTCC
*pcf1-d:*GGTCAAACCACTACAG
kanFor: GACTCACGTTTCGAGG

1.8. Fluorescence microscopy of living cells

A pre-culture of *S. pombe* cells was prepared in 10 ml of filtered EMM-Glu, implemented in amino acids. After 24 hours at 30°C, the cultures were diluted to obtain a maximum of cells in S-phase. The final cellular concentration should be between 5.10^6 and 1.10^7 cells/ml. If the

recruitment of proteins in the presence of the fork blockade at *RuraR* has to be analyzed, the cells from the pre-culture have to be washed twice in sterile water and splitted into two cultures with or without thiamine, for 24 hours.

1 ml of the culture was centrifugated at low speed (1.500 rpm for 1 min) and the pellet was resuspendend in 1 ml of fresh filtered EMM-Glu. 1 µl of cells were dropped onto the microscopy agarose-slide, previously prepared. The slides present a layer of 1.4% agarose dissolved in filtered EMM-Glu. Cells were observed with a LEICA DMRXA microscopy equipped of an oil immersion 100X objective, with numerical aperture corresponding to 1.4 and coupled to a COOLSNAP HQ camera (Roper Scientific, USA). The filters we used on the microscope were FITC filter for GFP, CFP filter for CFP and the YFP filter for YFP. Photos were taken with the Z-stack (3D) parameterized at 15 slices and were analyzed using METAMORPH (Roper Scientific, USA) and Image J software.

2. MOLECULAR BIOLOGY TECHNIQUES

2.1. Colony PCR

Colony PCR was performed disaggregating a fission yeast colony in 30 µl of sterile water, in a PCR tube. Then the PCR tubes were heated in the microwave oven (900W) for 3 minutes. For each tube 20 µl of PCR mix were added. The tubes were laid in the PCR machine and the appropriate PCR program was set.

The mix for one PCR tube was composed by: 1 µl of dNTPs (10 mM each), 0.5 µl Red Hot® *Taq* DNA Polymerase (ABgene), 0.25 µl for each primer (100µM), 5 µl of PCR buffer (10X), 9 µl of sterile water and 4 µl of $MgCl_2$ (25 mM).

2.2. Pulsed Field Gel Electrophoresis (PFGE)

Chromosomes can be separated depending on their size, by PFGE. This technic allows the detection of gross chromosomal rearrangements on the chromosome III, which are produced by the induction of the replication fork blockade at *ura4* locus. The rearrangements were visualized via specific ^{32}P marked probes.

Chromosome sizes were analyzed by pulse field gel electrophoresis as follows: 30 ml of culture (1.10^7 cells/ml) was washed twice in 30 ml CSE buffer (20 mM citrate/phosphate [pH 5.6], 40 mM EDTA, 1.2 M sorbitol) and then digested in 5 ml CSE containing 1.5 mg/ml Zymolyase 20T (MP biomedicals). Cells pellet was then resuspended in 300 µl of TSE buffer (10 mM tris-HCl [pH 7.5], 45 mM EDTA, 0.9 M sorbitol) and mixed with 400 µl of 1% agarose (Ultra Pure™ Agarose Invitrogen) in TSE and dispensed in 80 µl aliquots to plug molds. Then, cell lysis was performed by incubating agar plugs in 0.25 M EDTA, 50 mM Tris-HCl (pH 7.5), 1% SDS for 90 min at 55°C, following by twice 24 hours incubation in 1% lauryl sarcosine, 0.5 M EDTA (pH 9.5), 0.5 mg/ml proteinase K at 55°C (Euromedex). Electrophoresis was performed using the Bio-Rad CHEF Mapper apparatus for 48 hr in TAE buffer 1X (Biorad) (0.8% agarose Biorad megabase, Pulse time: 1800 s, 2V/cm, angle: 100°, temperature: 14°C). Finally, agarose gel was stained in 0.5 µg/ml Ethidium bromide for 30 minutes and chromosomes were transferred on a nylon membrane. The chromosomes were probed with ^{32}P-*rng3* probe (GE healthcare and Applied biosystem) and signals were screened with a Typhoon PhosphorImager. The intensity of the revealed bands was quantified by Imagequant software (Amersham Biosciences). The total chromosome III signal was calculated by summing up the 3.5Mb chromosome signal and the acentric signals (without the

background signal of each one). The percentage of acentric chromosome was obtained doing the ratio between the acentric signal and the signal corresponding to the total chromosome III.

2.3. Restriction Fragment Length Analysis (RFLA)

Recombination outcomes due to template exchange reactions are visualized and quantified by RFLA.

Chromosomal DNA was extracted using standard procedures. 40 µg of DNA were resuspendend in 100 µl of sterile water and digested overnight by 10 µl of AseI (10U/µl) or 5 µl of EcoRV (20 U/µl). Both reactions required also 50 µl of Buffer n.3 (10X), 5µl of BSA (100X) and sterile water until a final volume of 500 µl. The digested genomic DNA was precipitated with 50 µl of potassium acetate (5M), 550 µl of ice-cold isopropanol and 1 µl of glycogen (20 mg/ml) and washed once in 70% ethanol. Then, DNA was resuspendend in 25 µl of sterile water and loaded on 0.8% gel. Once the gel was migrated, the genetic material was transferred on a nylon membrane to perform Southern blot. Recombination outcomes were probed with ^{32}P-*ura4* probe (GE healthcare and Applied biosystem), signals were screened with a Typhoon PhosphorImager, and band intensities were quantified with Imagequant software (Amersham Biosciences). Quantification of rearrangements associated with one or two cross-over were performed using *ura4* as probe: % of global rearrangements (E*co*RV digestion: *ura4* inversion + acentric) = intensity of the (inversion+acentric) band/intensity of ((inversion+acentric band) + (*RuraR* locus band)); % of acentric isochromosome (A*se*I digestion) = intensity of lower band, corresponding to the acentric/intensity of (acentric band + *RuraR* locus band) % of *ura4* inversion = % of global rearrangements – % of acentric isochromosomes.

2.4. Replication intermediates (RIs) analysis: the 2D gel (2DGE)

2DGE is a powerful technique to visualize replication intermediates at the fork arrest site. Chromosomal DNA of cells embedded in agarose plug was digested with 30 units of A*se*I (60 units if DNA was previously cross-linked – See 2.5 for the DNA cross-link procedure), and RIs were enriched on BND cellulose columns using the following procedure: 2 g of BND (Sigma, B6385) was dissolved in 15 ml of 0.3 M NaCl, 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA for at least 4 hr and then, equally splitting in Poly-Prep Chromatography Columns (Biorad), was washed three times with 1 ml of the same buffer and dried. DNA samples were equilibrated at 0.3 M NaCl and loaded on BND cellulose columns.

Most of the double-stranded DNA was eluted by addition of 3 ml of 0.8 M NaCl, Tris-HCl (pH 7.5), and 1 mM EDTA. DNA containing single-stranded regions was eluted by addition of 3 ml of 1 M NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 1.8% (w/v) caffeine. Fractions containing RIs were precipitated and dissolved in 10 µl of 10 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 0.1 mM EDTA and were analyzed by 2D gels, run according to Brewer et al., (1992), using 0.35% and 0.9% agarose for the first and second dimensions, respectively. If DNA was cross-linked, before transfer onto a nitrocellulose membrane it was decross-linked 10 min at 280 nm.

Replication intermediates were visualized using ^{32}P-*ura4* probe. Analysis and quantification of ^{32}P-probed blots (using positively charged membrane) were performed by phosphor-imager using Imagequant software (as for PFGE). Quantification of 2DGE: the volume signal (background subtracted) for each RI was collected. The relative level of joint molecules (JM) was calculated by comparing to stalled forks signal (% of JM/stalled forks = volume JM/(volume JM + volume stalled fork) × 100).

2.5. Crosslink with 365-nm UV lamp

The DNA of certain strains was cross-linked before the 2DGE analysis, to demonstrate that joint molecules instability occurs *in vivo* and they are not particularly liable to dissolution *in vitro*.

1 to 2.10^9 cells were washed twice in 20 ml ultra-pure ice-cold water and were resuspended in 20 ml ice-cold water. Then the cells were transferred into an 8.5 cm-diameter pre-cooled glass-petri dish and placed on ice. 1 ml of 200µg/ml trimethylpsoralen (TMP) was added (Sigma). The petri dishes were incubated for 5 min in the dark mixing every minute. The cross-link was made by using a "Sellamed system Dr. sellmeier 4000" platform, in which the flux was adjusted at 50 mW/cm^2 and the cells were irradiated for 90 seconds. In these conditions the cells were submitted to 45 KJ.

2.6. Chromatin immunoprecipitation (ChIP)

The ChIP allows the detection of the recruitment of a specific protein, in a defined region in the genome. ChIP was performed using a protocol modified from Strahl-Bolsinger and colleagues (Strahl-Bolsinger, Hecht et al. 1997). Anti-GFP antibody (Molecular Probe) was used 1:300 and precipitated with G protein Dynabeads. The relative amounts of DNA

fragments were quantified by real-time PCR (qPCR) on 5 µl of each samples using QuantiTect SYBR Green PCR master mix (Qiagen).

Chromatin immunoprecipitation was performed as follow: 1.10^9 cells was centrifuged, washed in 10 ml in PBS1X, and incubated in 10 ml PBS1X containing 25 mg of DMA (Dimethyl adipimidate - Sigma) and 0.25% DMSO for 45 min at RT under agitation. Then, cells were washed in 10 ml PBS1X and incubated in PBS1X containing 1% formaldehyde for 15 min at RT under agitation. Then, 2 ml of 2.5 M glycine was added for 5 min incubation at RT with agitation. Cells pellet was resuspended in 400 µl ChIP lysis buffer (50 mM HEPES [pH 7.4], 140 mM NaCl, 1% tritonX100, 0.1% NaDeoxycholate, protease inhibitors, 1 mM PMSF) with glass beads and ribolysed 3 times 30 seconds at 6000 rpm, 1 minute pause at 4°C. Cell extract pellet was then washed in 1 ml ChIP lysis buffer, resuspended in 400 µl ChIP lysis buffer and sonicated during 15 cycles of 30 seconds on/30 seconds off at the higher amplitude (H level), on ice water. Cells extract was centrifuged, and 5 µl of supernatant was mixed with 100 µl of elution buffer (50 mM Tris, 1% SDS, 10 mM EDTA) as input control and 20 µl was kept for immunoblotting.

For immunoprecipitation, 300 µl of extract was incubated with 1 µl of anti-GFP antibody (Molecular Probe) for 1 hr at 4°C, following by incubation with 20 µl of previously washed magnetic G protein beads (Dynabeads Sigma) overnight at 4°C. Immunoprecipitation was then washed as follows: twice with 1 ml of ChIP lysis buffer for 5 min, twice with 1 ml of ChIP lysis buffer high salt (50 mM HEPES [pH 7.4], 500 mM NaCl, 1% tritonX100, 0.1% NaDeoxycholate) for 10 min, twice with 1 ml ChIP wash buffer (10 mM Tris [pH 8], 250 mM LiCl, 0,5% NP40, 0.5% NaDeoxycholate, 1 mM EDTA) for 5 min, and finally with 1 ml of TE for 1 min.

Immunoprecipitation was then eluted with 130 µl of elution buffer, following by 2 hr of incubation at 65°C. Input control was as well incubated in same condition and 20 µl of immunoprecipitation was kept for immunoblotting. Finally, DNA was recover using Qiagen PCR purification kit and eluted in 200 µl of water. The relative amounts of PCR products were quantified using real-time PCR (qPCR) performing using 5 µl of each samples (input control and immunoprecipitation) and QuantiTect SYBR Green PCR master mix (Qiagen). Fold enrichments were calculated using previously described method (Chakrabarti, James et al. 2002).

2.7. Total cellular extract (TCA) protocol

Add 1 ml of stop buffer (50 mM NaF, 10 mM NaN3 in PBS 1X) to 1.10^8 cells, mix and put on ice. Centrifuge 30 seconds 14.000 rpm, room temperature, and wash with 1 ml of stop buffer. Spin again and wash with 1 ml of TCA 20%. Spin, resuspend the pellet in 200 µl of TCA 20% and add glass beads to break the cell walls using a homogenizer machine (Precellys). The program: 6.000 rpm 3 times, 30 seconds with one minute of pause between the cycles. After the homogenization of cells, add 400 µl of TCA 5% and centrifuge 5 minutes 4.000 rpm at 4°C, to collect the surnatant in a new 1.5 ml tube. Centrifuge 5 minutes, 13.000 rpm at 4°C and, after the aspiration of the surnatant, dry well the pellet. Then, resuspend in 200 µl of loading blue (for 10 ml: 5 ml of 2X SDS loading buffer, 2 ml Tris-HCl 1M pH8 and 3 ml of sterile water) and heat 5 minutes at 100°C. Samples can be frozen at -20°C or loaded on acrylamide gel.

2.8. Protein complex immunoprecipitation (Co-IP)

Using the Co-IP technique we could investigate if the interaction of two proteins occurs in fission yeast cells and if this interaction is still present mutating particular amino acids residues of one of the two partners.

To 5.10^8 cells add 1/100 of volume of sodium azide 10% (Sigma) and centrifuge. Wash cells in cold water, resuspend pellet in 400 µL EB Buffer (50 mM HEPES High Salt, 50 mM KOAc pH 7.5; 5 mM EGTA, 1% triton X-100; 1 mM PMSF (Sigma) + 1 tablet anti-protease (Roche) and ribolyse cells with glass beads (Sigma- as for TCA extract). Centrifuge and recover surnatant.

- Keep 50 µl as INPUT to which add Laemmli 4X and boil 5 min. Keep samples at -20°C.
- Keep 300 µl to do IP: add 2 µl of anti-GFP/anti-Myc antibody (Invitrogen) and incubate for one hour, at 4°C.

Wash twice 40 µl of Dynabeads Protein-G (Sigma) for sample in 1 ml PBS 1X, 10 min at room temperature and resuspend in 40 µL de EB Buffer. Add the beads to the 300 µL of extract and incubate at 4°C overnight. The day after keep 50 µL of surnatant as UNBOUND FRACTION, then wash beads twice in EB Buffer, 10 min. Resuspend beads in Laemmli (30 µL) and boil for 5 min (IP FRACTION).

Western Blot: Samples (Input, Unbound and Ip) were loaded and migrated using an acrylamide gradient gel 4- 12% (NuPAGE® Gels). The proteins were transferred on nitrocellulose membrane (Amersham Biosciences) and stained with “Red Ponceau”. Cut the

membrane to incubate with anti-GFP/anti Myc antibody (Roche) and anti-PCNA antibody (Santa Cruz Biotech). The day after wash in TBSt 0.1% three times 15 min, incubate with appropriate secondary antibody (anti-rabbit for GFP/Myc tagged protein and anti-mouse for PCNA) for one hour and then wash again three times in TBSt 0.1%. The final step is the revelation with ECL-Plus kit (Amersham Biosciences).

Table n.4 – List of strains used in this study

Legend: RTS1b -> RTS1 sequences were integrated on both sides of ura4 locus

RTS1C -> RTS1 sequence was integrated at the centromeric side of ura4 locus

Strain name	***Genotype***	***Notes***
VP001	*h- smt0, rtf1nmt41::sup35, ade6-704, leu1-32, RTS1b:ura4+*	*Lambert*
VP002	*h- smt0, rad22::Kan, rtf1nmt41::sup35, ade6-704, leu1-32, RTS1b:ura4+*	*Lambert*
VP003	*h+, pcf1::Kan, ura4-D18, leu1-32, ade6 M-216*	*Francesconi*
VP005	*h+, pcf2::Kan, ura4-D18, leu1-32, ade6 M-216*	*Francesconi*
VP007	*h+, pcf3::Kan, ura4-D18, leu1-32, ade6 M-216*	*Francesconi*
VP009	*h- smt0, pcf2::Kan, rtf1nmt41::sup35, ade6-704, leu1-32, RTS1b:ura4+*	*This study*
VP015	*h- smt0, pcf1::Kan, rtf1nmt41::sup35, ade6-704, leu1-32, RTS1b:ura4+*	*This study*
VP016	*h- smt0, pcf3::Kan, rtf1nmt41::sup35, ade6-704, leu1-32, RTS1b:ura4+*	*This study*
VP018	*h-, ade6-704, leu1-32, ura4-D18*	*Lambert*
VP020	*h+, rad3::ura4+ , ade6-704, leu1-32, ura4-D18*	*Lambert*
VP021	*h+, rad22:GFP-Kan, rtf1nmt41::sup35, ade6-704, leu1-32, RTS1b:ura4+*	*Lambert*
VP023	*h- smt0, lsd1-W89IA-HA::Kan, ade6-704, leu1-32, ura4-D18*	*Arcangioli*
VP024	*h- mgs1::ura4+, leu1-32, ade6-704, ura4-D18*	*Lambert*
VP036	*h- smt0 swi6::Nat, ade6-704, leu1-32, ura4-D18*	*This study*
VP045	*h- smt0, pcf3::Kan, rad22:GFP-Kan, rtf1nmt41::sup35, ade6-704, leu1-32, RTS1b:ura4+*	*This study*
VP050	*h- smt0, pcf1::Kan, rtf1nmt41::sup35, ade6-704, leu1-32, RTS1C:ura4+*	*This study*
VP052	*h- smt0, pcf2::Kan, rtf1nmt41::sup35, ade6-704, leu1-32, RTS1C:ura4+*	*This study*
VP056	*h- smt0, pcf3::Kan, rtf1nmt41::sup35, ade6-704, leu1-32, RTS1C:ura4+*	*This study*
VP069	*h- smt0, swi6::Nat, rtf1nmt41::sup35, ade6-704, leu1-32, RTS1b:ura4+*	*This study*
VP077	*h- smt0, pcf2::Kan, srs2::Nat, rtf1nmt41::sup35, ade6-704, leu1-32, RTS1b:ura4+*	*This study*
VP087	*h- smt0, pcf1::Kan, srs2::Nat, rtf1nmt41::sup35, ade6-704, leu1-32, RTS1b:ura4+*	*This study*
VP094	*h- smt0, pcf1::Kan, rad22::Nat, rtf1nmt41::sup35, ade6-704, leu1-32, RTS1b:ura4+*	*This study*
VP098	*h- smt0, pcf2::Kan, rad22::Nat, rtf1nmt41::sup35, ade6-704, leu1-32, RTS1b:ura4+*	*This study*
VP103	*h- smt0, pcf1::Kan, rtf1nmt41::sup35, ade6-704, leu 1-32, RTS1b:ura4+*	*This study*
VP109	*h- pcf1::Kan, rtf1nmt41::sup35, ade6-704, leu 1-32, RTS1b:ura4+*	*This study*

VP111	*h- pcf3::Kan, rtf1nmt41::sup35, ade6-704, leu 1-32, RTS1b:ura4+*	*This study*
VP114	*h- pcf2::Kan, rtf1nmt41::sup35, ade6-704, leu 1-32, RTS1b:ura4+*	*This study*
VP120	*h- smt0, pcf1::Kan, pcf2::Kan, pcf3::Kan, rtf1nmt41::sup35, ade6-704, leu1-32, RTS1b:ura4+*	*This study*
VP133	*h-smt0, pcf2::Kan, rhp51::Kan, rtf1nmt41::sup35, ade6-704, leu1-32, RTS1b:ura4+*	*This study*
SL 917	*h- smt0, ura4-D18, ade6 M-375 int:puc8/ura4+/ade64-69*	*Hartsuiker*
VP152	*h- smt0, pcf1::Kan, ura4-D18, ade6 M-375 int:puc8/ura4+/ade64-69*	*This study*
VP155	*h- smt0, pcf2::Kan, ura4-D18 ade6 M-375 int:puc8/ura4+/ade64-69*	*This study*
VP158	*h- smt0, pcf3::Kan, ura4-D18 ade6 M-375 int:puc8/ura4+/ade64-69*	*This study*
VP161	*h- smt0, swi6::Nat, ura4-D18 ade 6M-375 int:puc8/ura4+/ade64-69*	*This study*
VP186	*h- smt0, ade6 M-210, ura4-D18/ade6 M-216*	*Hartsuiker*
VP190	*h- smt0, pcf1::Kan, ade6 M-210, ura4-D18/h- smt0, pcf1::Kan, ade6 M-216*	*This study*
VP198	*h- smt0, pcf2::Kan, ade6 M-210, ura4-D18/h- smt0, pcf2::Kan, ade6 M-216*	*This study*
VP203	*h- smt0, pcf3::Kan, ade6 M-210, ura4-D18/h- smt0, pcf3::Kan, ade6 M-216*	*This study*
VP208	*h- smt0, swi6::Nat, ade6 M-210, ura4-D18/h- smt0, swi6::Nat, ade6 M-216*	*This study*
VP217	*h-, ura4:pECFP-pcna, pcf1:YFP- Kan, ura4-D18*	*This study*
VP 219	*h- smt0, pcf2::Kan, rad22:GFP-Kan, rtf1nmt41::sup35, ade6-704, leu1-32, RTS1b:ura4+*	*This study*
VP229	*h- smt0, pcf1::Kan, rad22:GFP-Kan, rtf1nmt41::sup35, ade6- 704, leu1-32, RTS1b:ura4+*	*This study*
VP232	*h+, pcf1:YFP-Kan, leu1-32, ura4-D18, ade6 M-216*	*Meister*
VP236	*h- smt0, pcf1::Kan, rqh1::Kan, rtf1nmt41::sup35, ade6-704, leu 1-32, RTS1b:ura4+*	*This study*
VP241	*h- smt0, pcf2::Kan, rqh1::Kan, rtf1nmt41::sup35, ade6- 704, leu 1-32, RTS1b:ura4+*	*This study*
VP244	*h-, hip::Kan, leu1-32, ura 4 D-18, lys1*	*Saitoh*
VP245	*h+, pcf1:YFP-Kan, rtf1nmt41::sup35, ade6-704, leu1-32, RTS1b:ura4+*	*This study*
VP256	*h- smt0, pcf1:YFP-Kan, pcf2::ura4+, leu1-32, ade6-704*	*This study*
VP259	*h- smt0, pcf1:YFP-Kan, pcf3::Kan, leu1-32, ade6-704*	*This study*
VP266	*h- smt0, srs2::Nat, pcf1::ura4+, ade6-704, leu 1-32, ura4-D18*	*This study*
VP270	*h- smt0, srs2::Nat, pcf2::ura4+, ade6-704, leu1-32, ura4-D18*	*This study*
VP272	*h-, slm9::Kan, leu1-32, ura4-D18, lys1*	*Saitoh*
VP290	*h-smt0, rqh1::Kan, pcf2::ura4+, ade6-704, leu1-32, ura4-D18*	*This study*
VP294	*h-smt0, srs2::Nat, rqh1::Kan, ade 6-704, leu1-32, ura4-D18*	*This study*
VP300	*h- smt0, srs2::Nat, rqh1::Kan, pcf1::ura4+, ade6-704, leu1-32, ura4-D18*	*This study*
VP307	*h- smt0, pcf1-PIPboxMut, rtf1nmt41::sup35, ade6-704, leu 1-32, RTS1b:ura4+*	*This study*
VP313	*h-smt0, pcf1::Kan, rhp51::Kan, rtf1nmt41::sup35, ade6-704, leu1-32, RTS1b:ura4+*	*This study*
VP316	*h- smt0, rqh1::Kan, pcf1::ura4+, ade6-704, leu1-32, ura4-D18*	*This study*
VP 320	*h+ srs2::Nat, rqh1::Kan, pcf2::ura4+, ade6-704, leu1-32, ura4-D18.*	*This study*

VP338	*h-, pcf1-PIPboxMut, rtf1nmt41::sup35, ade6-704, leu1-32, RTS1b:ura4+*	*This study*
VP340	*P Δ17::leu2, ura4-D18, leu1-32*	*Arcangioli*
VP346	*M 2,3 Δ::leu2, ade6 M-210, ura4-D18, leu1-32*	*Arcangioli*
VP354	*h-, pcf1::Kan, rqh1::Kan, rtf1nmt41::sup35, ade6-704, leu1-32, RTS1b:ura4+*	*This study*
VP358	*h -, pcf1::ura4+, slm9::Kan, leu1-32, ura4-D18*	*This study*
VP385	*pcf1::Kan, P Δ17::leu2, ura4-D18, leu1-32, ade6 M-216*	*This study*
VP388	*h- smt0, pcf1-PIPboxMut:YFP-Kan, ade6-704, leu1-32, ura4-D18*	*This study*
VP409	*h- smto, pcf2-Myc (4.5rep)-KanR ade6-704, leu 1-32, ura 4-D18*	*This study*
VP394	*h- smt0, ade6-704, leu1-32, ura4-D18, pcf1:YFP-Kan*	*This study*
VP425	*h- smto, pcf2-Myc(4.5rep)-KanR, pcf1PIPbox-YFP-KanR, ade 6-704, leu 1-32, ura 4-D18*	*This study*
VP426	*h- smto, pcf2-Myc(4.5rep)-KanR, pcf1-YFP-KanR, ade 6-704, leu 1-32, ura 4-D18*	*This study*
SL80	*h+ rqh1::kan ade6-704 leu1-32 ura4D18*	*Lambert*
SL279	*h- smt0 rhp51::Kan rtf1nmt41::sup35 ade6-704 leu1-32 RTS1b::ura4*$^{+}$	*Lambert*
SL294	*h- rad22GFP::Kan rtf1 nmt41::sup35 ade6-704 leu1-32 RTS1b::ura4*$^{+}$	*Lambert*
SL511	*h- smt0 rqh1::Kan rtf1nmt41::sup35 ade6-704 leu1-32 RTS1b::ura4*$^{+}$	*Lambert*
SL350	*h- rtf1nmt41::sup35 ade6-704 leu1-32 RTS1C::ura4*$^{+}$	*Lambert*

RESULTS

1. CHARACTERIZATION OF STRAINS DEFICIENT FOR CAF-1

In an effort to investigate the role of CAF-1 in the DNA Damage Response (DDR) in fission yeast, Dr. Francesconi (UMR3348, Institut Curie) created three strains, each one deleted for one of CAF-1 subunits: *pcf1-d, pcf2-d* or *pcf3-d.* These three strains are viable, showing that CAF-1 is not an essential H3-H4 histone chaperone in fission yeast, in contrast to Asf1 (Dohke, Miyazaki et al. 2008; Tanae, Horiuchi et al. 2012).

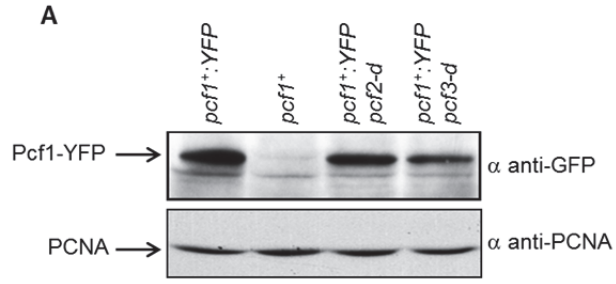
1.1. Stability of CAF-1 subunits

In budding yeast, deletion of one of the three CAF-1 subunits results in a loss of function of CAF-1 and each deletion shares common phenotypes (Kaufman, Kobayashi et al. 1997; Linger and Tyler 2005). These data suggest that, in budding yeast, the main function of each subunit is to assemble the active CAF-1 complex. This situation contrasts with the situation in mammals in which the largest subunit p150 has a function in the DDR, independently of the two other subunits. Moreover, the third subunit p48 interacts with chromatin modifier enzymes independently of CAF-1. Based on these data, I first analyzed the role of fission yeast CAF-1 subunits in the DDR. Moreover, as the absence of one subunit in the complex can destabilize the expression level or the stability of the others subunits (Schnaitman and McDonald 1984; Rodgers, Moser et al. 2000), I investigated the stability of the largest subunit Pcf1 in the absence of the two other subunits.

The C-terminal domain of Pcf1 was fused to the yellow fluorescent protein tag (Pcf1-YFP). The expression level of Pcf1-YFP was analyzed in *pcf2-d* or *pcf3-d* deleted strains by western-blot (Figure n.28A). As positive control, I used a strain expressing Pcf1-YFP with wild-type $pcf2^{+}$ and $pcf3^{+}$, and as negative control, a strain expressing untagged Pcf1. To quantify the expression level of Pcf1-YFP in these genetic backgrounds, the expression of PCNA was used as loading control.

I found that the deletion of *pcf2* or *pcf3* slightly decrease the expression level of Pcf1 (between 10% and 20%) (Figure n. 28B). This minor effect could be due to the asynchrony of cultures in which different amounts of S-phase cells could account for the observed fluctuation in Pcf1 expression level. **Thus, I concluded that deletion of Pcf2 or Pcf3 subunit of CAF-1 has little or no effect on the stability of the largest subunit Pcf1.** This does not presume of the functionality of CAF-1 and does not exclude potential functions of

each subunit, independently of CAF-1. Therefore, further experiments were performed using the three single deleted strains: *pcf1-d, pcf2-d* or *pcf3-d.*



B

Strain	Fold expression
pcf1⁺:YFP	1.00
pcf1⁺:YFP pcf2-d	0.80
pcf1⁺:YFP pcf3-d	0.91

Figure n. 28: Analysis of the stability of Pcf1 protein in the absence of Pcf2 or Pcf3 subunits. A) Total protein extracts from strains *pcf1⁺:YFP, pcf1⁺, pcf1⁺:YFP pcf2-d* and *pcf1⁺:YFP pcf3-d* were analyzed by western-blot. Pcf1-YFP (89.2 kDa) was detected using an anti-GFP antibody and PCNA (28.9 kDa) was detected using an anti-human PCNA antibody. B) The expression level of Pcf1-YFP and PCNA were estimated using ImageJ software. The ratio between Pcf1-YFP and PCNA expression level in *pcf1⁺:YFP* was considered as 1 and the expression of Pcf1-YFP in *pcf1⁺:YFP pcf2-d* and *pcf1⁺:YFP pcf3-d* was quantified relative to that of *pcf1⁺:YFP*.

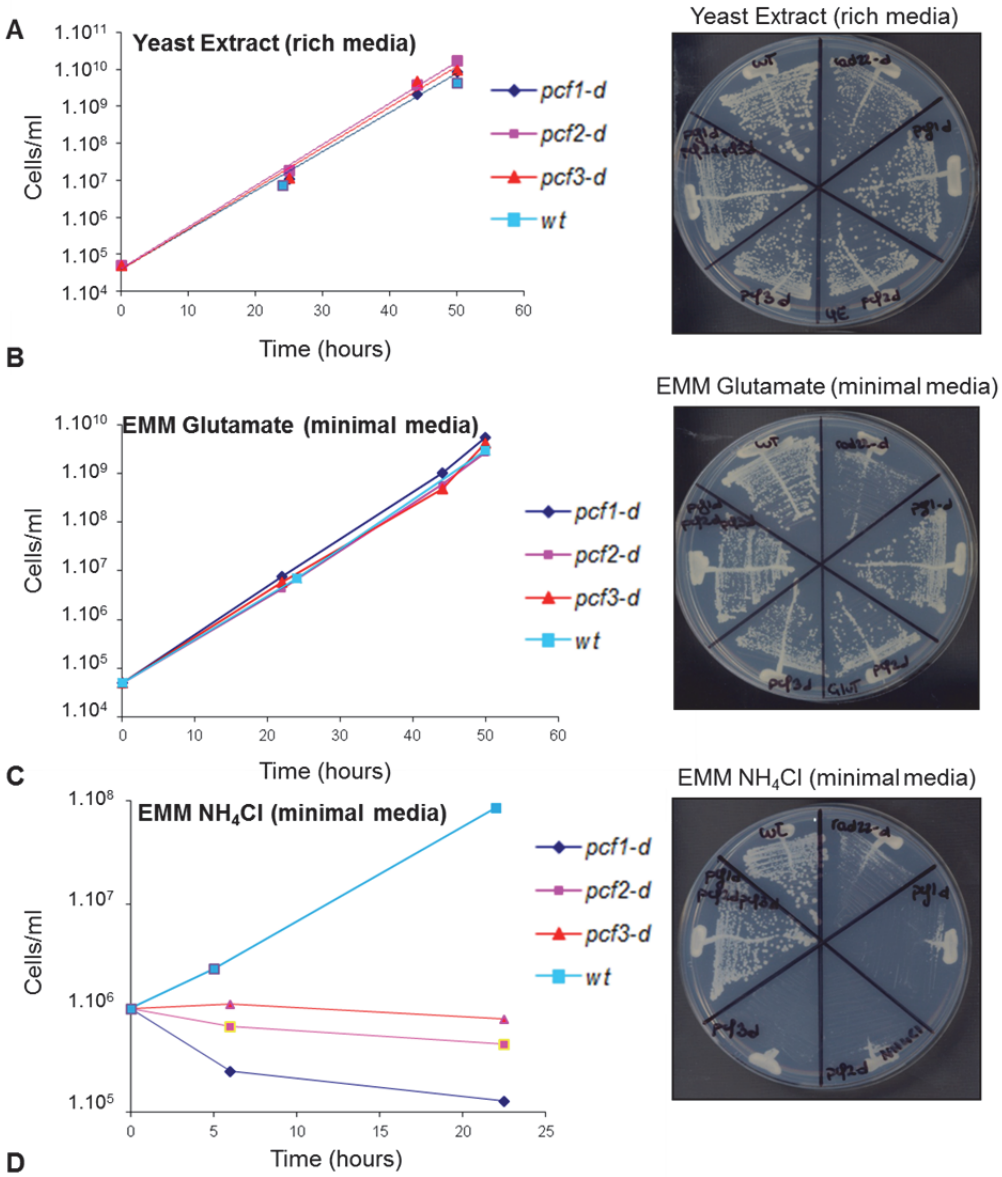
1.2. Strains defective for CAF-1 are sensitive to the nitrogen source

To investigate the role of CAF-1 on cell growth and fitness, I studied the ability of strains defective for CAF-1 to grow on different culture media. I compared the growth parameters of single *pcf1-d, pcf2-d* or *pcf3-d* null mutant with a wild-type (*wt*) strain.

I tested both liquid and solid rich media (YE) and two minimal media containing different nitrogen sources: ammonium chloride (EMM-NH_4Cl) and glutamate (EMM-Glu). These media were selected as they are commonly used to grow the yeast *S. pombe*, but also because my further experiments have required the use of conditional addition of particular substances, such as thiamine, which is already present in rich media.

I observed that, in both YE and EMM-Glu, the doubling time for each strain defective for CAF-1 is comparable to the doubling time of the *wt* strain (around 3 hours) (Figure n. 29A, B and D). In contrast, the three strains (*pcf1-d*, *pcf2-d* and *pcf3-d*) exhibited a very slow growth phenotype on agar plate containing EMM-NH_4Cl and were unable to divide on liquid media containing EMM-NH_4Cl (Figure 29C). These data showed that the absence of CAF-1 does not affect cell cycle length, but renders cells sensitive to the nitrogen source. I first supposed that the absence of CAF-1 might lead to a defect in nitrogen metabolism and/or in the transporters used to import ammonium chloride. This might be linked to a role of CAF-1, directly or indirectly, in transcription processes (Kim, Seol et al. 2009; Heyd, Chen et al.

2011). However, I was surprised to observe that the strain deleted for the three subunits of CAF-1 (*pcf1-d pcf2-d pcf3-d* triple mutant) was able to grow on solid media containing EMM-NH_4Cl, to the same extend than the *wt* strain (Figure n. 29C). **Therefore, I concluded that the high sensitivity of single null mutant to nitrogen source is rather a consequence of the formation of an aberrant CAF-1 complex, than the lack of its function.**



D

Strains	Doubling Time (in hours)	
	Rich media (YEA)	Minimal media (EMM-Glutamate)
wt	2.90	3.10
pcf1-d	2.89	3.01
pcf-2-d	2.77	3.19
pcf3-d	2.77	3.15

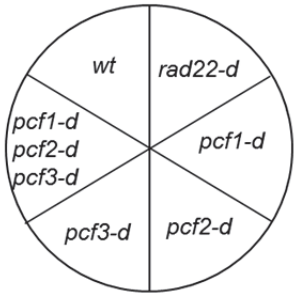


Figure n. 29: Analysis of growth parameters in strains defective for CAF-1. The growth temperature was 30°C for all experiments. A) Left panel: growth curves in YE (rich media) of indicated strains. Right panel: YE plate with *wt, rad22-d, pcf1-d, pcf2-d, pcf3-d* and *pcf1-d pcf2-d pcf3-d* strains. B) Left panel: growth curve in EMM-Glu (minimal media) of indicated strains. Right panel: corresponding plate. The *rad22-d* strain presents a slow growth phenotype C) Left panel: growth curve in EMM-NH_4Cl (minimal media) of indicated strains. Right panel: corresponding plate. The initial concentration of cells in the culture was higher (1.10^6) than in the previous two media. D) Doubling time of indicated strains and media.

1.3. Strains defective for CAF-1 are not sensitive to DNA-damaging and replication-blocking agents

Another key point was to investigate the implication of CAF-1 complex in the DDR. As previously mentioned (see Introduction, Chapter 4), CAF-1 is involved in DNA repair pathways, including DSBs repair, both by promoting chromatin restoration after completion of DNA repair and by acting at early steps to recruit repair enzymes. In support of this, lack of CAF-1 function leads to increase sensitivity to UV-C and DSB-inducing agents in budding yeast (Kaufman, Kobayashi et al. 1997; Linger and Tyler 2005). Thus, I investigated the sensitivity of strains defective for CAF-1 (*pcf1-d*, *pcf2-d* and *pcf3-d* single mutant) to DNA-damaging and replication-blocking agents, in fission yeast. The following drugs were used:

- camptothecin (CPT), a replication-blocking agent that inhibits the topoisomerase I and results in torsional stress and replicative DSBs.

- hydroxyurea (HU), an inhibitor of the ribonucleotide reductase, which leads to an inhibition of bulk dNTP synthesis in S-phase and thus, to a strong slowing down of the replication forks progression.

- UV-C radiation, that produces two predominant types of DNA damage: cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone (6-4 photoproducts).

- methyl methanesulfonate (MMS), an alkylating agents leading to base alkylation and to a strong delay in S-phase progression.

- cisplatine (CIS) and mitomycin-C (MMC), two cross-linking agents. The main lethal lesions induced by cross-linking agents are inter-strands cross-links (ICLs) that compromise the unwinding of parental duplex DNA, ahead of advancing forks.

- tetrabenazine (TBZ) that inhibits microtubule polymerisation and compromise kinetochore attachment to the mitotic spindle.

Data showed that strains defective for CAF-1 are not more sensitive to these DNA-damaging and replication-blocking agents than the *wt* strain (Figures n. 30, 31B-C, 32). Importantly, sensitivities to these agents were analyzed both on rich (YE) and EMM-Glu media and similar data were obtained.

However, I found that strains deleted for each subunit of CAF-1 were highly resistant to MMS (Figure n. 31A). Indeed, the three strains *pcf1-d*, *pcf2-d* or *pcf3-d* exhibit at least a 10 fold greater resistance to MMS than the *wt* strain. These data suggest that CAF-1 defect might play a direct role in conferring MMS resistance. Another possible explanation is that chronic exposure to MMS (one week) results in a longer G1-phase in the absence of CAF-1, therefore increasing the resistance to MMS. These two hypotheses are not mutually exclusives.

In contrast to the data reported in *S. cerevisiae* (Kaufman, Kobayashi et al. 1997; Linger and Tyler 2005), **my results suggest that none of CAF-1 subunits plays a major role in the DNA damage response**. Probably, the differences observed between *S. cerevisiae* and *S. pombe* are due to their cell cycle. Indeed, in fission yeast the longer phase of the cell cycle is the G2 phase (80% of the cell-cycle time), while it is not the case for the budding yeast. The higher sensitivity to DNA damaging agents occurs in S-phase, therefore in *S. pombe* the window of time in which cells can experience DNA damage is limited.

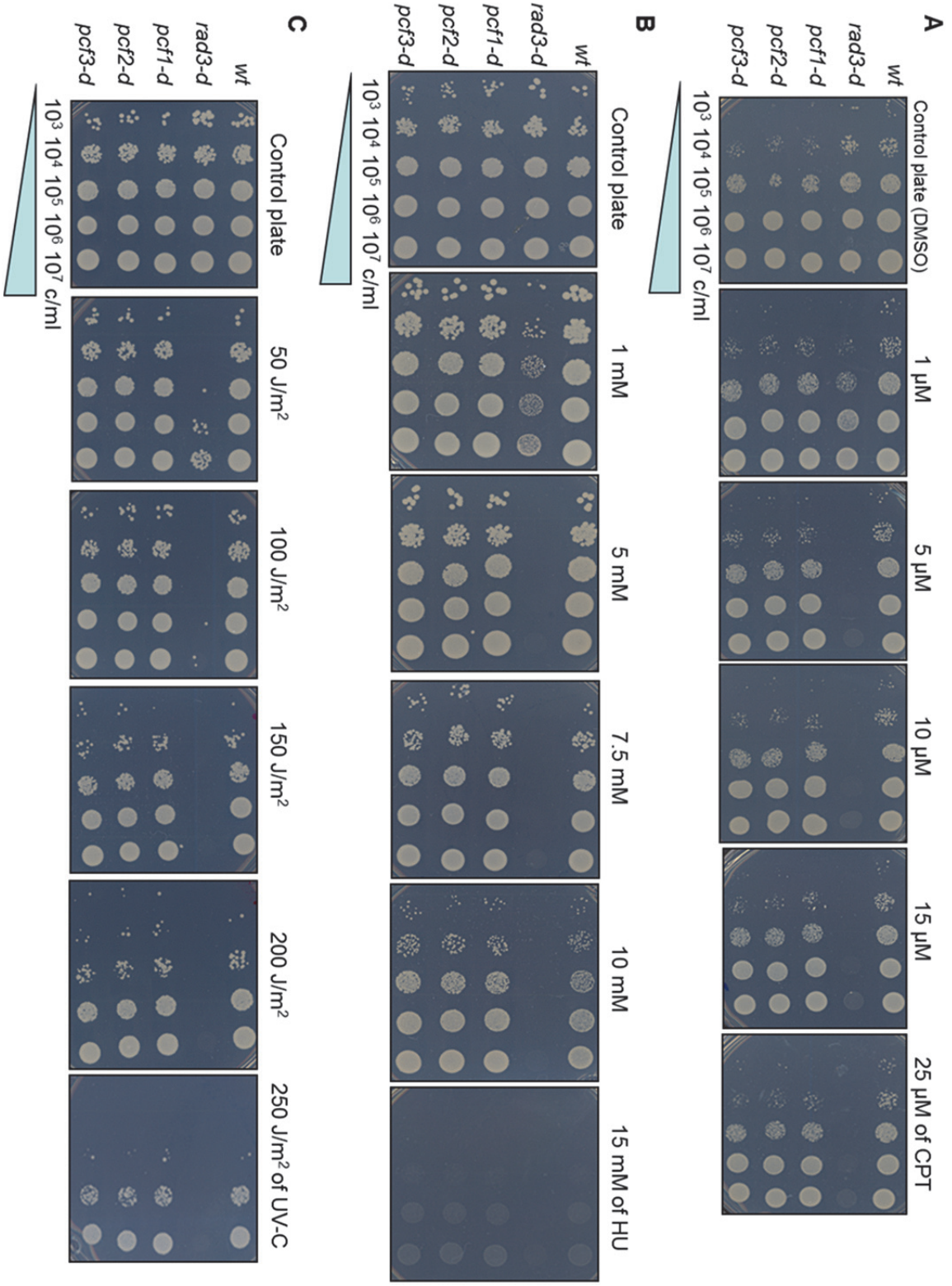


Figure n. 30: Strains defective for CAF-1 are not sensitive to CPT, HU or UV-C exposure. A) YE plates with different concentrations of CPT, on which serial tenfold-dilutions from indicated strains were spotted. B) YE plates containing different concentrations of HU. C) YE plates exposed to different doses of UV-C. *rad3* is the gene coding for the checkpoint kinase Rad3$^{ATR/Mec1}$ and the strain deleted for *rad3* was used as control for the quality of the plates, because of its hypersensitivity to DNA damaging and replication blocking agents.

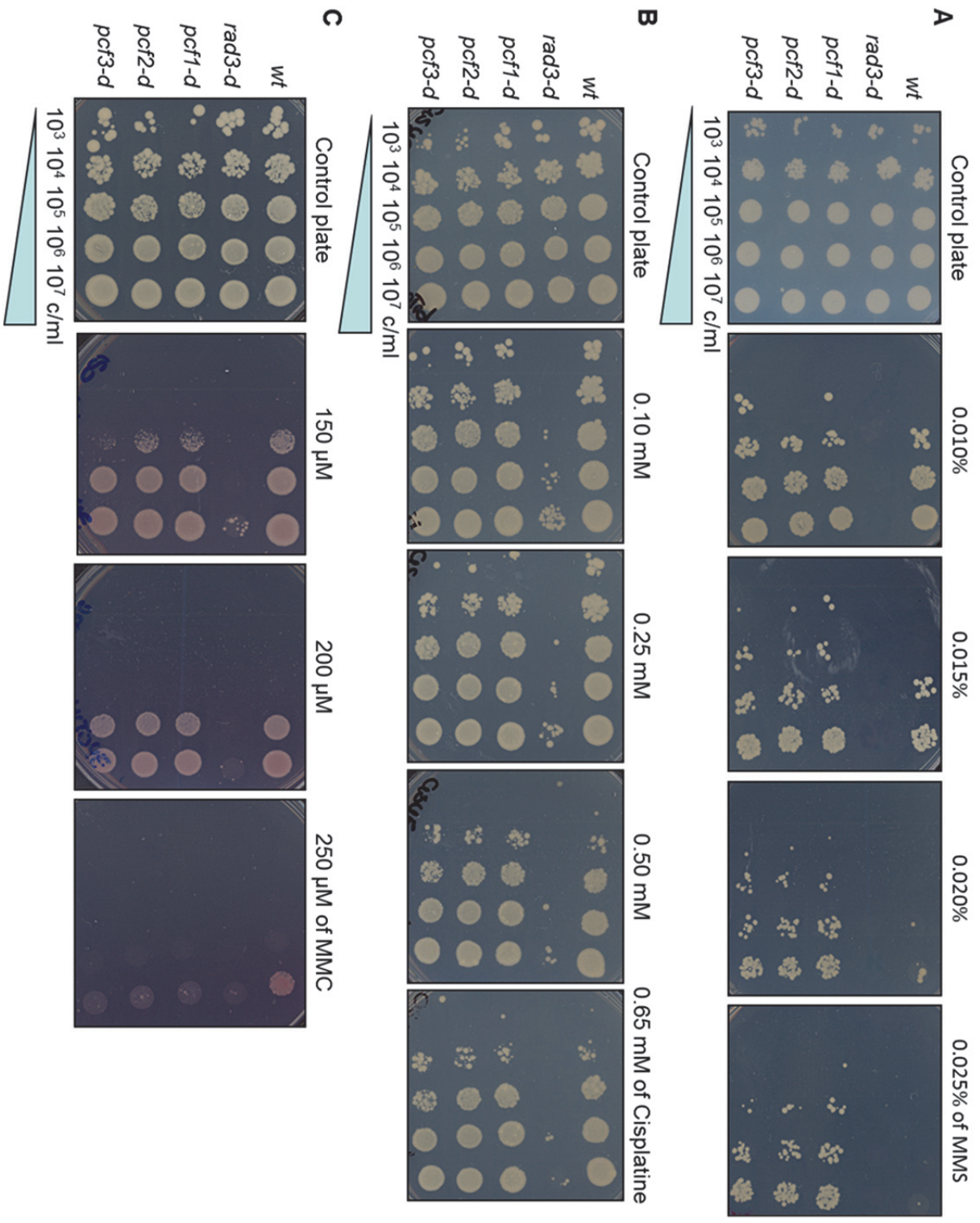


Figure n. 31: Strains defective for CAF-1 are not sensitive to MMC, Cisplatine or MMC exposure A) YE plates containing different concentrations of MMS, on which serial tenfold-dilutions from indicated strains were spotted. B) YE plates containing different concentrations of Cisplatine. C) YE plates containing different concentrations of MMC. The strain deleted for *rad3* was used as control for the quality of the plates, because it is hypersensitive to DNA damaging and replication blocking agents.

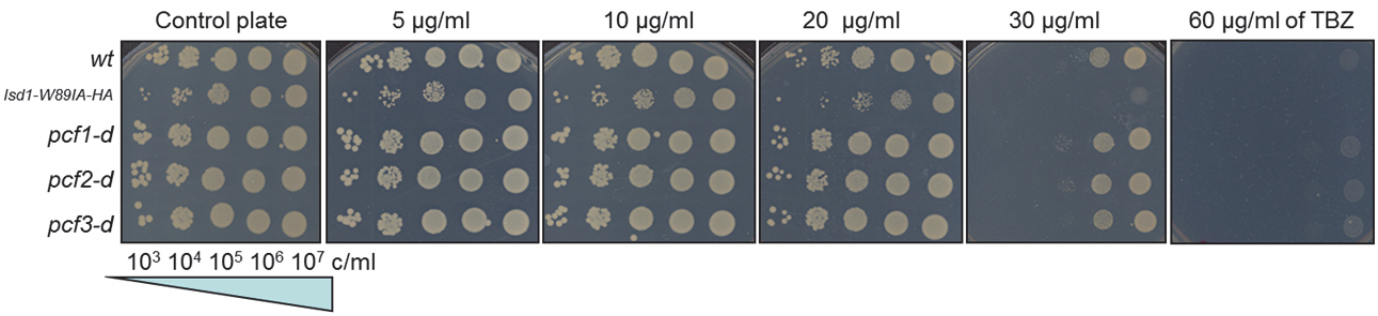


Figure n. 32: Strains defective for CAF-1 are not sensitive to TBZ. YE plates containing different concentrations of TBZ, on which serial tenfold-dilutions from indicated strains were spotted. The strains tested were *wt, lsd1-W89IA-HA, pcf1-d, pcf2-d* and *pcf3-d.* The strain *lsd1-W89IA-HA* harbour a mutation of the gene *lsd1*, whose product is the histone demethylase SWIRM1. The mutation confers a hypersensitivity of this strain to TBZ.

1.4. Genetic interactions with the histone chaperone HIRA

HIRA complex is a H3-H4 histone chaperone required for replication independent chromatin assembly, for example during gene transcription and gene silencing (See Introduction 3.2.3). In *S. pombe,* the Hir complex (HIRA homologue) is composed of three subunits: Hip3 (gene: SPBC31F10.14c), Hip1 (gene: SPBC31F10.13c) and Slm9 (gene: SPBC15D4.03). Deletion of either *hip1* or *slm9* leads to cell cycle delay, poor recovery from nitrogen starvation, increased chromosome loss, sensitivity to DNA damaging agents, increased sensitivity to spindle damage and decreased transcriptional silencing in the outer centromere repeats. In addition, deletion of *hip1* alone leads to derepression of core histone genes expression, outside S-phase. In *S. cerevisiae,* combining mutations in CAC genes (encoded the CAF-1 subunits) and HIR genes results in increased sensitivity to DNA-damaging agents and in a synergistic decrease in gene silencing at both mating type and telomeric loci (Kaufman, Cohen et al. 1998; Kanoh and Russell 2000; Sutton, Bucaria et al. 2001; Blackwell, Martin et al. 2004; Greenall, Williams et al. 2006; Anderson, Kagansky et al. 2010).

To test the genetic interactions between HIRA and CAF-1, I crossed a *pcf1-d* strain with two strains, respectively deleted for *hip1* or *slm9* genes (Figure n. 33). Regarding the *hip1-d* x *pcf1-d* cross, it appears that the double mutant *hip1-d pcf1-d* is unviable because only 2 spores out of 43 *hip1-d pcf1-d* expected were viable. This low frequency of viable *hip1-d pcf1-d* spores is not due to a bias in meiotic recombination as the two genes are not genetically linked. The two viable spores could be in fact heterozygous diploid.

Regarding the second cross, the double mutant *slm9-d pcf1-d* seems to be viable because 15 spores out of 36 were viable. **The co-lethality of the double mutant *pcf1-d hip1-d* could be**

due to redundant functions between CAF-1 and HIRA in maintaining the correct histone pool during the cell cycle, via the selective transcription of core histone genes. Indeed, CAF-1 seems to play a role, directly or indirectly in transcription processes (Kim, Seol et al. 2009; Heyd, Chen et al. 2011) and an excess of histones in the cell might have deleterious effects on cell survival.

I performed preliminary tests with the double mutant *pcf1-d slm9-d* to test its sensitivity to HU, CPT and UV-C treatment, compared to each single mutant. However, the double mutant does not present any significant increased sensitivity compared to the single mutant *slm9-d* (data not showed).

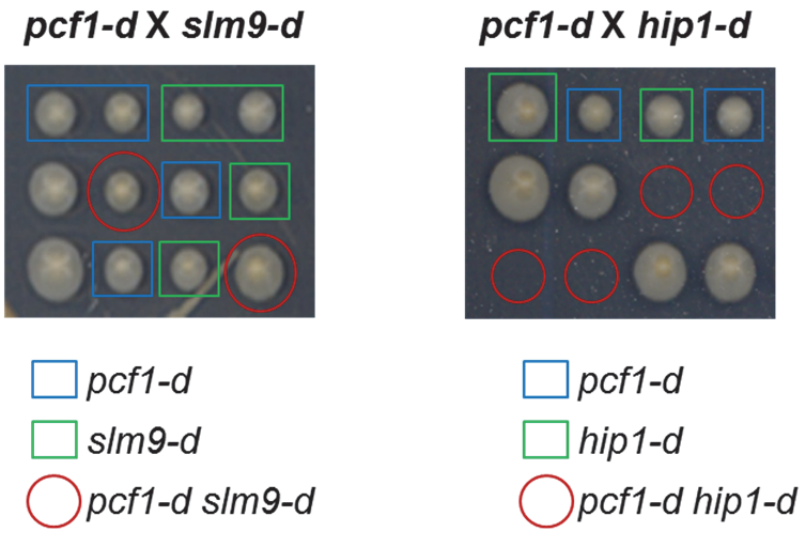


Figure n. 33: Genetic interaction between CAF-1 and HIRA Left panel: tetrads from the cross between *pcf1-d* and *slm9-d* strains are represented. Blue squares indicate *pcf1-d* spores, green squares indicate *slm9-d* spores and red circles indicate the double mutant *pcf1-d slm9-d.* Right panel: tetrads from the cross between *pcf1-d* and *hip1-d* strains are represented. Blue squares indicate *pcf1-d* spores, green squares indicate *hip1-d* spores and red circles indicate the double mutant *pcf1-d hip1-d.*

2. CAF-1 IS DISPENSABLE FOR ALLELIC RECOMBINATION BUT NOT FOR ECTOPIC RECOMBINATION

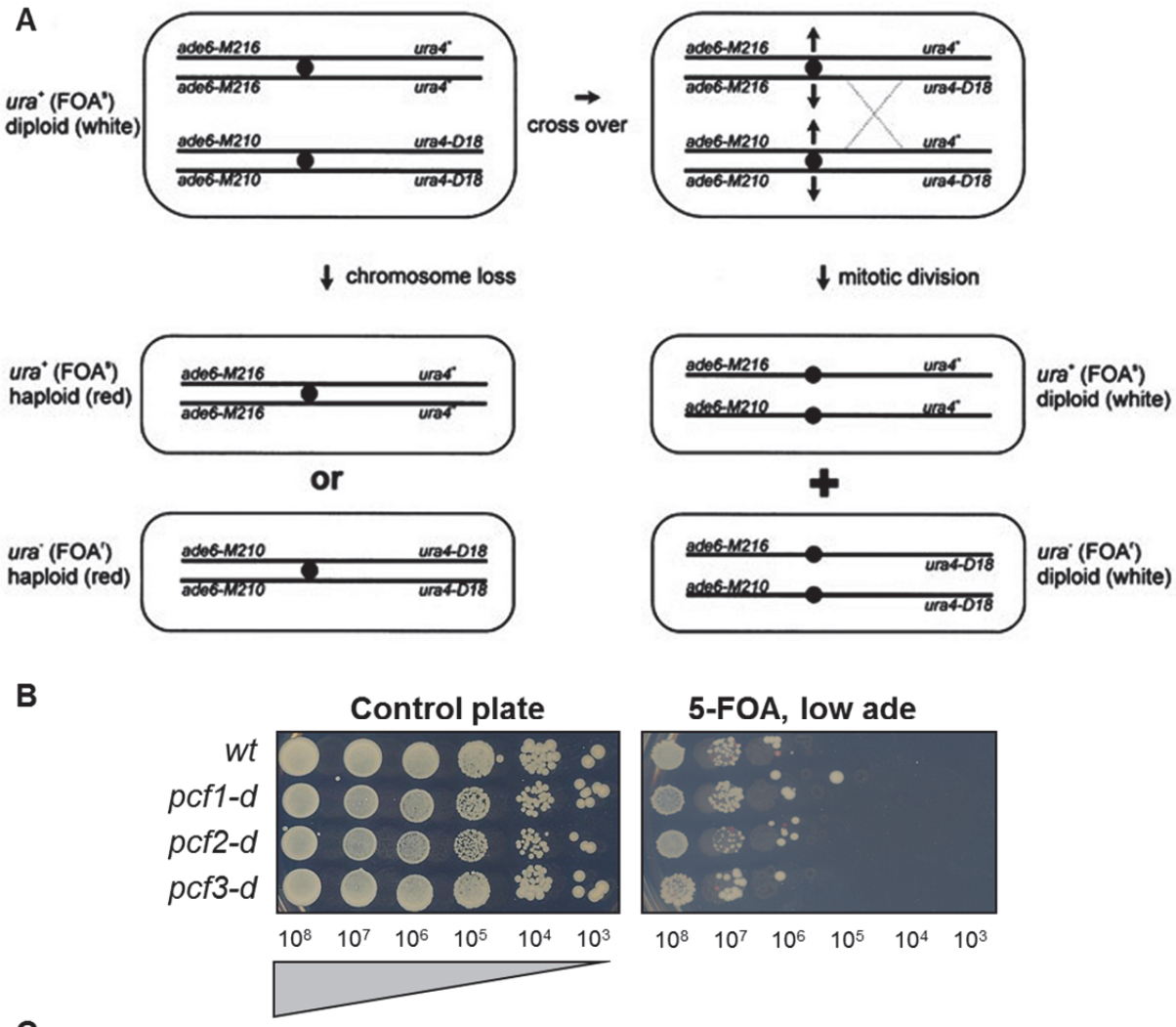
In this chapter, I investigated the contribution of CAF-1 to mechanisms of homologous recombination. Therefore, I analyzed the impact of CAF-1 defects on both allelic recombination (recombination between sister-chromatids and homologous chromosomes) and on ectopic recombination (recombination between dispersed repeated sequences), both spontaneously or induced by replication-blocks.

2.1. Allelic recombination between homologous chromosomes

To achieve the understanding of the role of CAF-1 in homologous recombination, I used an assay to analyze allelic recombination between homologous chromosomes in a non-sporulating diploid. This assay allows the score of cross-over events between the two homologous chromosomes III. Moreover, this assay also permits to score the frequency of loss of chromosome III (Figure n. 34A) (Hartsuiker, Vaessen et al. 2001).

The non-sporulating diploid harbors two different alleles of *ade6* on each chromosome III (*ade6-M216* and *ade6*-M210) that allow an intra-allelic complementation. Thus, the diploid is prototroph for the adenine (Ade^+). On the opposite chromosome arm, one homologue harbors the *wt* $ura4^+$ gene and the other one a deletion of $ura4^+$ (*ura4-D18*). Thus, the diploid is prototroph for the uracil and sensitive to the 5-fluoroorotic acid drug (Ura^+ $5\text{-}FOA^S$). A crossover event between the centromere and the *ura4* locus with subsequent mitotic division, leads to a diploid harbouring *ura4D-18* on the two chromosomes III, therefore leading to an Ade^+ $5\text{-}FOA^R$ diploid. In contrast, loss of one homologue chromosome III leads to an aneuploid harbouring only one chromosome III and conferring an Ade^- $5\text{-}FOA^R$ phenotype. Both Ade^+ and Ade^- cells can be selected on low-adenine media which confers white-color to Ade^+ diploid and red-color to Ade^- diploid.

All diploids deleted for *pcf1*, *pcf2* or *pcf3* showed a rate of cross-overs between homologous chromosomes and a rate of chromosome loss similar to the ones of the *wt* strain (Figure n. 34B and C). Furthermore, I have found a small but not significant increase of chromosome loss in *pcf1-d* (1.7 fold over *wt*) but not in *pcf2-d* or *pcf3-d* strains.



C

Strains	Rate of recombination event/cell/division x10^{-4}	Fold increase over *wt*	Rate of chromosome loss event/cell/division x 10^{-5}	Fold increase over *wt*
wt	1.17 (0.92 – 1.76)	--	4.86 (3.56 – 5.87)	--
pcf1-d	1.53 (1.03 – 1.63)	1.3	8.23 (4.71 – 16.10)	1.7
pcf2-d	1.39 (1.12 – 2.68)	1.2	4.46 (2.98 - 6.23)	0.9
pcf3-d	1.67 (1.26 – 2.26)	1.4	5.45 (5.16 – 9.74)	1.1

Figure n. 34: CAF-1 is dispensable for recombination between homologous chromosomes and chromosomes loss. A) Scheme of the non-sporulating diploid strain used for the assay and representation of the events (cross over and chromosome loss) that can occur. The resulting phenotypes in term of 5-FOA resistance and growth on low-adenine media are also reported. B) Serial tenfold-dilution from indicated strains, spotted onto YE plate (control plate) and in plate containing low- adenine and 5-FOA. C) Rate of recombination events and rate of chromosome loss in indicated strains. The values correspond to the mean of three independent rates, each one being median of 13 independent cultures. The confidence interval is calculated at 95% (enclosed in bracket) and the fold increase over *wt* is indicated for each strain.

2.2. Allelic recombination between sister-chromatids

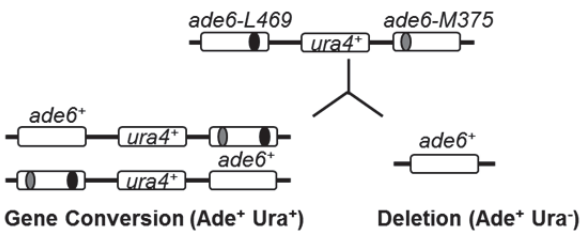
To further investigate the role of CAF-1 in allelic recombination, I analyzed CAF-1 involvement in sister-chromatid exchange. For this purpose, I used a strain harboring two inactivated *ade6* alleles orientated in direct repeats (*ade6-L469* and *ade6-M375* alleles), separated by the *ura4*$^+$ marker (Schuchert, Kohli et al. 1988; Hartsuiker, Vaessen et al. 2001) (Figure n. 35A). A wild-type *ade6*$^+$ gene can be restored by different intra or inter-chromatids recombination processes (Schuchert, Kohli et al. 1988):

- Gene conversion that gives rise to *ade6*$^+$ *ura4*$^+$ cells. The gene serving as donor is either on the same chromatid (intra-sister recombination) or on the other sister chromatid (inter-sister recombination).
- Gene conversion associated to cross-over, that leads to *ade6*$^+$ *ura4*$^-$ cells.
- Intra-chromatid Single Strand Annealing (SSA), that gives rise to *ade6*$^+$ *ura4*$^-$cells.

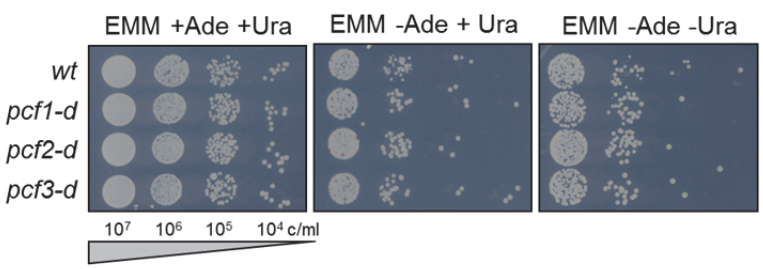
Strains deleted for *pcf1, pcf2* or *pcf3* did not present any significant differences in the rate of sister-chromatid exchange compared to *wt* strain, regardless the type of recombinant analyzed (gene conversion or deletion), (Figure n. 35B and C). Thus, I concluded that CAF-1 is dispensable for sister-chromatid recombination in *S. pombe*. These data are coherent with the fact that defects in CAF-1 do not lead to an increased sensitivity to DNA-damaging agents, suggesting that post-replication repair mediated by recombination between sister-chromatid remains functional in the absence of CAF-1.

To further reinforce this conclusion, I analyzed the frequency of sister-chromatid recombination induced by camptothecin (CPT). As previously reported by Ahn and colleagues, CPT-treatment induces a slight (1.5 fold over spontaneous events), but significant, increase in the frequency of gene conversion and a 3 fold increase in the frequency of deletions (Ahn, Osman et al. 2005) (Figure n. 35D). Nonetheless, a similar induction in sister-chromatid recombination by CPT-treatment was observed in *pcf1-d* cells (Figure n. 35D). Therefore, I concluded that, in the absence of CAF-1, the potential reduction in nucleosome density behind the fork does not lead to an increased level of sister chromatid exchanges, even in response to replication stress.

A



B



C

Strains	Rate of Gene Conversion (event/cell/division) x10^{-5}	Fold increase over *wt*	Rate of deletion (event/cell/division) x10^{-5}	Fold increase over *wt*
wild type	2.68 (1.94-5.27)	--	8.86 (5.42-17.2)	--
pcf1-d	4.13 (3.45-9.22)	**1.54**	13.8 (10.1-13.9)	**1.55**
pcf2-d	6.96 (3.81-7.85)	**2.59**	11.9 (9.65-18.8)	**1.40**
pcf3-d	4.86 (3.39-7.91)	**1.81**	10.5 (8.19- 14.2)	**1.18**

D

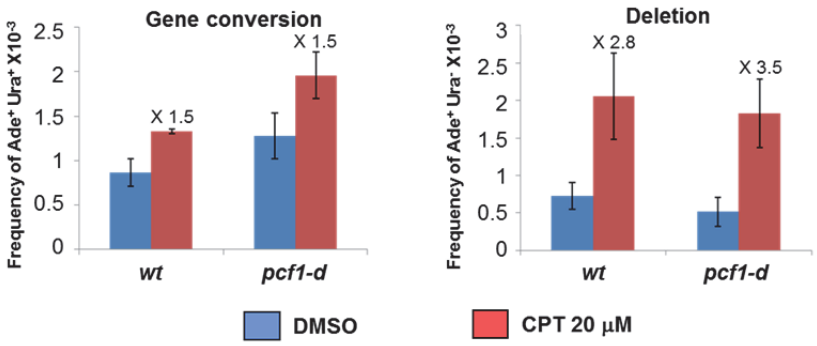


Figure n. 35: CAF-1 is dispensable for spontaneous and CPT-induced sister-chromatids recombination. A) Scheme of recombination substrate and recombinant products. The black and the gray circles indicate the location of the mutation present in *ade6-L469* and *ade6-M375* respectively. B) Serial tenfold-dilution from indicated strains, spotted on indicated media. C) Rate of gene conversion and of deletions in indicated strains. The numbers represent the mean of three independent rates, each one being median of 11 independent cultures. The confidence interval is calculated at 95% (enclosed in bracket) and fold increase over *wt* is indicated for each strain. D) Frequency of gene conversion and deletion in a *wt* and *pcf1-d* strain, after 4 hours treatment with 20µM of CPT. DMSO was used as control vehicle. The values reported in the histogram are the mean of two independent experiments and the error bars indicate the standard deviation. The numbers on the top of the bars indicate the induction of gene conversion and deletion by CPT-treatment.

2.3. Repair of a site-specific polar DSB by recombination between sister-chromatids

When the replication fork collides with an unrepaired single-strand break (SSB) or nick, a polar one-ended double-strand break (DSB) is formed. To repair such replicative DSB, homologous recombination uses the closer homologous sequence, the sister chromatid, thus restoring the fork structure and allowing replication to restart (Arcangioli 2000).

To confirm data obtained for the role of CAF-1 in the repair of replicative DSBs by homologous recombination, I made use of a genetic system that allows the repair of a unique broken replication fork at a specific locus to be analyzed (Roseaulin, Yamada et al. 2008). In *S. pombe*, a stable site-specific and strand-specific DNA lesion was identified at the *mat1* locus (Klar and Miglio 1986; Arcangioli 1998). The lesion was described as a Single Strand Break (SSB) with free 3′OH and 5′OH termini (Kaykov and Arcangioli, 2004). Arcangioli and colleagues showed that the SSB occurs on the neo-synthesized lagging strand during *mat1* DNA replication. This SSB remains stable throughout the next cell cycle. During the following round of DNA replication, fork passage through the SSB converts the SSB into a polar blunt-ended DSB on the leading strand (Kaykov, Holmes et al. 2004; Holmes, Kaykov et al. 2005). The mode of repair of the polar DSB depends on the presence or absence of homologous sequences for repair. In a *wt* strain harboring a switchable *mat* configuration, the two silent donor cassettes (*mat2P* and *mat3M*) are located in a heterocromatic region, and provides intact DNA templates for recombinational repair, thus allowing mating type switching (Egel 2005; Roseaulin, Yamada et al. 2008) (Figure n. 36A). In the absence of silent cassettes (*mat 2,3Δ*), the polar DSB is repaired by homologous recombination using the sister-chromatid. It was reported that inducing the strand-specific polar DSB at *mat1* is sufficient to induce cell death in the absence of homologous recombination (Roseaulin, Yamada et al. 2008). Thus, this genetic system is a powerful tool to analyze genetic requirement to repair replicative DSBs, by sister-chromatid recombination.

To investigate the role of CAF-1 in the repair of a replicative DSB by sister-chromatid recombination, I created a strain *mat1-PΔ17* (-SSB, + donors) deleted for *pcf1*. The *PΔ17* corresponds to small deletion in *mat1* that inhibits SSB formation, but leads to the expression of the P mating-type (Arcangioli and Klar 1991; Roseaulin, Yamada et al., 2008). I crossed the *h+ mat1-PΔ17 pcf1-d* strain with the h^- *mat1-M2,3*Δ (+SSB, - donors) strain. The resulted

diploid was selected and spores were analyzed for the presence of the SSB (h^-), the presence of the donors (h^+) and the deletion of *pcf1*.

The data showed that *pcf1-d* spores were viable, regardless of the presence or not of the SSB and the donors, indicating that CAF-1 is not essential to repair the polar DSB in the absence of donors (Figure n. 36B). Therefore, I concluded that CAF-1 is dispensable for the repair of replicative DSB by sister-chromatid recombination and these data are in agreement with the data presented above.

Altogether, the data obtained support that CAF-1 is dispensable for allelic recombination and for the repair of DSBs by sister-chromatid recombination.

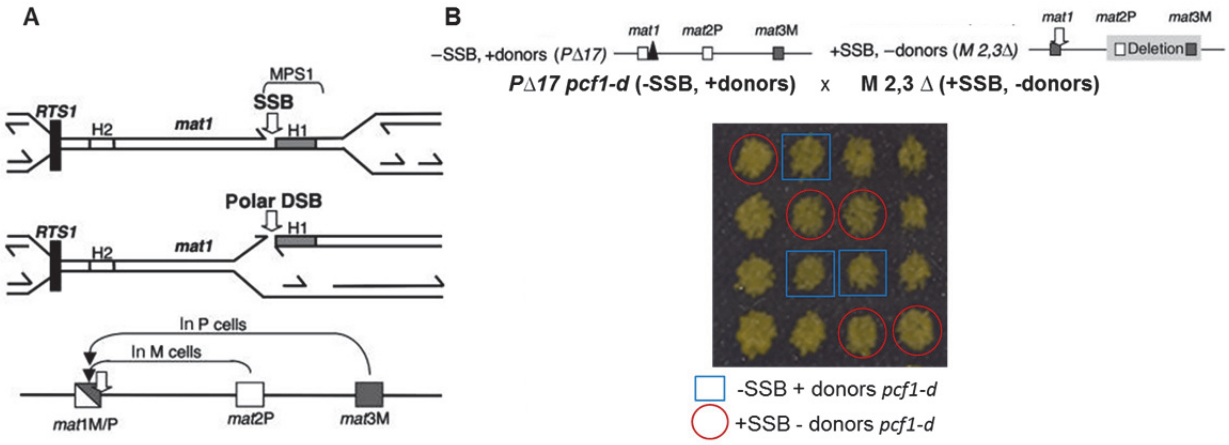


Figure n. 36: CAF-1 is dispensable for the repair of replicative DSB by sister-chromatid recombination. A) Scheme of a site-specific and strand-specific break at *mat1* locus. The white arrow indicates the SSB which is converted in a polar DSB. The black rectangle represents the *RTS1* sequence at the centromere-proximal side of *mat1* and MPS1 indicates *mat1* pause site. The last scheme on the bottom represents the MT switching in a wild-type strain. The white square is the *mat2P* locus and the grey square is the *mat3M*. The *mat2P* and the *mat3M* provide the intact DNA template, respectively in M and P cells (Roseaulin, Yamada et al., 2008). B) Tested strains: *PΔ17 pcf1-d* is deficient for the SSB (black triangle) but harbours the donors sequences (*mat2P* and *mat3M*); *M2,3*Δ is proficient for SSB (white arrow), but lacks the donors sequences (grey rectangle). Bottom panel: tetrads from the cross of these two strains. Blue squares indicate spores deleted for *pcf1* with no SSB and harbouring the donors sequences. Red circles indicate spores deleted for *pcf1* experiencing the SSB, but without donors sequences.

2.4. Fork-arrest-induced ectopic recombination

Then, I investigated the role of CAF-1 in ectopic recombination. For this purpose, I made use of the *RTS1*-RFB genetic system that allows the site-specific block of a replication fork to be induced. The activity of the Replication Fork Barrier (RFB) *RTS1* is mediated by the binding of the Rtf1 protein to the *RTS1* sequence (see introduction, Figure n. 25A). Thus, to control the activity of the *RTS1*-RFB, the transcription level of *rtf1* is regulated via the thiamine repressible promoter *nmt41*. In the presence of thiamine in the media, the *RTS1*-RFB is not active (defined as OFF condition) and in the absence of thiamine in the media, the *RTS1*-RFB is active and the replication fork is arrested at the *ura4* locus (defined as ON condition) (see Introduction, Figure n. 25B). The *RTS1*-RFB was previously shown to behave as hot spot of recombination and chromosomal rearrangements (Lambert, Watson et al. 2005).

It was previously reported that recombination mutants are sensitive to the induction of the *RTS1*-RFB. Therefore, I first analyzed the sensitivity of cells defective for CAF-1 to the induction of the *RTS1*-RFB. As strains defective for CAF-1 show a severe slow grow phenotype in EMM-NH_4Cl, all experiments were performed using EMM-Glu as minimal media. I performed both a serial dilution assay and a survival test in the presence (OFF condition) or in the absence (ON condition) of thiamine in the media. I observed that CAF-1 is not required to promote cell viability, upon induction of the *RTS1*-RFB. On the contrary, the survival of the *rad22* deleted strain was severely decreased when the fork-arrest at *ura4* was induced. The reason is the inability of the *rad22-d* strain to restart the blocked replication fork by recombination (Figure n 37A and B).

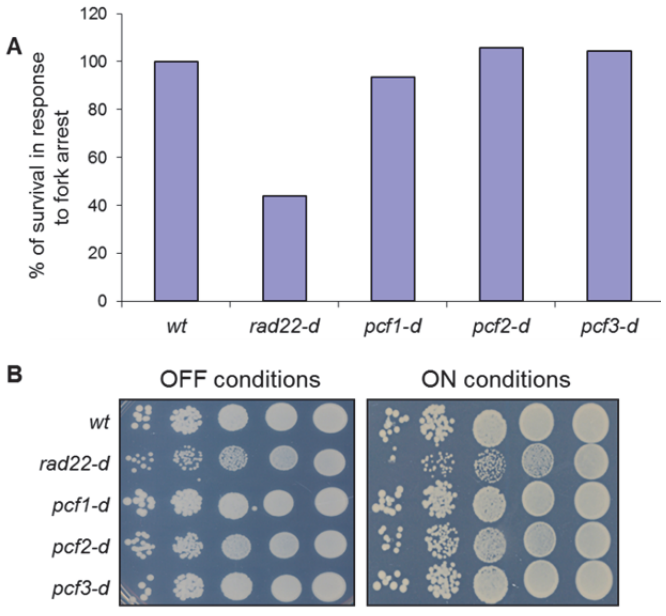


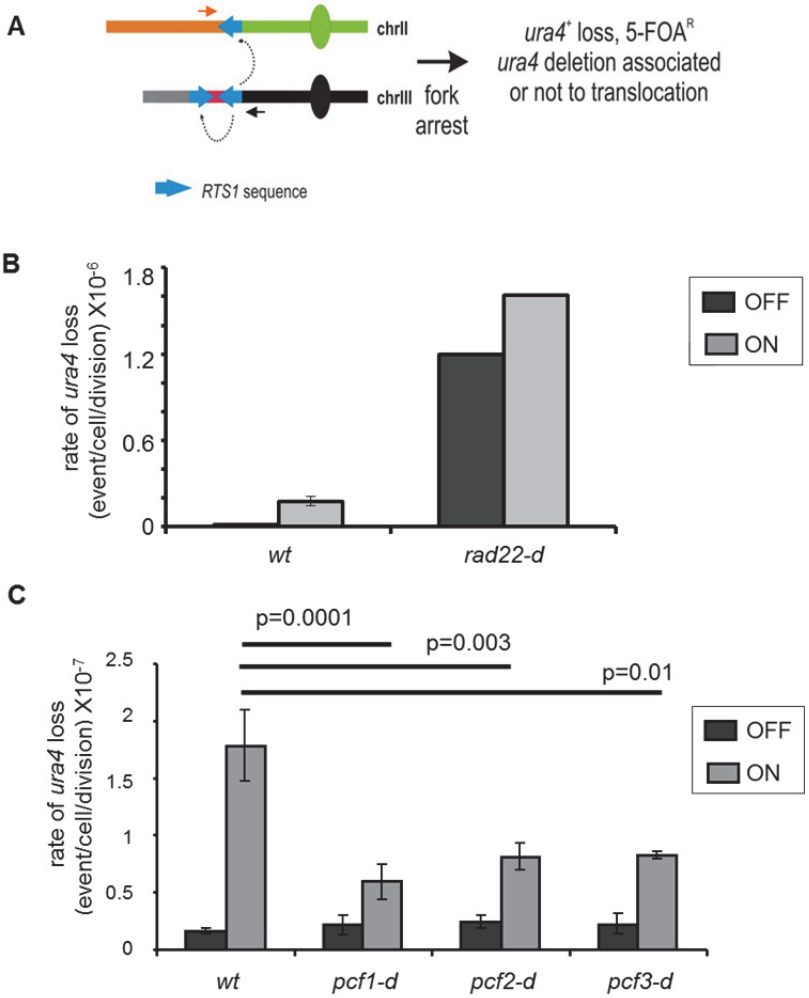
Figure n. 37: CAF-1 is dispensable for cell viability, upon induction of the *RTS1*-RFB. A) Cell survival of indicated strains experiencing fork arrest at *RuraR*. Values are the mean of two independent experiments. B) Serial 10-fold dilutions of indicated strains, spotted onto EMM-Glu containing thiamine (pause OFF) or not (pause ON).

Replication forks arrested at the *RTS1*-RFB are restarted by homologous recombination (Lambert et al., 2010). Fork-restart by recombination occasionally results in ectopic recombination with the *RTS1* sequence naturally located near the *mat1* locus on chromosome II, thus leading to the loss of *ura4*$^{+}$ marker (5-FOA^{R} cells). Analysis of chromosomal rearrangements by PCR showed that ectopic recombination induced by fork-arrest results in translocations and genomic deletions (Figure n. 38A; Iraqui, Chekkal et al. 2012 in press).

Exploiting this assay, my aim was to investigate the role of CAF-1 in ectopic recombination induced by fork-arrest. Induction of the *RTS1*-RFB resulted in 11 fold increased in the rate of *ura4* loss. As previously reported, the rate of *ura4* loss was not increased in *rad22-d* strain in response to fork-arrest (Figure n. 38B). If compared to the wt strain, *rad22-d* strain exhibits a 8 fold decrease in the rate of *ura4* loss upon fork arrest, thus confirming that loss of this marker is mediated by homologous recombination (Figure n. 38B). Strains deleted for *pcf1, pcf2* or *pcf3* showed a similar and significant 3 to 5 fold decrease in the rate of *ura4* loss, compared to *wt* strain, showing that CAF-1 promotes genetic instability induced by the *RTS1*-RFB (Figure n. 38 C and D).

Then, I analyzed by PCR the nature of chromosomal rearrangements mediated by ectopic recombination (Figure n. 39A). In the *wt* strain, the *RTS1*-RFB induced genomic deletion by 19.4 fold and translocations by 29.3 fold, over the OFF condition. In contrast, strains deleted for *pcf1-d*, *pcf2-d* or *pcf3-d* showed a 2.3 to 5.5 fold reduction in genomic deletion and a 3.5 to 9.9 fold reduction in translocation events, compared to *wt* strain (Figure n. 39B and C). Therefore, these data establish that CAF-1 promotes ectopic recombination induced by the *RTS1*-RFB.

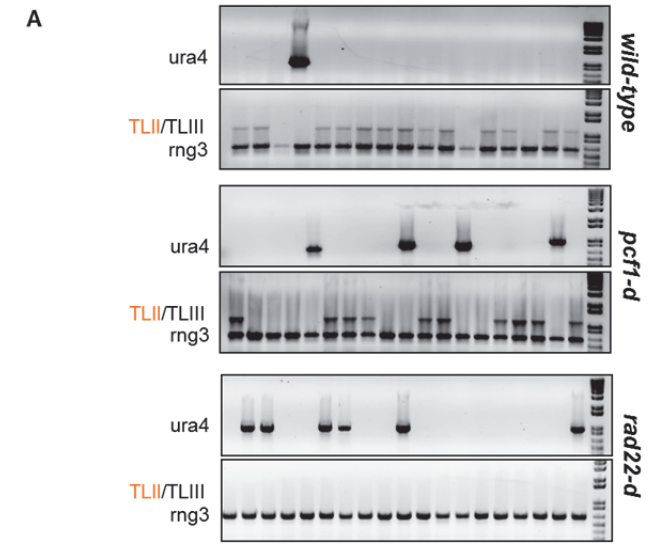
Altogether, my investigations on the role of CAF-1 in homologous recombination lead to the conclusion that, while CAF-1 is dispensable for allelic recombination even in response to replication stress, it promotes ectopic recombination induced by fork-arrest. The role of CAF-1 during homologous recombination is of particular importance when the homology between the donor and the acceptor is limited. Importantly, this pro-recombinogenic function of CAF-1 implies the three subunits, suggesting that the histone-chaperone function is involved in the regulation of recombination. **My data suggest that CAF-1 could be a new regulator of recombination outcomes, especially by acting in non-allelic recombination.**

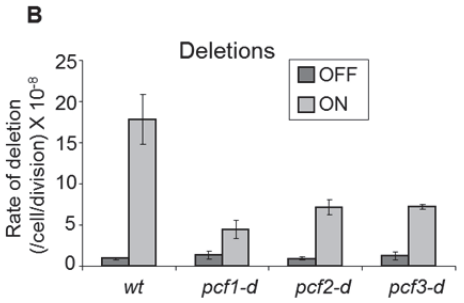


D

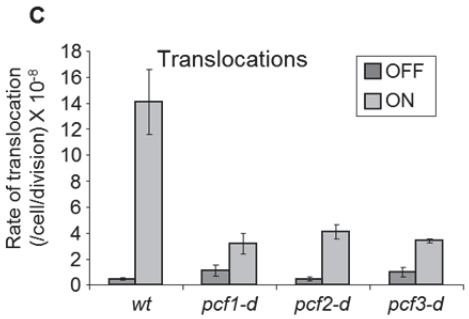
Strain	Induction by RFB (ON/OFF)	Decrease over *wt*
wt	11.1	-
pcf1-d	2.2	5.0
pcf2-d	3.4	3.3
pcf3-d	3.7	3
rad22-d	1.34	8.3

Figure n. 38: Assay to analyze fork-arrest-induced rearrangements between dispersed repeated sequences. A) Induction of the *RTS1*-RFB in *RuraR* cells leads to loss of *ura4* marker, as assayed by 5-FOA resistance. Blue arrows represent *RTS1* sequences and its polarity at *ura4* locus (chr. III) and at *mat* locus (chr. II). The *ura4* gene is represented in red. The black and orange arrows indicate the primers TLIII and TLII, to analyze translocation. Black and green circles represent the centromeres of chromosome III and chromosome II, respectively. B) Rate of *ura4* loss assayed by 5-FOA resistance. 20-30 cultures were analyzed by fluctuation analysis for wild-type and *rad22-d* strains. The rate of *ura4* loss was determined using the median method. C) Rate of *ura4* loss assayed by 5-FOA resistance in indicated strains. The values on the histogram correspond to the mean of at least three independent median rates (11 cultures for each experiment). The error bars represent the standard deviation and the statistical significance was calculated using the non-parametric Mann Whitney test. D) Table indicating the induction of *ura4* loss by the *RTS1*-RFB in indicated strains (rate of *ura4* loss in ON conditions/rate of *ura4* loss in OFF conditions), and the decrease over *wt.*





Strains	Induction in deletion by RFB (ON/OFF)	Decrease over *wt*
wt	19.4	-
pcf1-d	3.5	5.5
pcf2-d	8.4	2.3
pcf3-d	5.9	3.3



Strains	Induction in translocation by RFB (ON/OFF)	Decrease over *wt*
wt	29.3	-
pcf1-d	3.0	9.9
pcf2-d	8.4	3.5
pcf3-d	3.4	8.6

Figure n. 39: CAF-1 promotes ectopic recombination upon fork-arrest induction. A) Examples of colony PCR analysis of 5-FOA^R colonies obtained with *rtf1* induction (ON conditions) in *wt*, *pcf1-d* and *rad22-d* strains. TLII and TLIII primers flank *RTS1* at 120 bp and 160 bp on chr. II and III respectively and amplify a product of 1140 bp (black and orange arrows indicate the primers TLIII and TLII in the Figure 38A). Rng3 primers amplify *rng3* gene, which is an essential gene located between *RuraR* and the telomere, giving a PCR product of 647 bp. Ura4 primers flank *ura4* locus and amplify a product of 960 bp. B) The % of deletion events, as determined by the PCR assay, was used to balance the rate of *ura4* loss. The values reported in the histogram correspond to the mean of 3 independent median rates. Error bars indicate the standard deviation. Right table: induction of genomic deletion by the *RTS1*-RFB (rate of genomic deletion in ON conditions/rate of genomic deletion in OFF conditions) and the relative decrease over wild-type. C) The % of translocation events, as determined by the PCR assay, was used to balance the rate of *ura4* loss. The values reported are means of 3 independent median rates. Error bars correspond to the standard deviation. Right table: induction of genomic translocations by the *RTS1*-RFB (rate of genomic translocations in ON conditions/rate of genomic translocations in OFF conditions) and the relative decrease over wild-type.

3. CAF-1 PROMOTES D-LOOP STABILIZATION

In this chapter, I investigated the impact of CAF-1 defects on homologous recombination at the molecular level. For this purpose, I used the *RuraR* system, based on the *RTS1*-RFB, which allows replication-induced recombination to be analyzed. Fork restart at the *RTS1*-RFB is proposed to be initiated by the unwinding of stalled nascent strands, on which recombination proteins are recruited (see Introduction, Figure n. 26A). Once the initial replication-block is removed, fork-restart occurs without changing template. However, stalled nascent strand can occasionally switch template and anneal with the second *RTS1* sequence, in inverted orientation. This template exchange reaction results in joint-molecules formation (JMs) that are visualized through the analysis of replication intermediates by 2-dimensional gel electrophoresis (2DGE). Two kinds of JMs formed between *RTS1* repeats were reported: JM-A that is proposed to correspond to an extended D-loop (after initiation of DNA synthesis on the non-contiguous template) and JM-B that contains Holliday junction-like structures (HJs-like) formed between the two *RTS1* repeats (see Introduction, Figure n. 26C). D-loop intermediates are thought to be precursors of HJs-like structures. Both JMs are dependent on Rad22, while JM-A depends on both Rhp51 and Rad22. The resolution of HJs-like structures gives rise to three kinds of chromosomal rearrangements: the formation of acentric and dicentric iso-chromosomes (resolution associated to one cross-over) and the switch of *ura4* orientation (resolution associated to two cross-overs) (see Introduction, Figure n. 27A). Chromosomal rearrangements can be scored by restriction fragment length analysis (RFLA) and pulse field gel electrophoresis (PFGE). Therefore, the *RuraR* system allows the different steps of replication-induced recombination to be analyzed: the stability of the arrested fork, the recruitment of recombination factors, the fork-recovery efficiency, the formation and stability of JMs, and the products of their resolution.

3.1. CAF-1 promotes replication-induced recombination by template exchange.

I first studied the role of CAF-1 in the efficiency of replication-induced recombination by analyzing JMs by 2DGE (Figure n. 40A). As previously reported, JMs were efficiently formed in the *wt RuraR* strain upon induction of the *RTS1*-RFB (Figures n. 40B and C). Interestingly, strains defective for CAF-1 (*pcf1-d, pcf2-d* or *pcf3-d* strains) showed a similar 3 to 4 fold reduction in JM-A intensity and a 8 to 11 fold reduction in JM-B intensity, compared

to *wt* strain. These data show that **CAF-1 affects JMs formed between *RTS1* sequences, upon fork arrest, either by promoting their formation or by promoting their stability. Furthermore, as the strains deleted for each of the three subunits exhibit the same phenotype, then the role of CAF-1 in promoting or stabilizing JMs is likely related to its histone chaperone function.**

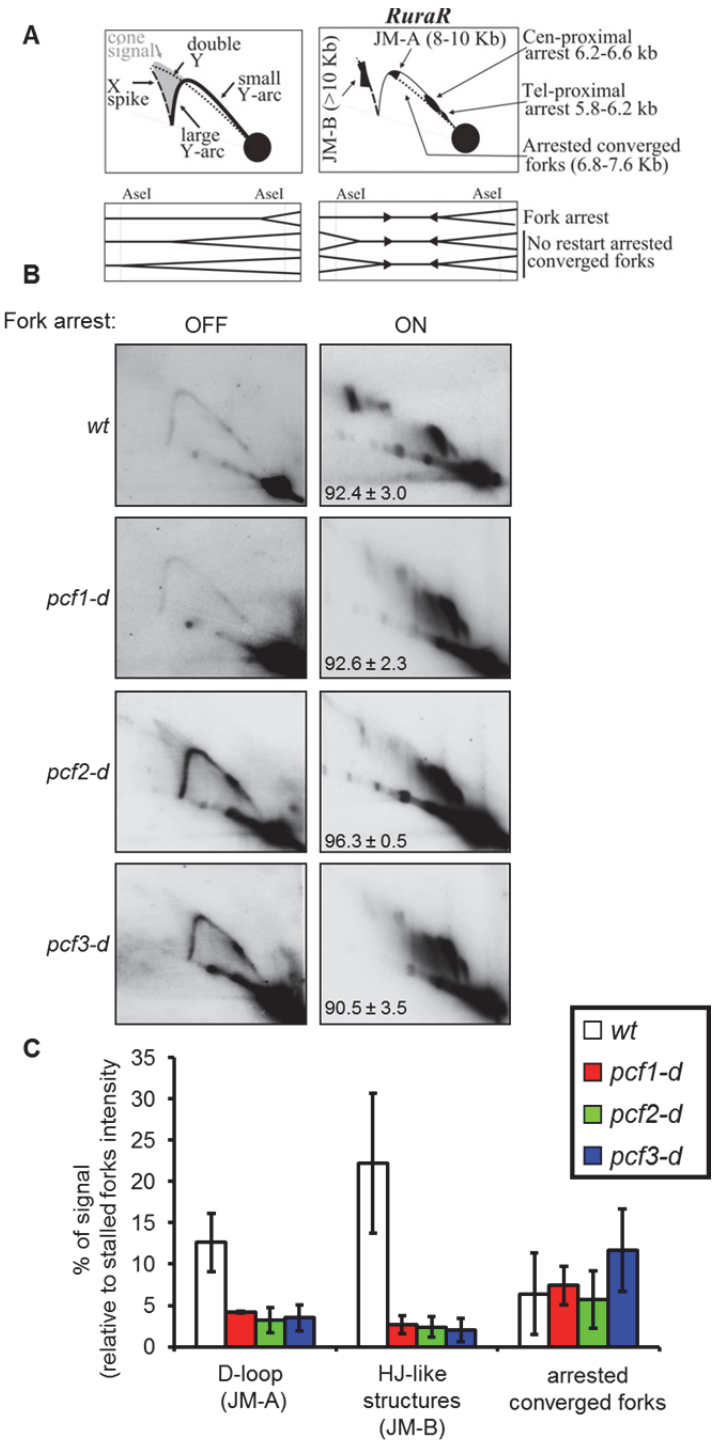


Figure n. 40: CAF-1 defects result in a decreased amount of JMs upon fork-arrest at *RuraR*. A) Diagrams of replication intermediates (RI) within AseI restriction fragment analyzed by 2DGE (top) and their structures (bottom: black arrowheads indicate *RTS1* orientation). Left: RIs observed when *ura4* locus is replicated passively (no fork arrest at *RTS1* barrier). Right: RIs observed in *RuraR* cells upon fork arrest. Expected mass of arrested forks and calculated mass of JMs are given. JM-A and JM-B indicate D-loop and HJs like structures, respectively. B) 2DGE analysis of RIs from indicated *RuraR* strains grown for 24 hours with (fork arrest OFF) or without thiamine (fork arrest ON). Numbers indicate the percentage of fork arrested by the *RTS1*-RFB (± standard deviation). C) Quantification of B. The values correspond to the mean of three independent experiments and the error bars correspond to the standard deviation.

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Many hypotheses could be drawn from these experiments (Figure n. 41). The decrease in JMs could be explained by the fact that JMs are resolved faster or by the fact that recombination factors such as Rad22 are not properly recruited at the blocked replication fork. Another possibility is that the stalled replication fork is restarted mainly by direct fork restart pathway, at expense of template exchange or CAF-1 might play a direct role during template exchange reactions. Therefore I investigated the different steps of replication-induced recombination at the molecular level, to identify at which steps CAF-1 acts to promote homologous recombination.

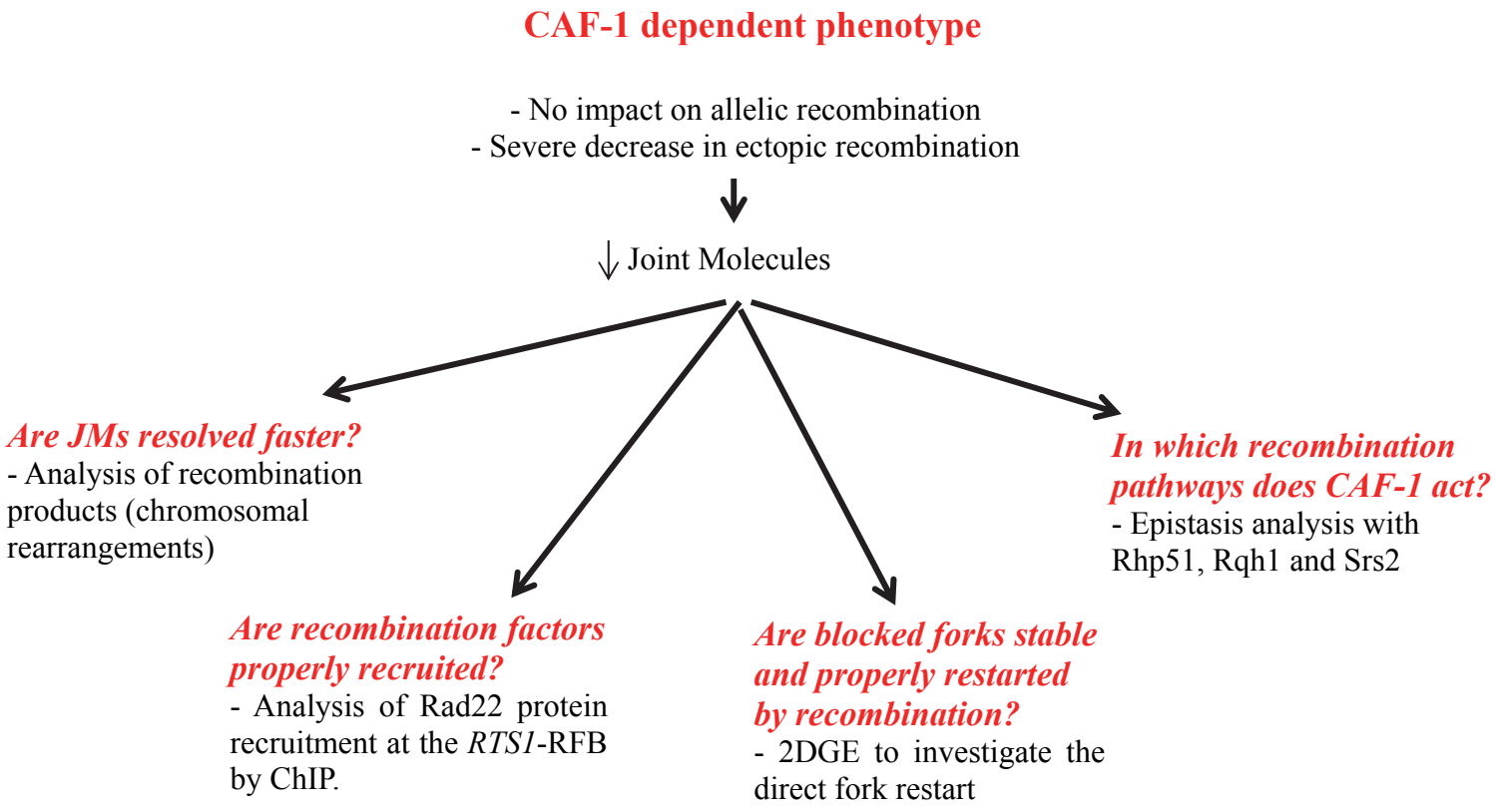


Figure n. 41: hypotheses drawn for CAF-1 dependent phenotype.

One possible explanation for the observed decrease in JMs intensity in the absence of CAF-1 is that JMs are resolved faster, due to a potential reduction in nucleosome density. To verify this hypothesis, I investigated the products of JMs resolution by analyzing chromosomal rearrangements both by RFLA and PFGE.

Firstly, genomic DNA was extracted from cells cultured with thiamine (OFF condition) or after 24 and 48 hours after thiamine removal (ON conditions). Genomic DNA was digested

either with EcoRV or AseI restriction enzymes. The digestion with EcoRV allows the analysis of acentric chromosome formation and the switch of *ura4* orientation. The AseI digestion allows the investigation of the formation of acentric and dicentric chromosomes. Chromosomal rearrangements were quantified as previously described (Lambert, Mizuno et al. 2010 and detailed in the material and methods) and presented as recombination events associated with one (acentric) or two cross-overs (switch of *ura4* orientation). Chromosomal rearrangements accumulated in the *wt RuraR* strain, upon induction of the *RTS1*-RFB (Figures n. 42A and B), as previously reported (Lambert, Mizuno et al. 2010). Compared to the *wt*, a significant reduction (a 2-3 fold decreased, $p<0.03$) in accumulation of both acentric and switch of *ura4* orientation was observed in each single mutant (*pcf1-d pcf2-d* or *pcf3-d)* (Figures n. 42A and B).

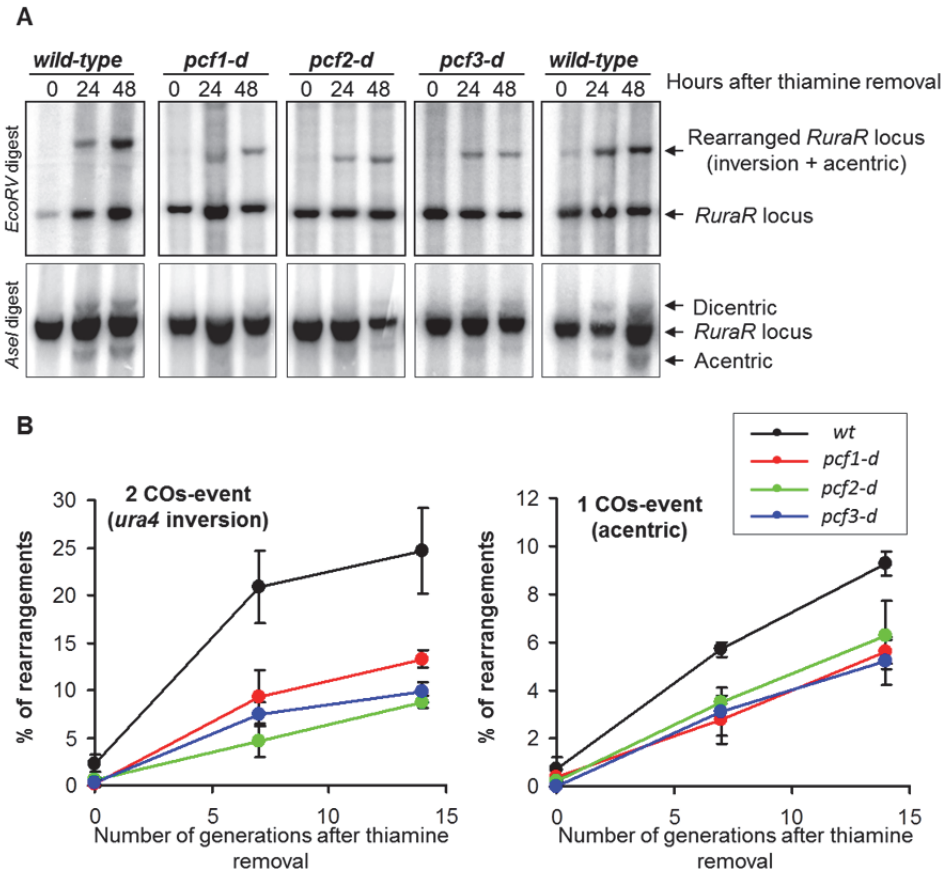


Figure n. 42: Decreased level of products of JMs resolution in the absence of CAF-1. A) RFLA of indicated *RuraR* strains grown in the presence of thiamine (OFF condition) and for 24h and 48h after thiamine removal. B) Kinetics of rearrangements formation according to the number of generation experiencing fork arrest at *RuraR*. The values correspond to the mean of three independent experiments and the error bars indicate the standard error of the mean.

To further confirm these results, I analyzed the formation of acentric iso-chromosome by PFGE in cells cultured with thiamine (OFF conditions) or at 24 h and 48 h after thiamine removal (ON conditions). As previously reported (Lambert, Mizuno et al. 2010), the acentric chromosome accumulated in the *wt RuraR* strain, upon fork arrest at the *RTS1*-RFB (Figures n. 43A and C). In agreement with the data shown in Figure n. 42, I observed a 2-3 fold ($p<0.0003$) reduction in the accumulation of acentric chromosomes in each single mutant, compared to the *wt* strain (Figures n. 43A and C). As this phenotype is common to each single mutated strain, this suggests that the histone chaperone function of CAF-1 is involved in this phenotype. To reinforce this hypothesis, I analyzed the *pcf1-d pcf2-d pcf3-d* triple mutant, in which the three genes encoding for CAF-1 subunits were inactivated. The triple mutant showed a reduction in the accumulation of acentric chromosome to the same extends than each single mutant, demonstrating that deletions of genes encoded CAF-1 subunits are epistatic for replication-induced recombination (Figure n. 43A and C).

Collectively, these data establish that the products of resolution of JMs are decreased in the absence of CAF-1, whatever the type of events (associated with one or with two cross-overs). Thus, I concluded that the decrease in JMs intensity in the absence of CAF-1 is not due to a faster resolution of JMs, but is rather due to defects in their formation or stability. Also, these data support the view that it is the histone chaperone function of CAF-1 that is essential to the formation or stability of JMs. However, further experiments are required to confirm this hypothesis.

Another important point is that defects in CAF-1 do not completely abolish replication-induced recombination (Figure n. 43). Therefore, I verified that the residual level of recombination products remains dependent on the main recombination factor, Rad22. I created two strains, *pcf1-d rad22-d* and *pcf2-d rad22-d* and analyzed the accumulation of acentric chromosomes by PFGE. The acentric chromosomes were not detected in *pcf1-d rad22-d RuraR*, *pcf2-d rad22-d RuraR* and *rad22-d RuraR* strains (Figure n. 43B), thus demonstrating that the remaining level of chromosomal rearrangements induced by the *RTS1*-RFB is indeed dependent on Rad22. Surprisingly, disomic chromosomes III were observed in the *pcf2-d rad22-d,* but not in *pcf1-d rad22-d* strain, independently of the induction of the *RTS1*-RFB. The presence of two chromosomes III could be due to gross chromosomal rearrangements, leading to the production of an abnormal chromosome III (maybe fusion with another one) or to the breakage of chromosome III. The difference between *pcf1-d rad22-d* and *pcf2-d rad22-d* strains could be related to a Pcf2 function that is not shared by the other

subunits of the complex. Nonetheless, these data highlight of a potential interplay between Rad22 and Pcf2 in the maintenance of genome stability.

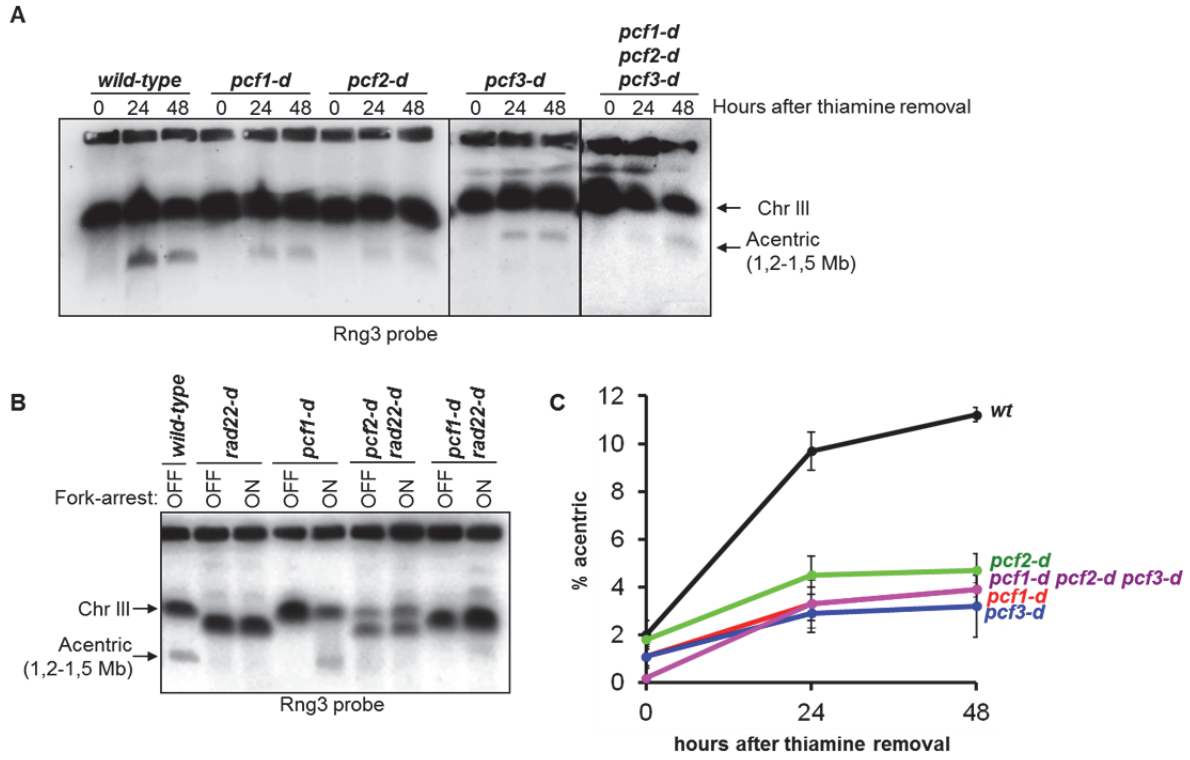


Figure n. 43: Analysis of acentric chromosome formation by PFGE. A) PFGE analysis of *wt, pcf1-d, pcf2-d, pcf3-d* and *pcf1-d pcf2-d pcf3-d* strains in OFF condition and in ON condition (24h and 48h after thiamine removal). The size of the chromosome III is 3.5 Mb and the size of the acentric chromosome is 1.2-1.5 Mb. B) PFGE analysis of *wt, rad22-d, pcf1-d, pcf1-d rad22-d* and *pcf2-d rad22-d* in OFF condition and in ON condition (24h and 48h after thiamine removal). The size of the chromosome III is 3.5 Mb and the size of the acentric chromosome is 1.2-1.5 Mb. C) Kinetic of acentric chromosome accumulation after thiamine removal in indicated *RuraR* strains. The values correspond to the mean of at least three independent experiments and error bars indicate the standard error of the mean.

3.2. Recruitment of Rad22 at arrested forks is not affected in the absence of CAF-1

To further understand the contribution of CAF-1 in replication-induced recombination, I studied the contribution of CAF-1 to early recombination steps, such as formation of recombination centers and Rad22 recruitment at arrested forks.

Firstly, I verified that the expression of Rad22 was not affected by defects in CAF-1. For this purpose, I used a strain in which the C-terminal of Rad22 was fused to the fluorescent tag

GFP. The expression level of Rad22-GFP was not affected by the deletion of *pcf1-d*, *pcf2-d* or *pcf3-d,* as verified by western-blot (Figure n. 44A).

Then, I investigated the formation of spontaneous recombination centers by analyzing Rad22 foci, using 3D-deconvolution fluorescence microscopy. In a *wt* asynchronous population (about 80% of G2 cells), around 10 % of cells show at least one Rad22 focus: 10 % of G2 cells and 10 % of S-phase cells (harbouring a septum as marker of S-phase cells). In the absence of each CAF-1 subunits, 20 to 25 % of cells showed at least one Rad22 focus, and this increased in recombination centers was particularly pronounced in S-phase cells, as 30 to 40 % of S-phase cells exhibited at least one Rad22 focus (Figures n. 44B an C). The accumulation of Rad22 foci, especially in S-phase, is coherent with data reported for other model organisms, in which defects in CAF-1 complex lead to impediments in S-phase progression (Hoek and Stillman 2003; Loyola and Almouzni 2004). Indeed, defective replication-coupled chromatin assembly was reported to cause accumulation of recombination centers in budding yeast, interpreted as accumulation of recombinogenic DNA lesions (Clemente-Ruiz, Gonzalez-Prieto et al. 2011). Nonetheless, such accumulation of recombinogenic DNA structures does not cause cell cycle delay in *S. pombe*, as strains deleted for each CAF-1 subunit exhibit a generation time similar to that of a *wt* strain. Moreover, these data indicate that defects in CAF-1 do not impede assembly of spontaneous recombination centers.

Afterwards, I concentrated on the recruitment of Rad22 at sites of replication forks arrested by the *RTS1*-RFB barrier, using the technique of chromatin immunoprecipitation (ChIP). As previously reported (Lambert, Watson et al., 2005), Rad22-GFP was found enriched at the *RuraR* locus when the *RTS1*-RFB is activated, in a *wt* strain (Figure n. 44D). A similar level of Rad22-GFP recruitment was observed in *pcf1-d* or *pcf2-d* strains. Moreover, Rad22-GFP is significantly recruited up to 1.4 Kb behind arrested forks whatever the genetic background analyzed. Thus, **Rad22 is recruited at arrested forks in the absence of CAF-1, to the same extend than the wild-type strain**. Therefore, the decreased in replication-induced recombination efficiency cannot be explained by a defect in recruiting recombination factors at arrested forks, in the absence of CAF-1.

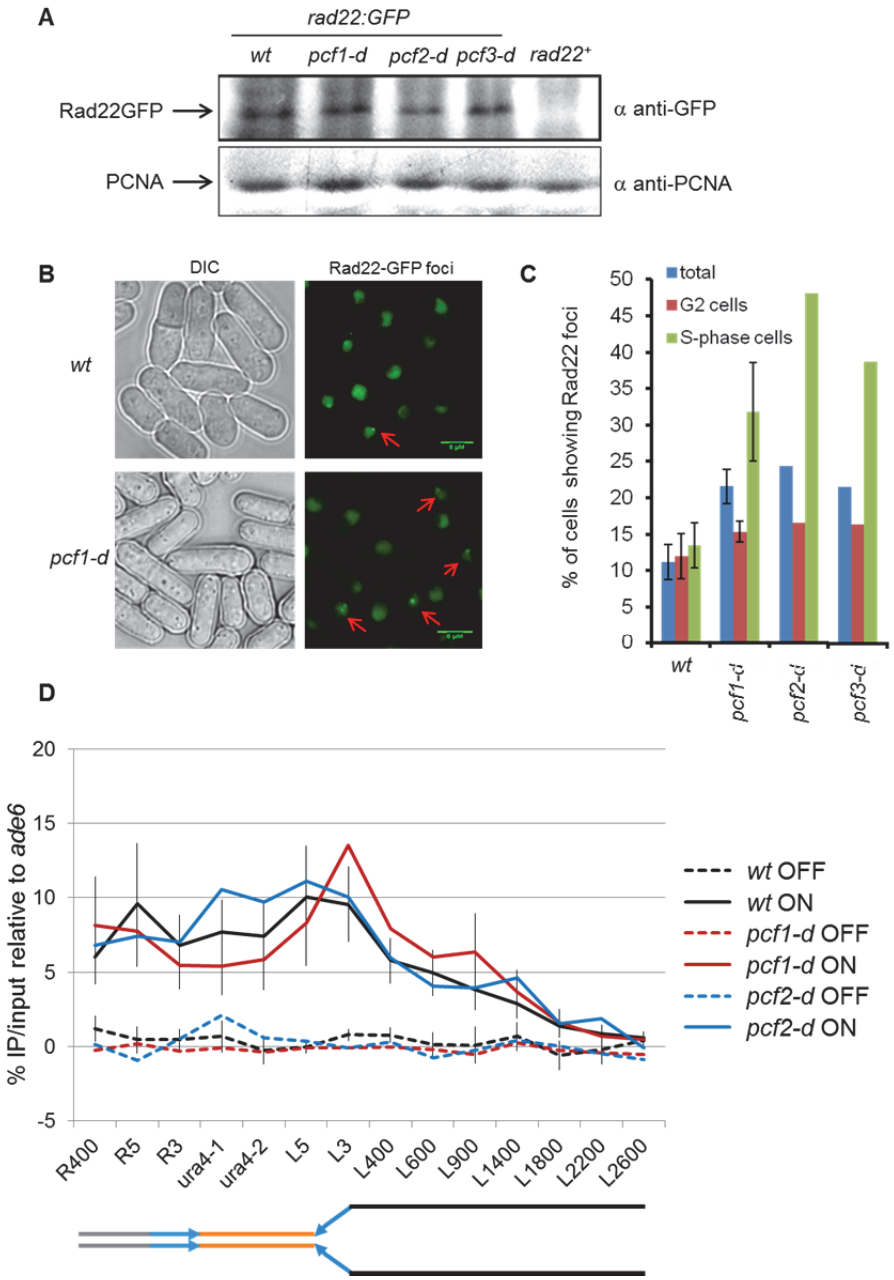


Figure n. 44: Recombination centres and Rad22 recruitment at arrested forks are not affected by the absence of CAF-1. A) Total protein extracts from indicated strains were analyzed by western-blot. Rad22-GFP (68.8 kDa) was detected using an anti-GFP antibody and PCNA (28.9 kDa) was detected using an anti-human PCNA antibody. B) Analysis of spontaneous Rad22-GFP foci in indicated strains. Differential Interference Contrast (DIC-grey) and GFP fluorescent signal (green) in wild-type (top panels) or *pcf1-d* cells (bottom panels), carrying *rad22-gfp*. Red arrows indicate the presence of Rad22-GFP foci. C) Quantification of B in indicated strains. Values are means of at three independent experiments (for *wt* and *pcf1*) or two (*pcf2-d* and *pcf3-d*). Errors bars correspond to standard deviation. At least 300 nuclei were analyzed per strain and experiment. D) Graphic representing the level of Rad22 recruitment at the R*ura4R* locus in indicated strains, grown for 24 hours in the presence (fork-arrest OFF) or the absence of thiamine (fork-arrest ON). Values correspond to the mean of three independent experiments and error bars correspond to the standard error of the mean.

3.3. CAF-1 is dispensable for fork-stability and recovery by recombination

To further understand the role of CAF-1 in replication-induced recombination, I analyzed the stability of arrested forks and their ability to be recovered by recombination, in the absence of CAF-1. Indeed, an increased instability of arrested forks and/or defects in their recovery could also explain the observed decreased intensity of JMs in the absence of CAF-1.

For this purpose, I created strains deleted for each CAF-1 subunits and harbouring a single *RTS1* sequence at *ura4* (*uraR*). In this case, template exchange cannot occur because of the absence of the second *RTS1* repeat; thus arrested forks are restarted exclusively on the initial template. Previous studies have shown that, in the absence of Rad22 or Rhp51, termination signal visualized by 2DGE accumulates at *uraR*, showing that the *RTS1*-RFB behaves as a hot spot of replication termination in the absence of recombination (see Introduction, Figure n. 25D). Therefore, termination signals are used as sign of impaired fork recovery. Analysis of replication intermediates by 2DGE showed that the accumulation of termination signals and the level of arrested forks in *uraR* strains defective for CAF-1 are at the same level than in the *wt* strain (Figures n. 45A-C). Therefore, I concluded that stabilization of arrested forks and their recovery by recombination is neither defective nor more efficient in the absence of CAF-1. Moreover, the data do not support the hypothesis that template exchange (in *RuraR* strain) is decreased in the absence of CAF-1 due to a more efficient engagement of fork restart on the initial template.

The aspect of stabilization of arrested forks in the absence of CAF-1 is a common theme in CAF-1 literature (Clemente-Ruiz, Gonzalez-Prieto et al. 2011; Clemente-Ruiz and Prado 2009; Introduction 3.2.4). A delay in histone deposition in the rear of advancing forks, due to CAF-1 defects, could result in a different chromatin context, favoring processes such as SCE and forks recovery by recombination. However, I demonstrated in S. *pombe* that CAF-1 defects do not affect in appreciable manner the stability of arrested forks. Moreover, I showed that replication-induced SCE are not significantly affected by the lack of CAF-1 function, confirming the marginal role of CAF-1 in maintaining integrity of impeded replication forks. **Altogether, these data suggest that CAF-1 is involved in the stability of JMs rather than in their formation.**

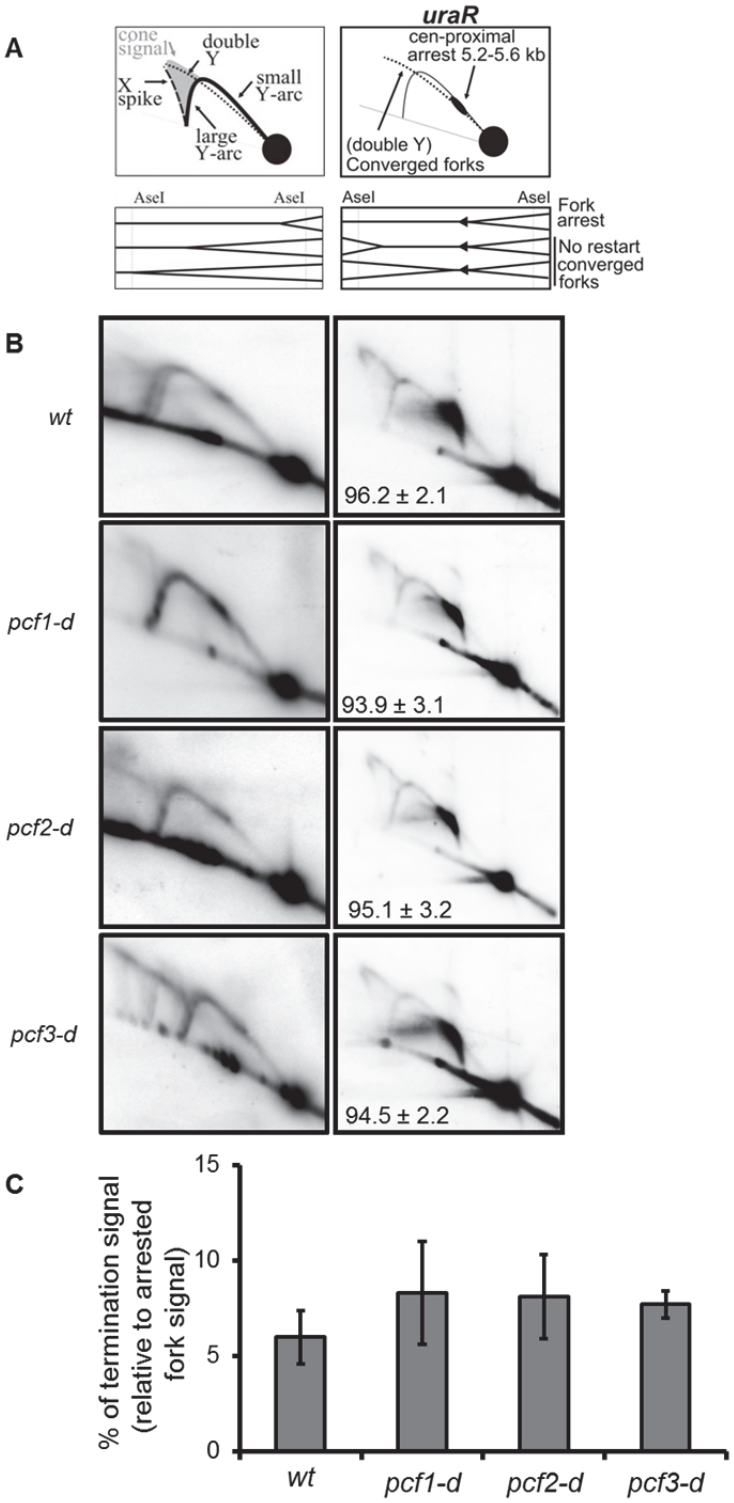


Figure n. 45: Arrested forks are stable and prone to recombination-dependent restart in the absence of CAF-1. A) Diagrams of replication intermediates (RI) within the AseI restriction fragment analyzed by 2DGE (top) and their structures (bottom: black arrowheads indicate *RTS1* orientation. Left: RIs observed when the *ura4* locus is replicated passively (no fork arrest at the *RTS1* barrier). Right: RIs observed in *uraR* cells, upon fork arrest. B) 2DGE analysis of RIs from indicated strains harbouring the *uraR* system, grown for 24 hours in the presence of thiamine (pause OFF) or not (pause ON). Numbers indicate the percentage of fork arrested at *uraR* ± standard deviation. C) Quantification of B. The values correspond to the mean of three independent experiments and the error bars correspond to the standard error of the mean.

3.4. CAF-1 stabilizes Rhp51-dependent Joint-molecules (D-loop)

In the *RuraR* system, template exchange occurs by two pathways:

- A Rad22- and Rhp51-dependent pathway, resulting in D-loop and HJs-like structures formation,
- A Rad22-dependent but Rhp51-independent pathway, resulting in HJs-like structures without D-loop formation (Lambert, Mizuno et al. 2010).

Indeed, the *rhp51-d* strain does not exhibit the signal corresponding to JM-A (D-loop), while HJs-like structures and acentric chromosomes are efficiently detected (Figure n. 46A; Lambert, Watson et al. 2005). Therefore, I decided to study genetic interactions, to identify on which recombination pathway CAF-1 acts.

For this purpose, I created *pcf1-d rhp51-d* and *pcf2-d rhp51-d* double mutants, harbouring the *RuraR* construct, and I analyzed the level of chromosomal rearrangements by PFGE (Figures n. 46B and C). It is noteworthy that 48 hours after induction of the *RTS1*-RFB, the level of acentric chromosome in *pcf1-d rhp51-d* and *pcf2-d rhp51-d* was similar to that of the *wt* strain. Thus, the deletion of *rhp51* rescues the defect of CAF-1 in promoting replication-induced recombination, demonstrating that **CAF-1 acts in the Rhp51 pathway, downstream of Rhp51.** In other words, CAF-1 has no function in promoting replication-induced recombination by template exchange, in the absence of the recombinase Rhp51. These data support the main hypothesis that CAF-1 regulates the stability of Rhp51-dependent JMs (thus D-loop intermediates). To further confirm this, it is necessary to analyze JMs by 2DGE in *pcf1-d rhp51-d* and *pcf2-d rhp51-d* double mutant, in which HJs-like structures are expected to be formed as in *rhp51-d* strain, but without D-loop formation.

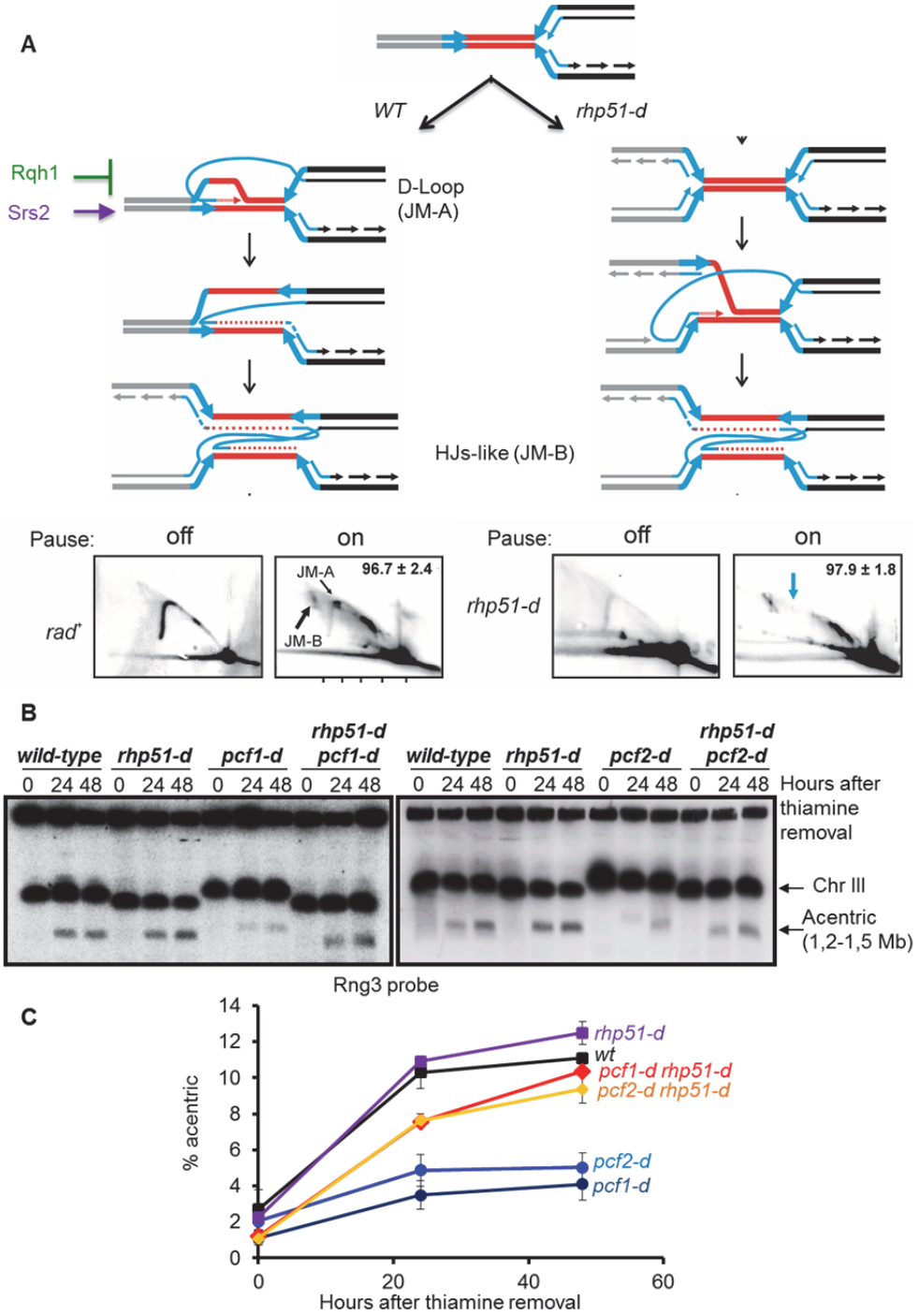


Figure n. 46 CAF-1 acts in the Rhp51-dependent pathway. A) Scheme representing the two pathways of replication-induced recombination: Rad22- and Rhp51-dependent (left panel) or Rad22-dependent and Rhp51-independent (right panel). Examples of RIs analysis by 2DGE in *wt* and *rhp51-d* strains are shown on the bottom part. Note that, in the absence of Rhp51, D-loop intermediates are not present while HJs-like structures are still detectable. B) PFGE analysis of indicated strains in the presence of thiamine (OFF condition) or 24h and 48h after thiamine removal (ON conditions). The size of the chromosome III is of 3.5 Mb and the size of the acentric chromosome is of 1.2-1.5 Mb. C) Kinetic of acentric chromosome accumulation after thiamine removal in indicated *RuraR* strains. The values correspond to the mean of at least three independent experiments and error bars indicate the standard error of the mean.

3.5. CAF-1 stabilizes D-loop structures by counteracting their dissociation by Rqh1.

Knowing that CAF-1 affects replication-induced recombination by acting on the Rhp51 pathway, I hypothesized that CAF-1 might regulate the stability of D-loop structures by preventing their dissolution by DNA helicases, such as Srs2 or Rqh1. If my hypothesis was correct, deletion of helicase genes could rescue the instability of JMs observed in the absence of CAF-1. Both Srs2 and Rqh1 are DNA helicases proposed to act during early steps of homologous recombination, by either dismantling the Rad51 filament or by dissolving D-loop intermediates, thus promoting SDSA pathway (Fabre, Chan et al. 2002; Aylon, Liefshitz et al. 2003; Ira, Malkova et al. 2003; Robert, Dervins et al. 2006; Oh, Lao et al. 2007; Oh, Lao et al. 2008; Lambert, Mizuno et al. 2010; see Introduction 2.3.2). In addition, Rqh1 plays a crucial role in preventing mitotic cross-overs by promoting the dissolution of double Holliday-junctions (Doe, Dixon et al. 2000).

To investigate genetic interactions between *srs2* and *pcf1* or *pcf2*, I created *pcf1-d srs2-d* and *pcf2-d srs2-d* strains, harbouring the *RuraR* construct, and I analyzed the level of acentric chromosome by PFGE upon induction of the *RTS1*-RFB (Figure n. 47A). As previously reported (Lambert, Mizuno et al. 2010), accumulation of acentric chromosomes was reduced by 2 fold in *srs2-d* strain compared to the *wt* level. Moreover, similar reduction in acentric level was found in *pcf1-d srs2-d* and *pcf2-d srs2-d* strains (Figure n. 47B). These data show that, in the *RuraR* system, Srs2 and CAF-1 act in a same pathway to promote replication-induced recombination.

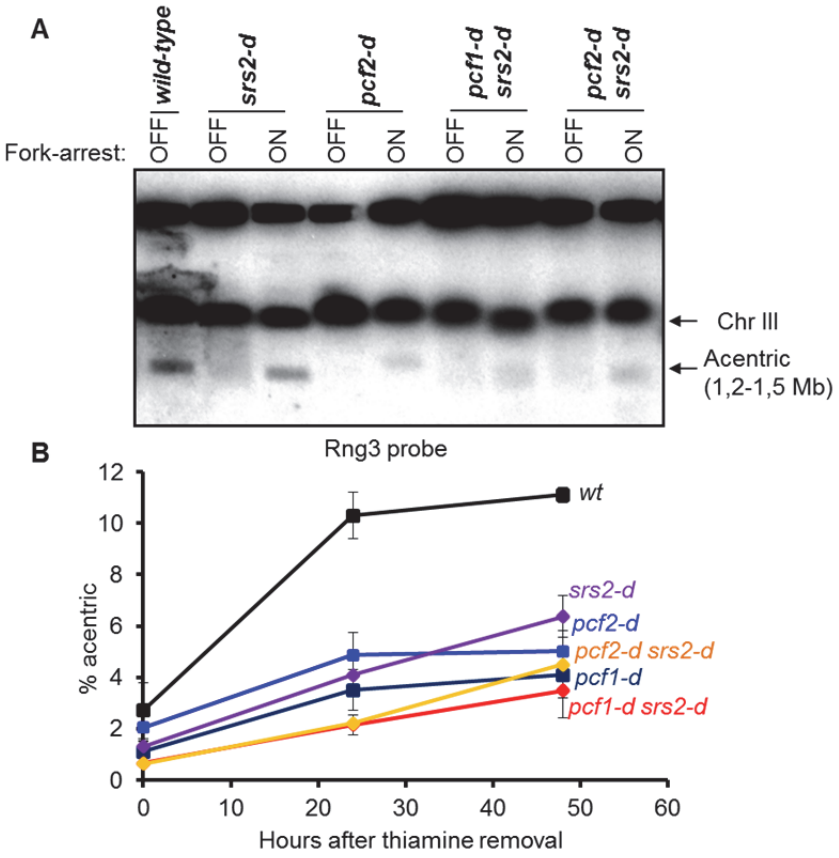


Figure n. 47: CAF-1 and Srs2 act in a same pathway of replication-induced recombination. A) PFGE analysis in indicated strains in OFF condition and in ON condition (48h after thiamine removal). The size of the chromosome III is of 3.5 Mb and the size of the acentric chromosome is of 1.2-1.5 Mb. B) Kinetic of acentric chromosome accumulation after thiamine removal in indicated *RuraR* strains. The values correspond to the mean of at least three independent experiments and error bars indicate the standard error of the mean.

To investigate genetic interactions between *rqh1* and *pcf1* or *pcf2*, I created *pcf1-d rqh1-d* and *pcf2-d rqh1-d* strains, harbouring the *RuraR* construct, and I analyzed the level of acentric chromosome by PFGE upon induction of the *RTS1*-RFB (Figure n. 48A). It was previously reported that Rqh1 prevents high level of chromosomal rearrangements upon induction of the *RTS1*-RFB at the *RuraR* locus, without affecting the efficiency of fork recovery by recombination (Lambert, Mizuno et al. 2010). This effect results in an increased level of recombination products associated to two cross-overs (switch of *ura4* orientation) at the expense of recombination products associated to one cross-over (acentric chromosome). Moreover, Rqh1 does not prevent chromosomal rearrangements by dissolution of JMs-B containing HJs, as these HJs were shown to be not branch-migrable *in vitro*. It was rather proposed that Rqh1 prevents chromosomal rearrangements by promoting D-loop dissociation (Lambert, Mizuno et al. 2010). As previously described, the level of acentric chromosome

was reduced by 2 times in the *rqh1-d RuraR* strain, compared to wt level, upon induction of the *RTS1*-RFB. Interestingly, the level of acentric chromosome in *pcf1-d rqh1-d* and *pcf2-d rqh1-d* strains was similar to that of the *wt* strain (Figure n. 48B). Thus, the deletion of *rqh1* rescues CAF-1 defects in promoting replication-induced recombination.

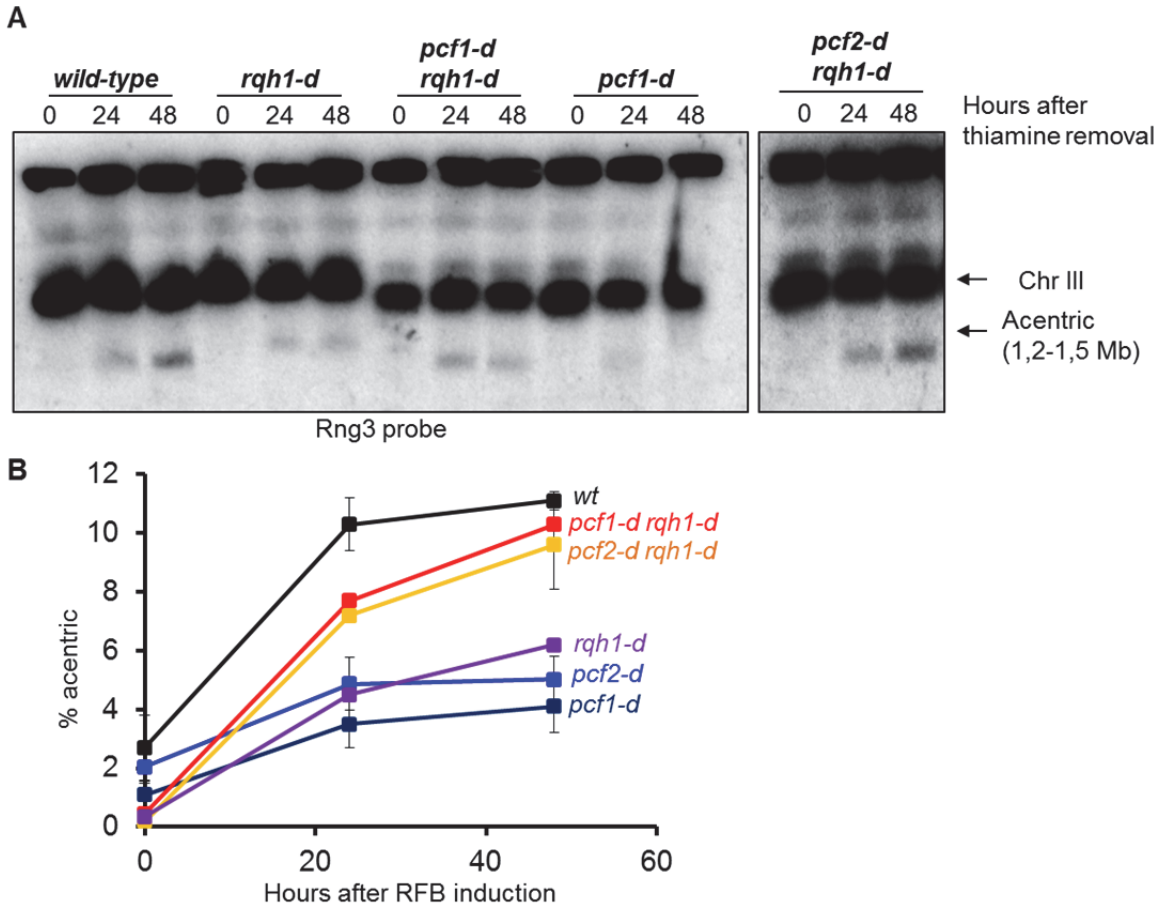


Figure n. 48: Genetic interaction between genes encoded CAF-1 subunits and *rqh1*. A) PFGE analysis of indicated strains in OFF condition and in ON condition (24h and 48h after thiamine removal) The size of the chromosome III is of 3.5 Mb and the size of the acentric chromosome is of 1.2-1.5 Mb. B) Kinetic of acentric chromosome accumulation after thiamine removal in indicated *RuraR* strains. The values correspond to the mean of at least three independent experiments and error bars indicate the standard error of the mean.

To directly test the hypothesis that JMs are unstable in the absence of CAF-1, because of the dissolution of D-loop intermediates by Rqh1, I analyzed JMs by 2DGE in single and double mutants. For this purpose, DNA structures were cross-linked *in vivo* by treating living cells with Tri-Methyl Psoralen (TMP) followed by ultraviolet A (UV-A) radiation to induce the cross-links. As previously mentioned, the intensity of JMs was decreased by 2-4 times in *pcf1-d* compared to *wt* cells, showing that JMs are unstable *in vivo* and not particularly liable to dissolution *in vitro*. Remarkably, the intensity of both D-loop intermediates (JMs-A) and

HJs-like structures (JMs-B) were restored to that of *wt* level in *pcf1-d rqh1-d* and *pcf2-d rqh1-d* strains (Figures n. 49A, B). As D-loop intermediates are the precursors of HJs-like structures that cannot be resolved by branch migration via Rqh1 activity, restored level of JMs can be explained by an increased stability of D-loop structures. **Thus, these data establish that CAF-1 stabilizes D-loop intermediates, by counteracting their dissociation by Rqh1.**

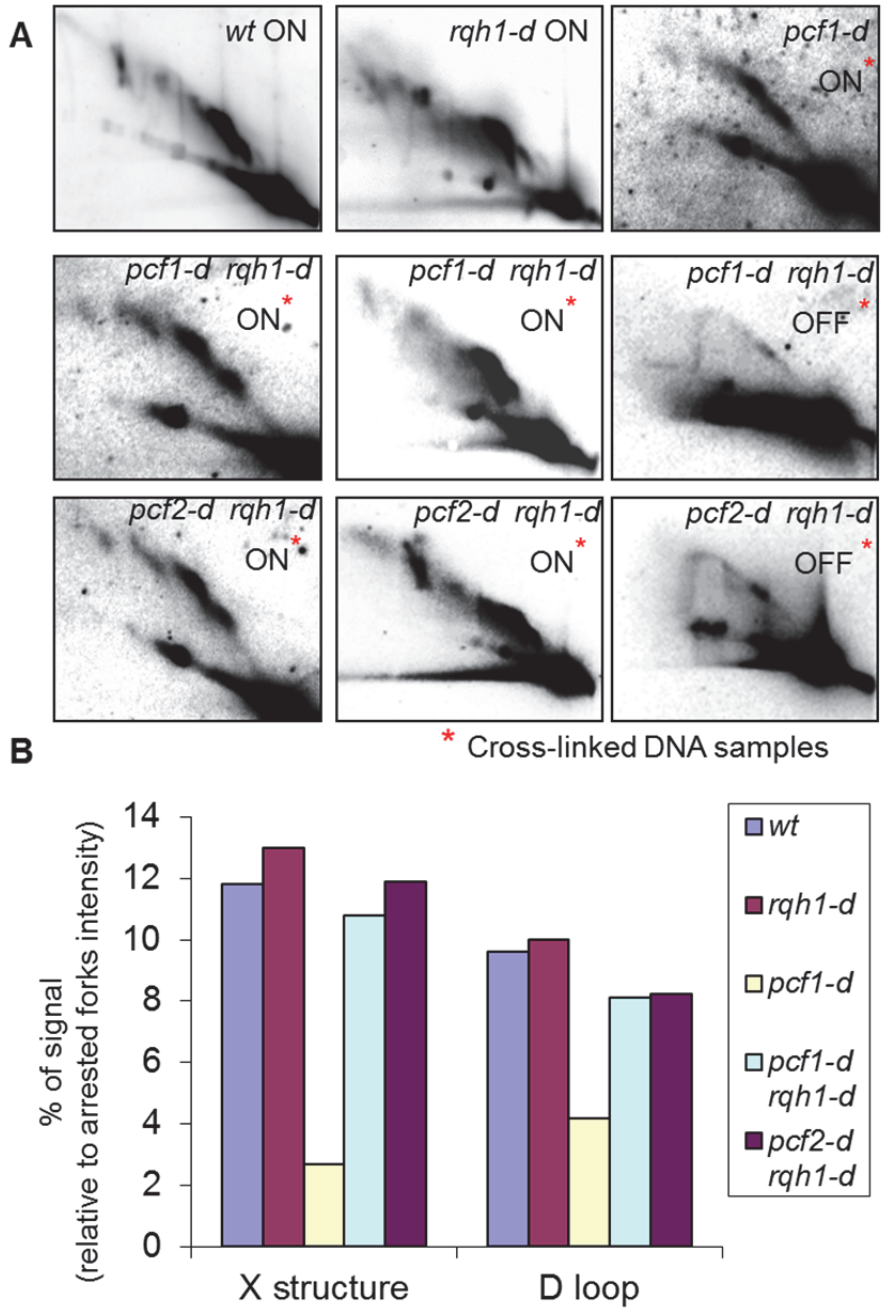
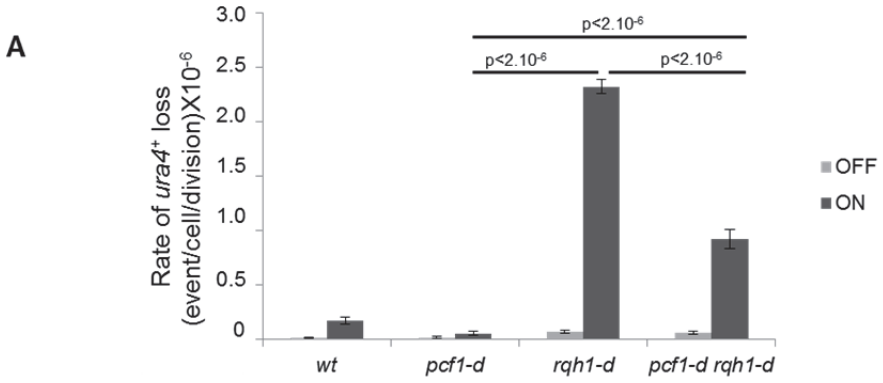
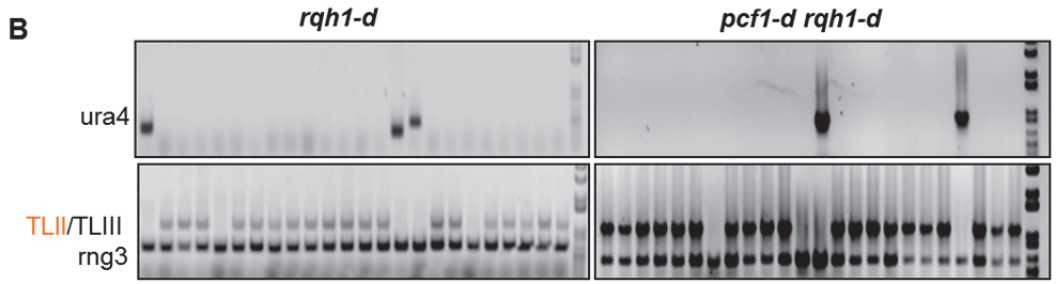


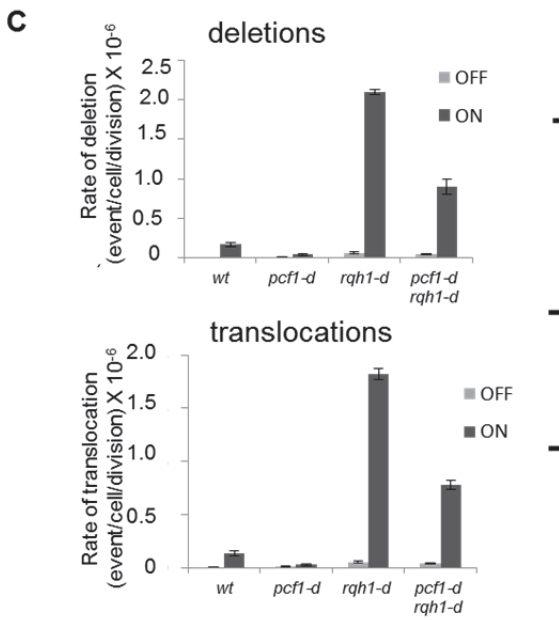
Figure n. 49: CAF-1 stabilizes D-loop intermediates by counteracting the activity of Rqh1. A) Analysis of RIs by 2DGE analysis in indicated *RuraR* strains grown for 24 hours in the presence of thiamine (pause OFF) or not (pause ON). The red star indicates cross-linked DNA samples. C) Quantification of A. The values correspond to the mean of two independent experiments.

To further support this conclusion, I analyzed the genetic interaction between *pcf1* and *rqh1* in ectopic recombination induced by the *RTS1*-RFB at the *RuraR* locus. As previously reported (Lambert, Mizuno et al. 2010), the induction of *ura4* loss by fork-arrest was 3 times higher in *rqh1-d* than in *wt* cells (Figure n.50A). Remarkably, the deletion of *rqh1* suppressed the decreased level of *ura4* loss in *pcf1-d*. Indeed, the induction of *ura4* loss by fork-arrest in *pcf1-d rqh1-d* was similar to that of *wt* strain (Figure n. 50A). Notably, both genomic deletion and translocation induced by the *RTS1*-RFB were nearly restored to that of *wt* level in *pcf1-d rqh1-d* double mutant (Figures n. 50B and C). **Thus, these data establish that CAF-1 promotes ectopic recombination by counteracting the anti-recombinase activity of Rqh1.** Nonetheless, deletion of *pcf1* did not fully abolish the high level of *ura4* loss observed in *rqh1-d* strain, especially regarding the level of genomic deletion. Therefore, Rqh1 has additional functions in preventing genome instability at arrested forks, not counteracted by CAF-1.



Strains	Fold induction by RFB (ON/OFF)	Increase over *wt*
wt	11.1	-
pcf1-d	2.2	0.2
rqh1-d	31.7	2.9
pcf1-d rqh1-d	14.6	1.3





Strains	Induction in deletion by RFB (ON/OFF)	Increase over *wt*
wt	19.40	-
pcf1-d	3.50	0.18
rqh1-d	32.10	1.65
pcf1-d rqh1-d	18.50	0.95

Strains	Induction in translocation by RFB (ON/OFF)	Increase over *wt*
wt	29.3	-
pcf1-d	3.0	0.10
rqh1-d	32.1	1.09
pcf1-d rqh1-d	18.2	0.62

Figure n. 50 CAF-1 promotes ectopic recombination induced by fork-arrest by counteracting the anti-recombinase Rqh1. A) Top panel: rate of *ura4* loss assayed by 5-FOA resistance in indicated strains and conditions. Values correspond to the mean of three independent median rates (each one determined from 9 independent cultures using the median method). The error bars represent the standard deviation and the statistical significance was calculated using the non-parametric Mann Whitney test. Bottom panel: table representing the induction of *ura4* loss by the *RTS1*-RFB in

indicated strains (rate of *ura4* loss in ON condition/ rate of *ura4* loss in OFF condition), and the increase over *wt*. B) Examples of PCR analysis of 5-FOA^R colonies obtained upon *rtf1* induction (ON condition) in indicated strains. Primers used were: TLII and TLIII, Rng3 and Ura4 primers. C) Top: The % of deletion events, as determined by the PCR assay, was used to balance the rate of *ura4* loss. The values reported are means of 3 independent median rates. Error bars correspond to the standard deviation. Bottom: The % of translocation events, as determined by the PCR assay, was used to balance the rate of *ura4* loss. The values reported are means of 3 independent median rates. Error bars correspond to the standard deviation. Right tables: induction of genomic deletions or translocations by the *RTS1*-RFB (rate of genomic deletions or translocations in ON conditions/rate of genomic deletions or translocations in OFF conditions) and the relative increase over wild-type.

4. THE CHROMATIN ASSEMBLY FUNCTION OF CAF-1 IS REQUIRED TO STABILIZE D-LOOP INTERMEDIATES

In this chapter, I investigated by which function CAF-1 promotes D-loop stabilization. One hypothesis was that CAF-1 promotes histone deposition during the elongation of D-loop intermediates, thus counteracting their dissolution by Rqh1.

4.1. The silencing / heterochromatin function of CAF-1 is dispensable for D-loop stabilization

The *RTS1*-RFB is based on a direct interaction between the Rtf1 protein and the *RTS1* sequence, to block the progression of replication forks. It has been recently reported that replication stress arising from tight protein-DNA complexes favours heterochromatin formation. This occurs through the ectopic recruitment of the SIR complex which promotes gene silencing and possible changes in subnuclear localization (Dubarry, Loiodice et al. 2011).

To investigate if gene silencing occurs at the *RTS1*-RFB, I took advantage of the counter-selectable marker *ura4* to select for *ura4* loss (resistance to 5-FOA) after short induction of fork-arrest (in contrast to the ectopic recombination assay that requires prolonged induction of fork-arrest). The strains used were a *wt* and a *pcf1-d* harbouring the *RuraR* construct. As positive control I used a strain deleted for *ura4 (ura4-D18),* which can grow on 5-FOA. I tested also a *swi6-d* strain harbouring the construct *RuraR*. In *S. pombe,* Swi6 (SPAC664.01c) is involved in heterochromatin maintenance. It directly interacts with Pcf1, to be displaced from the heterochromatin ahead the replication fork and delivered to methylated histones, behind the replication fork, at the centromeric region. This transfer is necessary to facilitate

the progress of the replication fork through heterochromatin and to ensure the self-perpetuation of the epigenetic state of the domain (Dohke, Miyazaki et al. 2008; Introduction 3.3).

After 72 hours of induction of the *RTS1*-RFB, there was no significant increase in the amount of cells resistant to 5-FOA, whatever the genetic background analyzed (*wt*, *pcf1-d* or *swi6-d*) (Figure n. 51A and B). Thus, these data do not support a potential gene silencing effect upon fork-arrest, in the *RTS1*-RFB system.

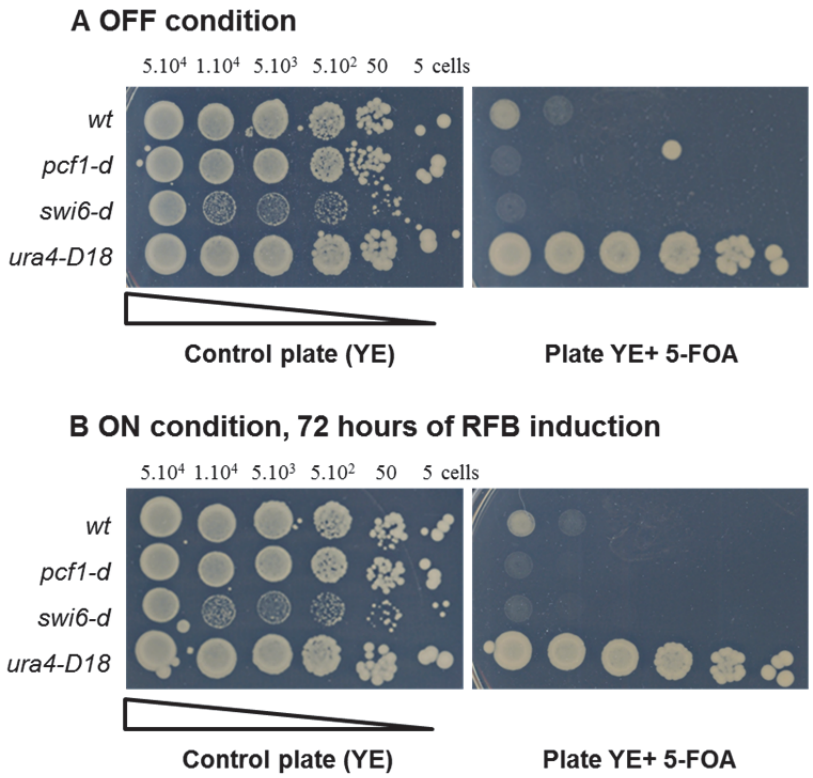


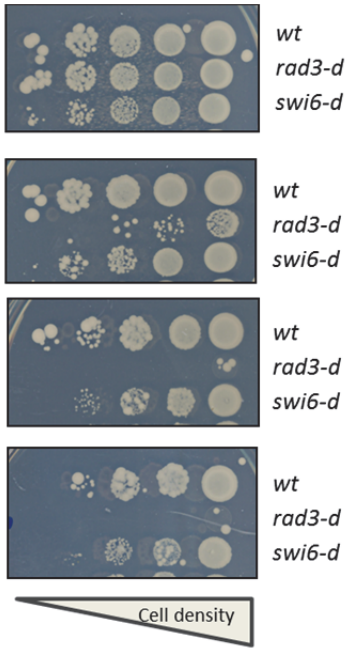
Figure n. 51: Absence of gene silencing after short induction of fork arrest by the *RTS1*-RFB. A) Serial ten-fold dilutions of indicated strains, previously grown in presence of thiamine (OFF condition), spotted onto complete YE and YE containing 5FOA plates. B) Serial ten-fold dilutions of indicated strains, previously grown in the absence of thiamine for 72 hours (ON condition), spotted onto complete YE and YE containing 5FOA plates. The liquid cultures in OFF and ON conditions, from which dilutions were obtained, were diluted every 24 hours to avoid cell saturation.

In mammals, HP1 is the homologue of Swi6 and it has similar functions, interacting with the largest subunit of CAF-1 (p150). Depletion of HP1 leads to defects in HR-mediated repair and it also compromises the recruitment of factors involved in the DNA damage response (Baldeyron, Soria et al. 2011). Thus, it seems likely that HP1 is involved in recombination processes and I decided to test if the role of CAF-1 in promoting replication-induced

recombination could be linked to its Swi6-dependent function in maintaining heterochromatin.

To achieve my purpose, I studied if a *swi6-d* strain could phenocopy strains defective for CAF-1, using previously described recombination assays. The strain deleted for *swi6* presented a slow growth phenotype compared to the *wt* strain and a slight increase in UV-C sensitivity, as previously reported (Figure n. 52A) (Ekwall, Cranston et al. 1999).

A



B

Strains	Rate of Gene Conversion (event/cell/division) x10^{-5}	Fold increase over *wt*	Rate of deletion (event/cell/division) x10^{-5}	Fold increase over *wt*
wild type	2.68 (1.94-5.27)	--	8.86 (5.42-17.2)	--
pcf1-d	4.13 (3.45-9.22)	1.54	13.8 (10.1-13.9)	1.55
swi6-d	2.72 (2.36-3.39)	1.01	6.39 (5.17-8.17)	0.72

C

Strains	Rate of recombination event/cell/division x10^{-4}	Fold increase over *wt*	Rate of chromosome loss event/cell/division x 10^{-5}	Fold increase over *wt*
wt	1.17 (0.92 – 1.76)	--	4.86 (3.56 – 5.87)	--
pcf1-d	1.53 (1.03 – 1.63)	1.3	8.23 (4.71 – 16.10)	1.7
swi6-d	0.35 (0.18-1.15)	0.3	104 (45.4-251)	21.4

Figure n. 52: Contribution of Swi6 to allelic homologous recombination A) Serial ten-fold dilutions of indicated strains, spotted on YE plates and exposed to increased doses of UV-C. B) Analysis of SCE: the rate of gene conversion and the rate of deletion is reported for indicated strains. The values represent the mean of three independent rates, each one being median of 11 independent cultures. The confidence interval is calculated at 95% (enclosed in bracket). Fold increase over *wt* is indicated for each strain. C) Analysis of recombination between homologous chromosomes: the rate of recombination events and rate of chromosome loss is reported for indicated strains. The values correspond to the mean of three independent rates, each one being median of 13 independent cultures. The confidence interval is calculated at 95% (enclosed in bracket) and the fold increase over *wt* is indicated for each strain.

I also analyzed the contribution of Swi6 to sister-chromatid exchange and found that the strain *swi6-d* exhibited no particular defect or increase in SCE, whatever the type of recombinant scored (gene conversion or deletion) (Figure n. 52B). In contrast, the rate of cross-overs between homologue chromosomes was decreased by 3 times in *swi6-d,* compared to *wt* strain. This decrease in allelic recombination between homologue chromosomes was accompanied by a 20 fold increase in chromosome loss (Figure n. 52C). The significant increase in chromosome loss could be due to a defect in maintaining centromeric heterochromatin, which could explain also the increased sensitivity to TBZ reported in the literature (Ekwall, Cranston et al. 1999). As mentioned above, Swi6 has a critical role in supporting accurate chromosome segregation, through heterochromatin maintenance at the centromere. Indeed, it has been demonstrated that localization of cohesins at the centromeric repeats is Swi6-dependent (Nonaka, Kitajima et al. 2002).

Finally, I analyzed the level of acentric chromosome in *swi6-d RuraR* cells, upon induction of the *RTS1*-RFB. In contrast to *pcf1-d* strain, *swi6-d* cells accumulated acentric chromosome to the same extent as the *wt* cells (Figure n. 53A and B).

Therefore **the deletion of *swi6* gene does not mimic CAF-1 defects in replication-induced recombination,** strongly suggesting that CAF-1 function in heterochromatin maintenance is not required to stabilize D-loop intermediates.

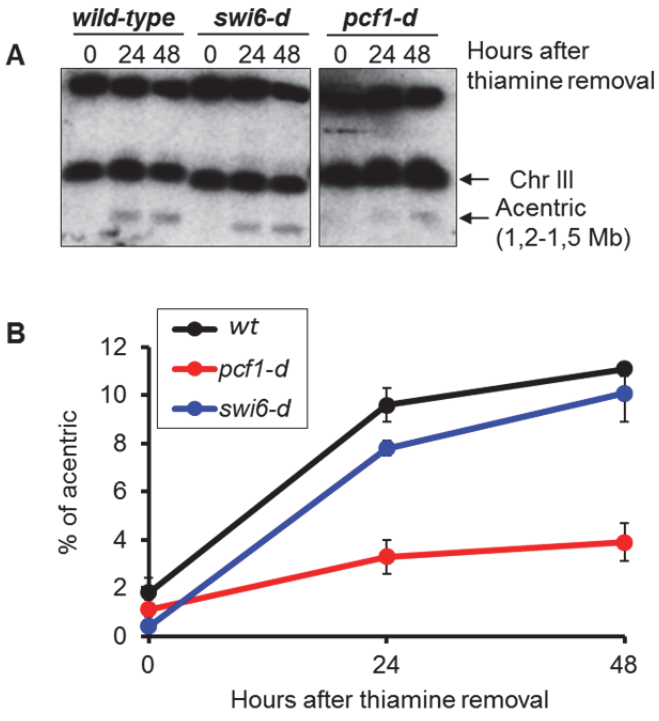


Figure n. 53: The deletion of *swi6* gene does not phenocopy the deletion of *pcf1* gene in replication-induced recombination. A) PFGE analysis of wild-type, *swi6-d* and *pcf1-d* strains in OFF condition and in ON condition (after 24h and 48h of fork-arrest induction). The chromosome III and the acentric chromosome were visualized using the *rng3* probe. The size of the chromosome III is 3.5 Mb and the size of the acentric chromosome is 1.2-1.5 Mb. B) Kinetic of acentric chromosome accumulation after thaimine removal in indicated *RuraR* strains. The values represent the mean of the % of acentric chromosome, obtained in three independent experiments and error bars represent the standard error of the mean.

4.2. The interaction between PCNA and Pcf1 is required to stabilize D-loop intermediates

During DNA replication, CAF-1 is targeted to replication foci through the interaction between the largest subunit of CAF-1 with PCNA. Moreover, the histone deposition function of CAF-1 requires its recruitment at the replication fork, through its interaction with PCNA (Moggs, Grandi et al. 2000; Krawitz, Kama et al. 2002; Rolef Ben-Shahar, Castillo et al. 2009). The inability of CAF-1 to interact with PCNA results in an inability to promote nucleosomal assembly *in vitro* (Krawitz, Kama et al., 2002).

Firstly, I confirmed that CAF-1 is targeted to replication foci in *S. pombe* (Figure n. 54A and B). For this purpose, I used a strain in which PCNA was already tagged with a cyan fluorescent protein (PCNA-CFP) (Meister, Poidevin et al. 2003) and I tagged Pcf1 in this strain with a yellow fluorescent protein (Pcf1-YFP). Using a fluorescent deconvolution 3D-microscopy, I investigated the co-localization between Pcf1 and PCNA and found that 74% of S-phase cells present at least one focus, containing both Pcf1 and PCNA. Remarkably, no co-localization was detected in G2-cells (Figure n. 54A).

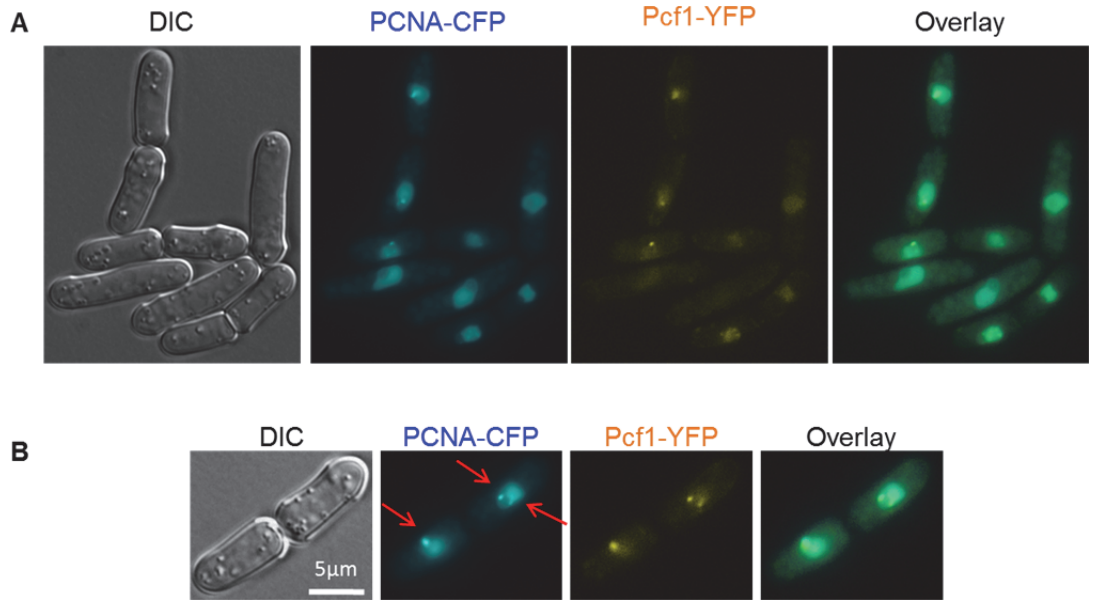


Figure n. 54: *In vivo* co-localization between PCNA and Pcf1 in S-phase cells. A) The co-localization between PCNA and Pcf1 was analyzed and quantified by fluorescence of Pcf-YFP and PCNA-CFP *in vivo*. Differential Interferential Contrast (DIC-grey), CFP (cyan), YFP (yellow) and overlay of those two fluorescent signals in wild-type cells, harbouring *pcna-cfp* and *pcf1-yfp*. B) Example of a typical late S-phase cell, presenting 3 foci (red arrows) corresponding to PCNA-Pcf1 co-localization.

My hypothesis was that CAF-1 could stabilize D-loop intermediates via its function in replication-coupled chromatin assembly. This was suggested by the shared phenotypes among *pcf1-d, pcf2-d, pcf3-d* and the triple mutant *pcf1-d pcf2-d pcf3-d*. To test this hypothesis, the unique canonical PIP-box of Pcf1 was mutated. Thus, the wild-type sequence QLKLNNFF was modified to ALKANNAA to create the *pcf1-PIP** allele (Figure n. 55A).

Co-ImmunoPrecipitations (Co-IP) were performed to confirm that the mutated PIP-box abolishes the interaction between Pcf1 and PCNA without affecting the formation of the trimeric complex CAF-1. PCNA was found interacting with Pcf1, but not with Pcf1-PIP* (mutated for the PIP-box) (Figure n. 55B). Moreover, interaction between PCNA and Pcf2 was found to be mediated by the PIP-box of Pcf1 (Figure n. 55C). Finally, mutation of the PIP-box did not affect the interaction between Pcf1 and Pcf2 (Figure n. 55D). Thus, the data indicate that Pcf1-PIP* is unable to interact with PCNA, but remains able to interact with Pcf2.

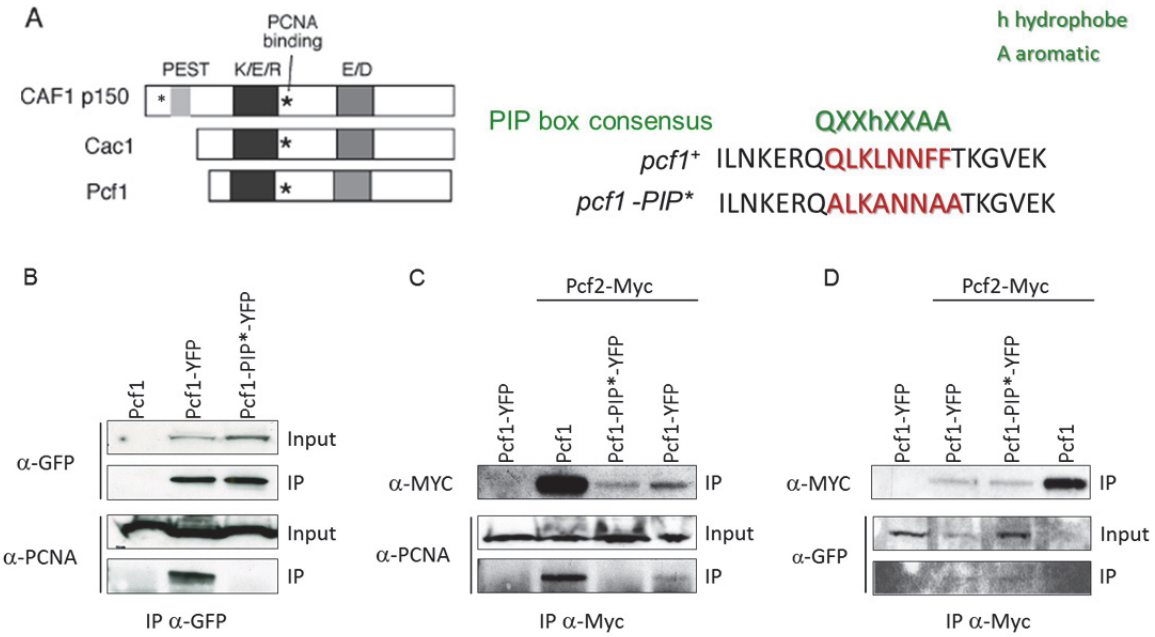


Figure n. 55: Mutations in the PIP-box of Pcf1 abolished its interaction with PCNA, but not with Pcf2. A) Left panel: alignment of human p150, budding yeast Cac1 and fission yeast Pcf1. Conserved domains are represented in grey (PEST, E/D) and black (KER). Stars indicate PCNA binding site (PIP box). Right panel: PIP-box consensus sequence (in green) and PIP-box sequence in *pcf1*$^{+}$ (in red) and its mutation in *pcf1-PIP**. In green, hydrophobic "h" and aromatic "A" amino acids are defined. B) Immunoprecipitation of Pcf1-YFP, harbouring or not the mutation in the PIP-box sequence, was performed using an anti-GFP antibody. C) Immunoprecipitation of Pcf2-MYC, harbouring or not the mutation in the PIP-box sequence of Pcf1, was performed using an anti-MYC antibody. The Input for α-MYC is absent because the concentration of the Pcf2-MYC was not enough to be detected by western blot. D) Immunoprecipitation of Pcf2-MYC, harbouring or not the mutation in the PIP-box sequence of Pcf1, was performed using an anti-MYC antibody. The Input for α-MYC is absent because the concentration of the Pcf2-MYC was not enough to be detected by western blot. Subsequent western blot revelation of Pcf1, Pcf2 and PCNA was achieved via anti-GFP, anti-MYC, and anti-human PCNA antibodies, respectively.

Then, I analyzed if the interaction between CAF-1 and PCNA is required to stabilize D-loop intermediates. First, *pcf1-PIP* RuraR* cells showed a reduction in the accumulation of acentric chromosome and in the intensity of JMs, to the same extent as *pcf1-d* cells. (Figure n. 56). Moreover, mutation in the PIP-box of Pcf1 resulted in a similar decreased in ectopic recombination induced by fork-arrest than the deletion of *pcf1* (Figure n. 57A). After analyzing the nature of ectopic recombination by PCR, genomic deletion was decreased by 3.4 times and translocation by 9 times, compared to *wt,* in the *pcf1-PIP** strain. In the *pcf1-d* strain the reduction was respectively of 5.5 fold and 9.9 fold (Figure n. 57B and C).

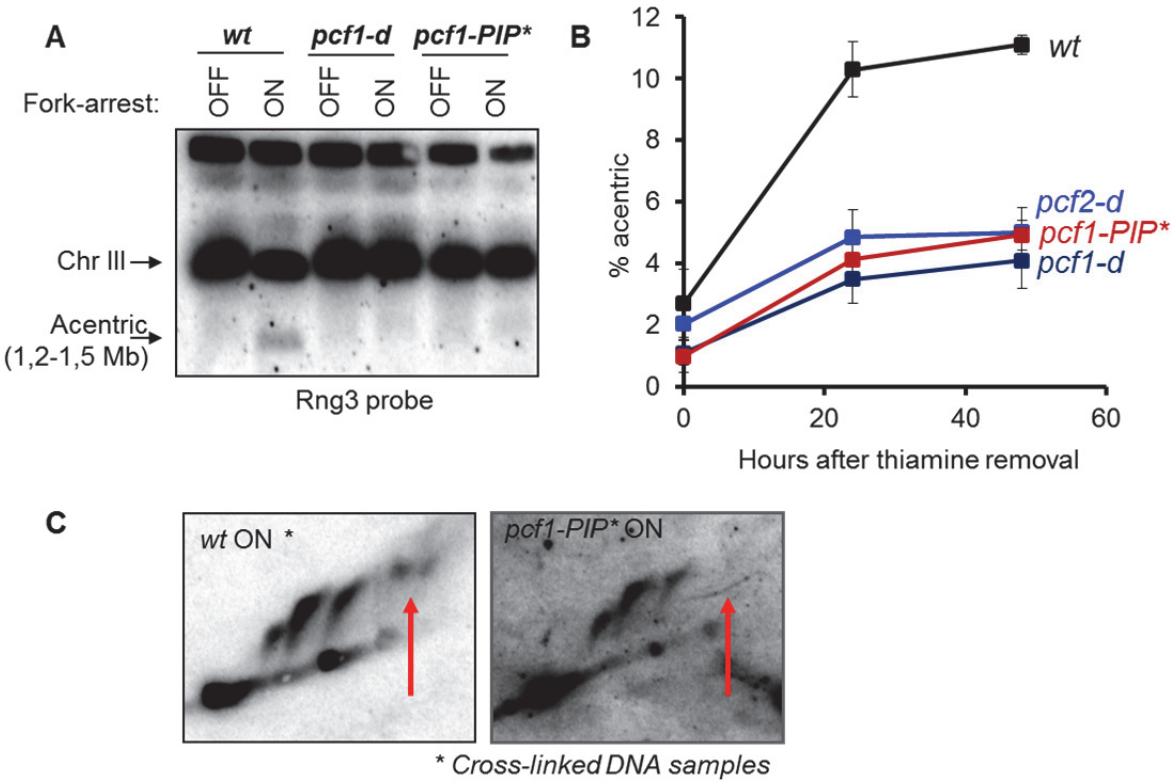
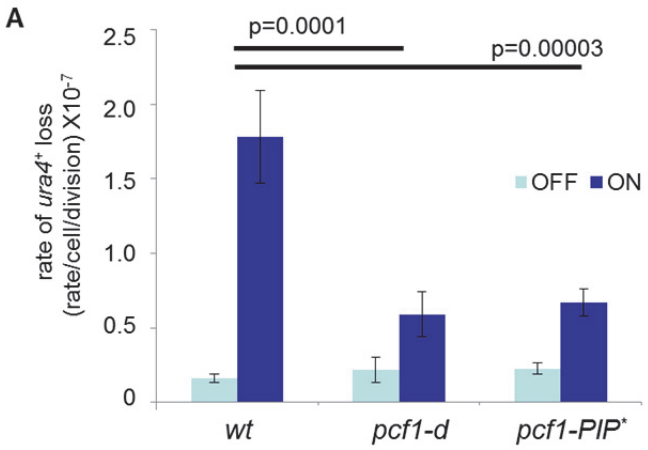
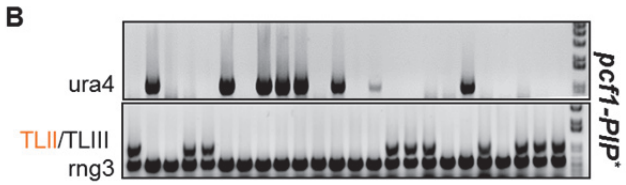
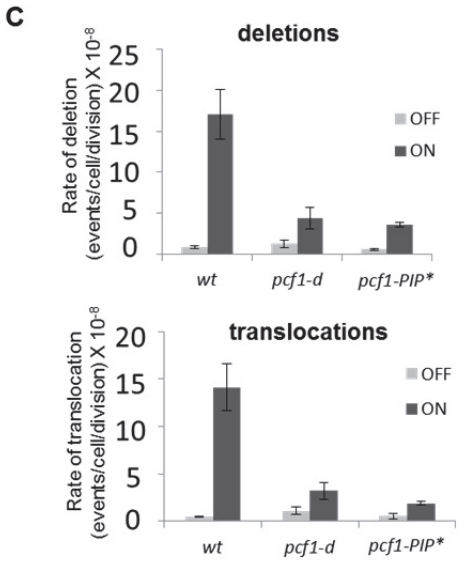


Figure n. 56: The interaction between PCNA and Pcf1 is required to promote replication-induced recombination. A) PFGE analysis of the wild-type, *pcf1-PIP** and *pcf1-d* strains in OFF condition and in ON condition (48h after thiamine removal). The chromosome III and the acentric chromosome were visualized using the *rng3* probe. The size of the chromosome III is 3.5 Mb while the size of the acentric chromosome is 1.2-1.5 Mb. B) Kinetic of acentric chromosome accumulation after thiamine removal in indicated strains. The values represent the mean of the % of acentric chromosome, obtained from at least three independent experiments and the error bars indicate the standard error of the mean. C) 2DGE analysis of replication intermediates within the AseI-restriction fragment in indicated *wt RuraR* and *pcf1-PIP* RuraR* strains, upon induction of fork-arrest. All DNA samples were cross-linked.



Strains	Fold induction by RFB (ON/OFF)	Decrease over *wt*
wt	11.1	-
pcf1-d	2.2	5.0
pcf1-PIP*	2.9	3.8





Strains	Induction in deletion by RFB (ON/OFF)	Decrease over *wt*
wt	19.4	-
pcf1-d	3.5	5.5
*pcf1-PIP**	5.7	3.4

Strains	Induction in translocations by RFB (ON/OFF)	Decrease over *wt*
wt	29.3	-
pcf1-d	3.0	9.9
*pcf1-PIP**	3.3	9.0

Figure n. 57: Pcf1 promotes ectopic recombination induced by fork-arrest via its interaction with PCNA. A) Top panel: Rate of *ura4* loss assayed by 5-FOA resistance in wild-type, *pcf1-d* and *pcf1-PIP**. The rate of *ura4* loss was determined doing the mean of the media obtained from at least three different experiments (9 cultures were analyzed by fluctuation analysis for each experiment). Error bars define standard deviation and statistical significance was calculated using the non-parametric Mann Whitney test. Bottom panel: table reporting the induction of *ura4* loss by the *RTS1*-RFB (rate of *ura4* loss in ON conditions/ rate of *ura4* loss in OFF conditions) in indicated strains. Decrease over the wild-type is reported. B) Examples of PCR analysis of 5-FOA^R colonies obtained followed *rtf1* induction (ON conditions) in *pcf1-PIP** strains. The primers used are: TLII and TLIII, Rng3 and Ura4.

C) Top: The % of deletion events, as determined by the PCR assay, was used to balance the rate of *ura4* loss. The values reported are means of 3 independent median rates. Error bars correspond to the standard deviation. Bottom: The % of translocation events, as determined by the PCR assay, was used to balance the rate of *ura4* loss. The values reported are means of 3 independent median rates. Error bars correspond to the standard deviation. On the right, tables reporting the induction of genomic deletion or translocation by the *RTS1*-RFB (rate of genomic deletion or translocation in ON conditions/ rate of genomic deletion or translocation in OFF conditions) in indicated strains. Decrease over the wild-type is reported.

Thus, these data establish that the interaction between Pcf1 and PCNA is crucial to promote replication-induced recombination and D-loop stabilization. This supports the hypothesis that CAF-1 promotes D-loop stabilization via its replication-coupled chromatin assembly function.

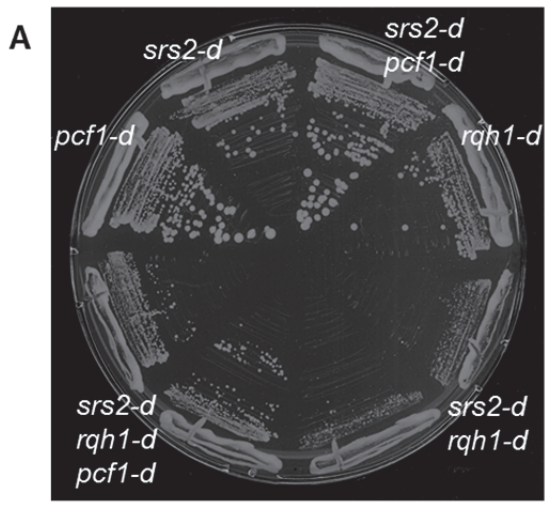
5. CAF-1 PROMOTES THE STABILIZATION OF TOXIC RECOMBINATION INTERMEDIATES

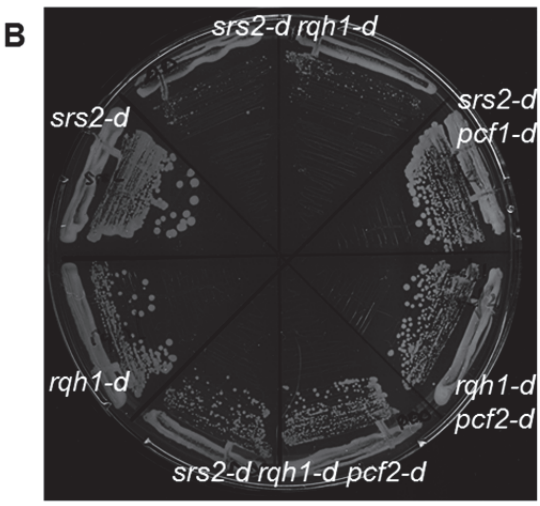
Exploiting the *RTS1*-RFB-based system, data obtained support the view that CAF-1 stabilizes D-loop intermediates by counteracting the anti-recombinase activity of Rqh1 helicase. CAF-1 function is achieved by promoting chromatin assembly during DNA synthesis, associated to homologous recombination.

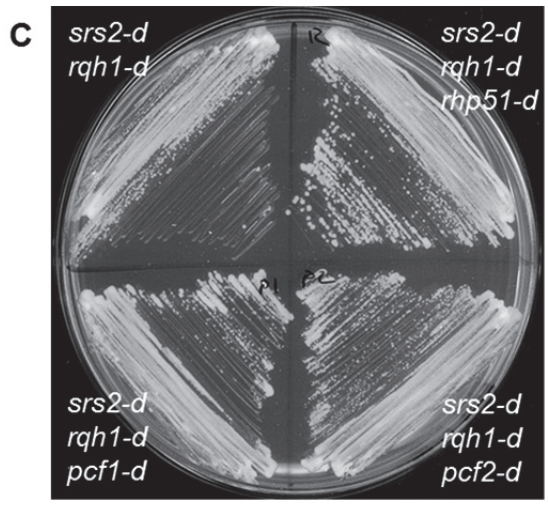
Defects in processing JMs and/or in the disassembly of Rad51- filaments result in accumulation of toxic recombination intermediates, explaining the severe slow growth phenotype of the double mutant *srs2-d rqh1-d*. This negative genetic interaction is suppressed by the deletion of *rhp51*, as Rhp51 is responsible for JMs formation which are normally resolved by either Srs2 or Rqh1 (Chanet, Heude et al. 1996; Gangloff, Soustelle et al. 2000; Doe and Whitby 2004; Burgess, Lisby et al. 2009).

To confirm the conclusion that CAF-1 regulates homologous recombination by acting in the Rhp51-dependent pathway and by counteracting the anti-recombinase activity of Rqh1, I tested if inactivation of CAF-1 subunits could also rescue the very slow growth phenotype of *srs2-d rqh1-d*. Therefore, triple mutant *srs2-d rqh1-d pcf1-d* and *srs2-d rqh1-d pcf2-d* were created and their ability to support cell growth was analyzed. As expected, both *pcf1* and *pcf2* deletions suppress the slow growth phenotype of *srs2-d rqh1-d*, but not to the same extent as the deletion of *rhp51* (Figures n. 58A-D). Indeed, the generation time of *srs2-d rqh1-d pcf1-d* or *srs2-d rqh1-d pcf2-d* was of 6.7 hours, compared to 5.7 hours for *srs2-d rqh1-d rph51-d*.

These data provide further strong evidences that CAF-1 complex acts in the same pathway than Rhp51 and that CAF-1 affects the stability of joint molecules produced by Rhp51.







D

Strains	Generation time (hours)
srs2-d rqh1-d	8.6 ± 2.3
srs2-d rqh1-d rhp51-d	5.7 ± 0.7
srs2-d rqh1-d pcf1-d	6.7 ± 0.1
srs2-d rqh1-d pcf2-d	6.7 ± 0.2

Figure n. 58: The deletions of *pcf1* or *pcf2* rescue the slow growth phenotype of *srs2-d rqh1-d*. A) Genetic interaction with *pcf1*. Indicated strains were streaked on plates containing EMM-Glu media. B) Genetic interaction with *pcf2*. Indicated strains were streaked on plates containing EMM-Glu media. C-D) Deletion of *pcf1* or *pcf2* does not rescue the slow growth phenotype of *srs2-d rqh1-d,* to the same extent as the deletion of *rhp51*. D) Generation time in hours of indicated strains.

DISCUSSION AND PERSPECTIVES

Chromatin Assembly Factor 1 promotes homologous recombination by counteracting joint molecules dissolution by Rqh1

1. *The histone chaperone function of CAF-1 stabilizes D-loop intermediates*

Using the *RuraR* system, I established that CAF-1 promotes fork-arrest-induced chromosomal rearrangements. Two alternatives explanations were hypothesized.

Firstly, defects in replication-coupled chromatin assembly through CAF-1 activity could lead to a transient decrease in nucleosome density behind the advancing fork, therefore affecting its stability and channeling its recovery towards a restart on the same template to the detriment of restart by template exchange (Figure n. 59A).

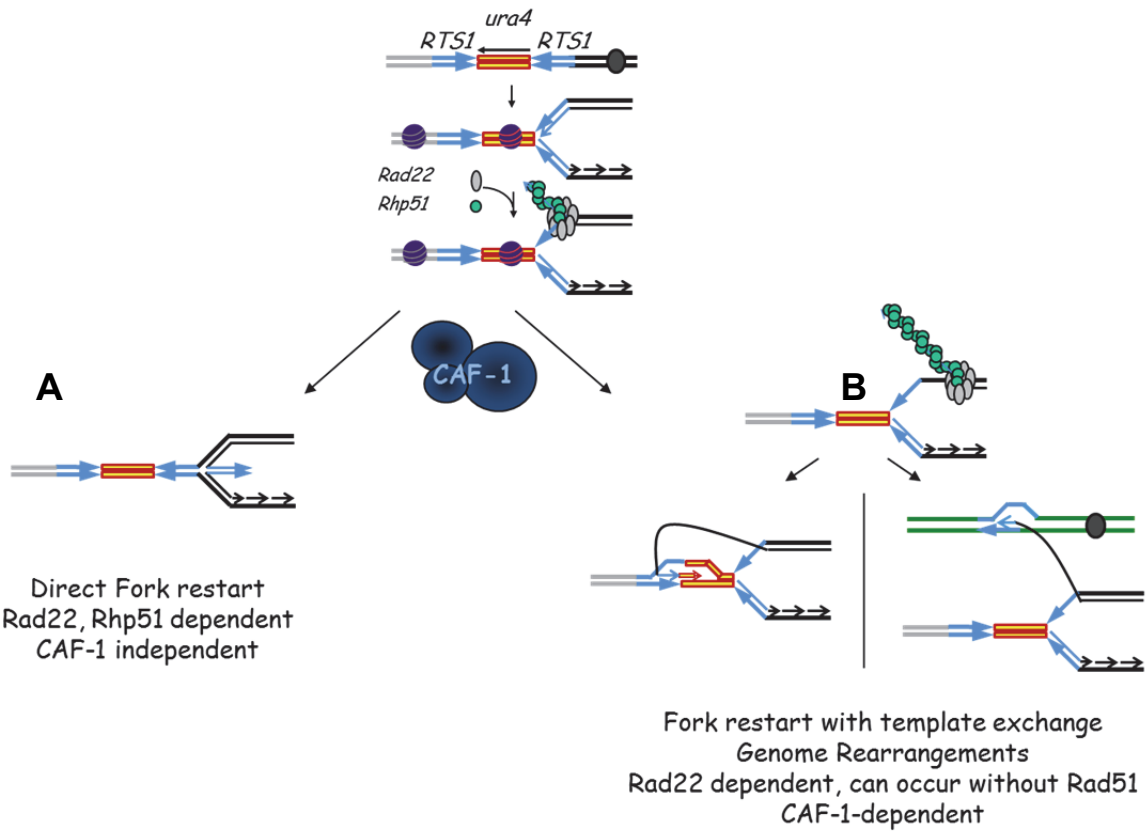


Figure n. 59: Two hypothesis to explain CAF-1 role in promoting fork arrest-induced chromosomal rearrangements. A) CAF-1 might increase fork restart on the initial template to the detriment of template exchange. B) CAF-1 might have a direct role in promoting template exchange of nascent strands.

As reported in the literature, defects in replication-coupled chromatin assembly lead to the instability of advancing forks and a need for homologous recombination to process them. This results in a general increase in genome instability (increased level of recombination and

genome rearrangements) caused by the deficiency of two main histones chaperones involved in DNA metabolism: CAF-1 and Asf1 (Myung, Pennaneach et al. 2003; Prado, Cortes-Ledesma et al. 2004 Endo, Ishikawa et al. 2006; Clemente-Ruiz and Prado 2009; Clemente-Ruiz, Gonzalez-Prieto et al. 2011). Moreover, defects in the deposition of the histone mark H3K56Ac during chromatin assembly lead to genetic instability due to a higher susceptibility of fork stalling, damage or breakage. The absence of H3K56Ac or the simultaneous knock-out of CAF-1 and Rtt106 affect the integrity of advancing replication forks, increasing the level of homologous recombination (Han, Zhou et al. 2007; Clemente-Ruiz, Gonzalez-Prieto et al. 2011; Su, Hu et al. 2012). Thus, to investigate the truthfulness of the supposition that forks arrested at the RTS1-barrier are unstable and more liable to restart on the initial template, I analyzed the level of recombination between sister chromatids and the stability of halted replication forks, in the absence of CAF-1. Defects in CAF-1 histone chaperone activity do not lead to a general genomic instability, as it was reported for *S. cerevisiae, Arabidopsis* and human cells. (Myung, Pennaneach et al. 2003; Prado, Cortes- Ledesma et al. 2004 Endo, Ishikawa et al. 2006; Clemente-Ruiz and Prado 2009; Clemente-Ruiz, Gonzalez-Prieto et al. 2011). Strains deleted for *pcf1, pcf2* or *pcf3* accumulate spontaneously Rad22 foci in S-phase, suggesting that recombinogenic DNA structures arise in the absence of CAF-1. Nonetheless, the level of spontaneous or replication-stress-induced SCE is not affected by the absence of CAF-1. Moreover, strains defective for CAF-1, do not present an increased sensitivity to replication blocking agents. I also found that arrested forks are stable and prone to recombination-dependent restart: restart of arrested forks on the initial template is neither defective nor more efficient, in the absence of CAF-1. Thus my data exclude that the deficiency of CAF-1 could threaten the stability of the arrested fork and channel a preferential recovery of the fork on the same template to the detriment of template exchange.

The second hypothesis was that CAF-1 could be involved directly in promoting template exchange reactions (Figure n. 59B). Indeed, I found that, in the absence of CAF-1, both JMs and the product of their resolution (chromosomal rearrangements) were reduced. I supposed that CAF-1 complex could be involved at different steps in template exchange pathway, therefore I investigated:

1. an eventual role of CAF-1 in the recruitment of Rad22, during early steps of homologous recombination.
2. the possibility that JMs could be solved faster in the absence of CAF-1.
3. a direct role for CAF-1 in the stabilization of JMs.

The data I obtained during the investigation of the three hypotheses mentioned above, lead to propose that CAF-1 has a direct role in homologous recombination mechanisms: CAF-1 promotes homologous recombination by counteracting the dissolution of D-loop intermediates by Rqh1 (Figure n. 60). Indeed, CAF-1 could be recruited via its interaction with PCNA, to promote histones deposition during extension of the D-loop by DNA synthesis. Histones deposition might then stabilize D-loop intermediates by preventing their dissolution by the helicase Rqh1. Thus, by counteracting Rqh1 function, CAF-1 might be part of an equilibrium regulating stabilization/dissociation of early recombination intermediates.

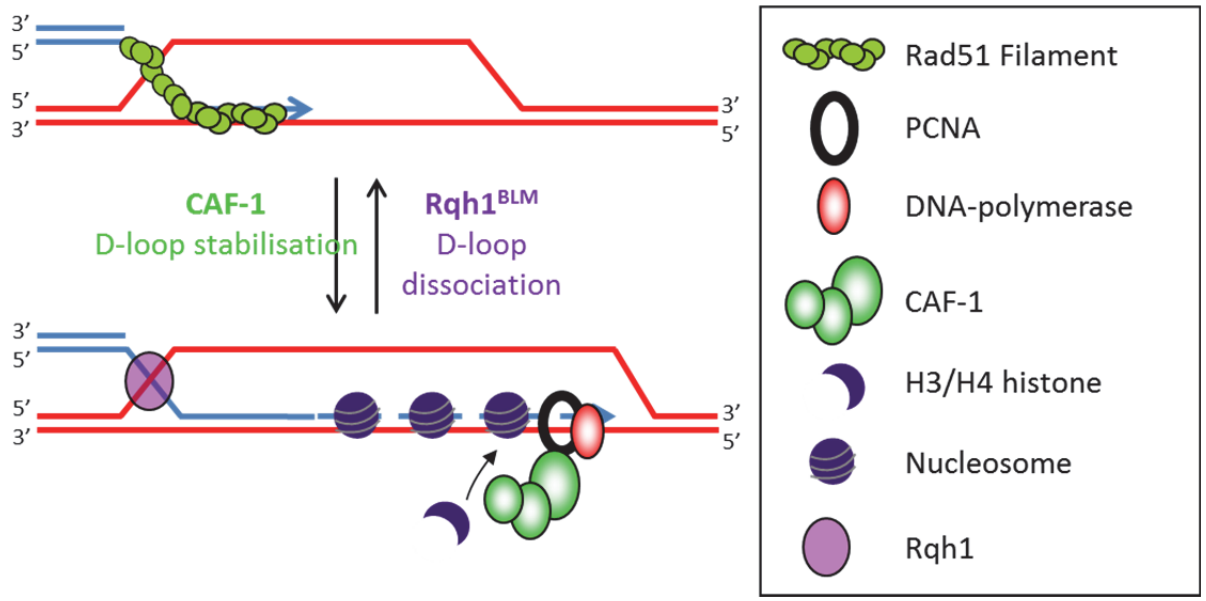


Figure n. 60: A model for D-loops stabilization by CAF-1 dependent nucleosome assembly. CAF-1 is recruited via the interaction between Pcf1 and PCNA during D-loops extension, to promote the chromatinization of early recombination intermediates (D-loops). Doing so, CAF-1 counteracts Rqh1 helicase activity and prevents the dissolution of D-loop intermediates.

2. *CAF-1 stabilizes D-loop intermediates by counteracting their dissolution by Rqh1 activity.*

CAF-1 appears to be part of an equilibrium that regulates the stability/dissociation of early steps of recombination events, acting as an antagonist of the DNA helicase Rqh1 (Figure n. 60). My data evidenced that, in the absence of both CAF-1 and Rqh1, JMs and the product of their resolution are properly formed. Moreover, the deletion of *rqh1* gene rescues the decreased level of fork-arrest induced ectopic recombination due to CAF-1 defects. Rqh1 but

not Srs2 helicase, was shown to antagonize CAF-1 function during homologous recombination mechanism.

Both Srs2 and Rqh1 are DNA helicases proposed to act during early steps of homologous recombination, by either dismantling the Rad51 filament or by dissolving D-loop intermediates, thus promoting the SDSA pathway (Fabre, Chan et al. 2002; Aylon, Liefshitz et al. 2003; Ira, Malkova et al. 2003; Robert, Dervins et al. 2006; Oh, Lao et al. 2007; Oh, Lao et al. 2008; Lambert, Mizuno et al., 2010; see Introduction 2.3.2). In addition, Rqh1 plays a crucial role in preventing mitotic cross-overs by promoting the dissolution of double Holliday-junctions (Doe, Dixon et al. 2000).

Considering the results obtained, it will be of interest to test the DNA helicase Fml1 (SPAC9.05), which deletion phenotype is viable. Fml1 is an orthologoue of human FANCM, a family of DNA helicases/translocases playing key roles in homologous recombination (HR) and DNA repair in archaea and eukaryotes (Whitby 2010). FANCM family members have a general ability to unwind and/or branch migrate DNA junctions that resemble intermediates of HR, in particular to branch migrate Holliday junctions (HJs) and unwinding of D-loops structures that are formed by Rad51-mediated strand invasion (Prakash, Satory et al. 2009; Sun, Nandi et al. 2008; Zheng, Prakash et al. 2011). It is proposed that Fml1 prevents crossing-over events during DSB repair by its ability to dissociate D-loop intermediates (Lorenz, Osman et al. 2012; Sun, Nandi et al. 2008), thus it could be a good candidate to counteract the stabilization function of CAF-1. Similarly, the orthologoue of Fml1 in *S. cerevisiae*, Mph1, is thought to channel recombination intermediates into a non-crossover pathway by dissociating D-loop structures (Prakash, Satory et al. 2009).

Fission yeast *rqh1* mutants are hypersensitive to agents such as UV light, CPT and MMS, that cause replication forks to stall (Boddy, Lopez-Girona et al., 2000; Stewart, Chapman et al. 1997). It has been proposed by Lambert and colleagues that Rqh1 helicase regulates Rhp51-dependent recombination at the *RTS1* barrier, and this limits the likelihood of rearrangements without affecting the efficiency of fork restart (Lambert, Mizuno et al. 2010). This might be achieved by Rqh1 function in processing the D-loop (Stewart, Chapman et al. 1997; Doe, Ahn et al. 2002). It has also been demonstrated that the double mutant *srs2-d rqh1-d* presents a very slow growth phenotype, probably due to the accumulation of toxic recombination intermediates. Indeed, this phenotype is rescued by the deletion of *rhp51*, *rhp55* or *rhp57* preventing the formation of the D-loop (Doe and Whitby 2004- Figure n. 58C). Deletion of *pcf1* or *pcf2* rescues this slow growth phenotype as well (Figure n. 58C). These data support

the view that CAF-1 acts in the same pathway than Rhp51, stabilizing the D-loop and promoting the formation of recombination intermediates that cannot be dissolved by helicases in the *srs2-d rqh1-d* strain.

The fact that CAF-1 stabilizes Rhp51-dependent JMs, could also provide an explanation for MMS resistance of strains deleted for *pcf1* or *pcf2* or *pcf3*. MMS methylates DNA on N^7-deoxyguanine and N^3-deoxyadenine and causes a variety of DNA damages, including stalling of replication forks, where HR is essential for fork restart (Lundin, North et al. 2005). Therefore, a possible explanation for MMS resistance of *pcf1-d* or *pcf2-d or pcf3-d* strains could be that Rad51-dependent joint molecules are not stable in the absence of CAF-1, leading to a decrease in the amount of toxic recombination intermediates. These toxic recombination intermediates are normally dissolved by Rqh1. An experiment to demonstrate that the resistance of CAF-1 defective strains to MMS is dependent on Rqh1 activity will be to test the the sensitivity to MMS of double mutants *pcf1-d rqh1-d* and *pcf2-d rqh1-d*, A loss of resistance to MMS in these double mutants will provide further evidences of the interplay between Rqh1 and CAF-1.

Another interesting point to explore is whether other histone chaperones are also involved in D-loop stabilization. Other H3-H4 and H2A-H2B histone chaperones could be involved in D-loop stabilization. In *S. pombe,* other histone chaperones could be tested:

- Histone chaperone Rtt106-like (H3-H4) is encoded by *mug183* gene (SPAC6G9.03c) and the strain deleted for this gene is viable. Rtt106 acts in replication-coupled chromatin assembly, therefore it could be a good candidate.
- Histone chaperone Asf1 (H3-H4) is encoded by *cia1* gene (SPCC663.05c), but the strain deleted for this gene is inviable. Termosensitive mutants were created in *S. pombe* (Tanae, Horiuchi et al. 2012) and also a mutant that cannot interact with HIRA and histone H3 and H4 (Yamane, Mizuguchi et al. 2011). However, Asf1 is known to donate H3-H4 tetramers to Rtt106, CAF-1 and HIRA and not to load them directly on the DNA.
- HIRA histone chaperone complex (H3-H4), which is composed of three subunits encoded by: *hip1* (SPBC31F10.13c) which deletion phenotype is viable; *slm9* (SPBC15D4.03) which deletion phenotype is viable and *hip3* (SPBC31F10.14c) which deletion phenotype is viable. However, HIRA is known to act during DNA replication-independent chromatin assembly pathways, such as during transcription and gene silencing.

- CENP-A chaperone Scm3 encoded by *scm3* gene (SPAPB1A10.02), but the deletion phenotype is inviable. Scm3 is involved in replication-coupled chromatin assembly at centromeric regions.
- Nap1 (SPCC364.06) is a H2A-H2B histone chaperone, which deletion phenotype is viable. However this histone chaperone is involved in replication-independent chromatin assembly (transcription-coupled nucleosome assembly).
- Nap2 (SPBC2D10.11C) is a H2A-H2B histone chaperone, which deletion does not affect the viability of the cells. This histone chaperone acts in replication-coupled chromatin assembly.
- Histone chaperone Chz1 (H2A-H2B) is encoded by *chz1* gene (SPAC4G9.06c) and the strain deleted for this gene is viable. Chz1 histone chaperone has overlapping functions with Nap1.

3. CAF-1 promotes D-loop stabilization via its replication-coupled chromatin assembly function.

My data also established that a tight interaction between Pcf1 and PCNA is crucial to promote replication-induced recombination and D-loop stabilization. This supports the hypothesis that CAF-1 promotes D-loop stabilization via its replication-coupled chromatin assembly function (Figure n. 60). Indeed, it has been shown that the interaction between PCNA and the largest subunit of CAF-1 is necessary to promote histone deposition during DNA replication (Krawitz, Kama et al. 2002).

After Rad51-filament invasion, a delay of 40 minutes is observed before extension of the D-loop, in budding yeast (Hicks, Yamaguchi et al. 2011; Introduction 2.3.2). During this timeframe, PCNA is recruited together with the DNA polymerases delta and eta to promote initiation of DNA synthesis from D-loop intermediates. At this step, PCNA could be also responsible for CAF-1 recruitment, necessary for the chromatinization of the extended D-loop. However evidences reported in the literature suggest that, PCNA might have an early role in homologous recombination during the formation of the D-loop (Hashimoto, Puddu et al. 2011). Therefore the recruitment of CAF-1 could be achieved in two steps, as suggested from Baldeyron and colleagues, in human cells (2011). Indeed, they propose that the larger subunit of CAF-1 could have a role independent on its function of histone chaperone during early steps of homologous recombination and, after the DNA damage repair is completed,

p150 could switch its function toward an active histone chaperone mode, together with the other subunits of CAF-1 complex.

In an effort to demonstrate that Pcf1 is recruited on D-loop intermediates, I investigated the recruitment of Pcf1 at the *RuraR* locus, by performing ChIP analysis using a strain expressing Pcf1-YFP. Unfortunately, I was not able to achieve my purpose maybe because the recruitment of Pcf1 at a specific locus (*RuraR)*, in specific conditions (fork arrest system ON) and in an asynchronous population, is below the resolution limit of the ChIP technique. Therefore, I focused on the possibility to detect a co-localization between the fluorescently tagged *RuraR* locus and Pcf1-YFP. Indeed, Dr. Costes, developed strains in which the *RuraR* locus is fluorescently tagged: LacO sequences were integrated 7.9 Kb away from *ura4* locus. LacO sequences are bound by the repressor LacI fused to a fluorescent tag (LacI-GFP or LacI-mCherry). LacI binding to LacO was shown to impede fork progression, and to avoid this, Dr Costes made use of a LacI variant isolated by Dr. Dubarry in Dr A. Taddei's lab (Dubarry, Loiodice et al. 2011). Based on strains developed by Dr. Costes, I created a strain harbouring the fluorescently tagged *RuraR* locus (expressing LacI-mCherry) and expressing the Pcf1-YFP. If an increase in the co-localization between LacI-mCherry and Pcf1-YFP in presence of the fork blockade at *RuraR* (ON condition), could be showed, this will be an evidence of Pcf1 recruitment at the arrested fork. To show that this recruitment is linked to recombination intermediates, Pcf1 recruitment is expected to be dependent on recombination proteins, at least Rad22.

Despite that D-loop stabilization by CAF-1 requires its interaction with PCNA, this does not provide evidences that histones are indeed deposited onto extended D-loop. A caveat of my work was the difficulty to create a strain in which CAF-1 does not interact with histones. Indeed, CAF-1 interacts with H3-H4 dimers via the three subunits and there are no point mutations, described in the literature, that allow abolishing CAF-1 interaction with H3/H4. Furthermore, there are not clear mapped region of interaction between the different CAF-1 subunits and the dimer H3-H4. To investigate nucleosome assembly on D-loop intermediates, a possible option is a biochemical approach: synthetic D-loop substrates could be incubated with nuclear extracts, to promote D-loops extension by DNA synthesis *in vitro*. Western-blot analysis could reveal the presence of histones on synthetic D-loops. Coupled with genetic, these kinds of assays could be used to demonstrate that nucleosome deposition occurs on D-loop intermediates, in a CAF-1 dependent manner. Such biochemical assays were already

exploited by McIIwraith and colleagues in 2005, to study DNA polymerases involved in DNA synthesis from strand invasion intermediates (McIIwraith, Vaisman et al. 2005).

To score JMs and the product of their resolution, I exploited the conditional fork arrest substrate *RuraR* at *ura4* locus. To widen the knowledge horizons of CAF-1 involvement in D-loop stabilization, it will be of interest to create various *RuraR* constructs, in which the distance between *RTS1* repeats will be decreased, to determine how much of DNA synthesis is needed to observe the stabilizer function of CAF-1. The length of the DNA wrapped around the nucleosome is about 147 bp and the length of the DNA linker between nucleosomes can be very different from region to region in the genome. Considering that the actual size between *RTS1* repeats is 1.7 Kb, the maximal number of nucleosomes can be deposit on the D-loop is of 11. Modifying the distance between *RTS1* repeats might be useful also to investigate if the ability of Rqh1 to dissociate D-loop is sensitive to the number of nucleosomes that are loaded, or if just one nucleosome might be enough to block its dissociative function.

CAF-1 deposits preferentially newly synthesized histones on DNA and the mark of such histones is the acetylation of H3 on lysine 56 (H3K56Ac). Considering the importance of this mark during DNA replication and repair (Introduction 3.2.4 and 4.2), further perspectives could be to investigate the function of H3K56Ac in D-loop stabilization by CAF-1, using a strain expressing histones H3 that cannot be acetylated on lysine 56. Dr. Xemalce provided me of a strain in which H3.3 and H4.3 encoding genes are deleted by *arg3,* H3.1 and H4.1 encoding genes are deleted by *his3* and the lysine 56 of histone H3.2 is mutated in arginine *(H3.1/H4.1::his3 + H3.3/H4.3::arg3, H3.2K56R* – Xemalce, Miller et al. 2007). The strain will be crossed with a second one, harbouring the *RuraR* construct at *ura4* locus to investigate if the absence of acetylation on K56 could mimics defects in D-loop stabilization, such as those due to the loss of CAF-1 complex. My hypothesis is that, D-loop dissociation by Rqh1 might be sensitive to histone acetylated on H3K56Ac and not to deposition of histones per se.

This model implies that CAF-1, as reported in the literature, could be a hub to couple genetic and epigenetic inheritance during DNA repair, as it does during DNA replication (Polo, Almouzni 2006; Groth, Rocha et al. 2007). Indeed, the histone deposition onto D-loop intermediates undergoing DNA synthesis could facilitate the maintenance of the memory of the epigenetic state. Parental histones harbouring specific marks could be evicted ahead of the replication fork and, together with newly synthesized histones, might be deposited onto the D-loop. Therefore, CAF-1 could be responsible for the maintenance of specific chromatin states

during DNA repair and, especially, homologous recombination. During DNA replication, PCNA interacts with several enzymes involved in epigenetic inheritance, such as the DNA methyltransferase DNMT1 in human cells (Chuang, Ian et al. 1997; Warbrick 1998). A further speculation is that the interaction between PCNA and such enzymes could facilitate the role of CAF-1 in maintaining the epigenetic memory, in mammals. Indeed, parental histones might be used as fingerprint, to promote specific modifications on newly synthesized histones before being taken in charge by CAF-1.

4. The role of CAF-1 in channelling the recombination pathway choice

As detailed in the introduction, homologous recombination is a very dynamic process, in which a variety of recombination mediators (Rad22, Rhp54, Rhp55-Rhp57, Rlp1-Rdlp1-Sws1) and negative regulators (Srs2, Fml1, Rqh1) act in a synergistic or antagonistic manner, to process Rad51 filament and D-loop intermediates, displacing the equilibrium of the balance toward different possible recombination pathways, such as DSBR and SDSA.

Once the D-loop is formed, DNA polymerases delta and eta initiate DNA synthesis to elongate this early recombination intermediate and different mechanisms are proposed for the stabilization of the extended D-loop. During allelic recombination, the long sequence homology, allows CAF-1 independent stabilization mechanisms to occur, such as Rad51-dependent branch migration (Murayama, Kurokawa et al. 2008). Indeed, my data support a dispensable function for CAF-1 in supporting allelic recombination. The rate of recombination between homologous chromosomes and chromosome loss in strains defective for CAF-1 are the same than in the wild-type strain. Considering also the experiment to score spontaneous sister chromatid exchanges, all my data are coherent with the fact that CAF-1 is dispensable for allelic recombination, in *S. pombe.*

The other side of the coin consists of the involvement of CAF-1 in ectopic (non-allelic) recombination. Non-allelic recombination occurs between dispersed repeated sequences in the genome, leading to aberrant outcomes threatening genome stability (Introduction 2.5.1). During ectopic recombination, the shorter length of homology between donor and acceptor DNA molecules renders Rad51-dependent branch migration impossible and the role of CAF-1 in D-loop stabilization might be crucial. These innovative data reveal a novel regulation level of homologous recombination mechanisms and outcomes (allelic versus non-allelic recombination), by CAF-1 dependent chromatin assembly, affirming the importance of CAF-1 in genome stability maintenance. Indeed, the role of CAF-1 in D-loop stabilization implies a

channelling of the recombination pathways toward the DSBR model and double HJs formation, to the detriment of the synthesis-dependent strand annealing (SDSA) pathway. To confirm this speculation, it is envisageable to use a plasmid gap repair assay, which measures the repair of a linearized plasmid through recombination with homologous DNA on the chromosome. Exploiting this assay, it will be possible to evaluate how CAF-1 influences the rate of mitotic crossing-overs (Orr-Weaver and Szostak 1983; Sun, Nandy et al. 2008). Another implication of my model is that, during the switch of nascent strands from one template to another one, invoked for example during Micro-homology Mediate BIR (MMBIR – see Introduction 2.5.2), the deposition of nucleosomes onto newly synthesized strand, could constitute a physical barrier to the re-annealing of the filament with the initial template.

It would have been interesting to dissect the role of CAF-1 during homologous recombination also in meiosis and not only in vegetative growing cells. Unfortunately *S. pombe* diploids, homozygous for *pcf1-d* or *pcf2-d* or *pcf3-d* present defects in meiosis leading to abnormal ascus morphology, abnormal number of spores containing in the ascus and severely reduced spore viability (data not shown; Dohke, Miyazaki et al. 2008). Thus, I concentrated on mitotic recombination in vegetative cells, where the deletion of *pcf1* or *pcf2* or *pcf3* genes do not present any significant growth phenotype, except for their sensitivity to the nitrogen source.

Paradoxically, even if CAF-1 could be considered as a sort of genome-keeper due to its function in DNA metabolic processes, it promotes recombination pathways associated with genome rearrangements. Thus, my data reveal a novel role for CAF-1, which could be considered as the first component of a regulator factor class, orchestrating ectopic but not allelic recombination.

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ANNEX

Violena Pietrobon, PhD.

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➢ PERSONAL DATA

Date/Place of birth: 5th of September 1985, Montebelluna (TV) Italy.

Languages: Italian - Mother tongue.
English - Optimal level for spoken and written English. First certificate in English, Council of Europe Level B2 obtained at Feltre seat of the Cambridge University in March 2004.
French - Good level for spoken and written French.

Citizenship: Italian, Canadian

➢ ACADEMIC BACKGROUND

September 2009 – December 2012 PhD student at the Institute Curie, Orsay (Paris) UMR3348 – Sarah Lambert laboratory affiliated to "Gènes, Génomes, Cellules" doctoral school – Paris Sud XI.
Subject: "Chromatin assembly by CAF-1 during homologous recombination: a novel step of regulation".

21st July 2009 Master degree in sanitary biology, achieved at the University of Padua (110/110), Italy.

28th September 2007 Bachelor degree in molecular biology, Class 12 of Biological Sciences, achieved at the University of Padua (laurea cum laude), Italy.

July 2007: High school leaving qualifications (98/100) achieved at "Primo Levi" scientific high school in Montebelluna, Italy.

➢ FORMATIONS AND STAGES

6th-10th of February 2012: Microscopy course "Frontiers in Imaging: DNA dynamics and repair" at the Friederich Miescher Institute for Biomedical Research, in Basel (Switzerland).

- *Training in using different kinds of microscope for in vivo imaging and techniques of deconvolution and de-noising.*

9th-13th of May 2011: Secondment in Molecular Combing and Industrial experience at Genomic Vision in Paris (France).

- *Training in practical DNA combing and interpretation of results.*

7th-12th of March 2011: Workshop on Single Molecules microscopy techniques and Total Internal reflection Microscopy (TIRFM) at the University of Sussex – Genome Damage and Stability Center (UK).

- *Training in the visualization of DNA molecules, TIRFM and application for FRET.*

20th-26th of February 2010: Workshop on Fluorescence Correlation spectroscopy at the Ben Gurion University (Israel).

- *Theorical lessons on the mathematical basis of microscopy and on how to build a microscope.*

October - July 2009: Stage at Pr. Russo's laboratory on "Characterization of replication pattern at the frataxin region (9q13)" at the University of Padua (Italy).

- *Use of molecular combing to study the DNA replication at 9q13 in lymphoid cells, to analyse particular features that could lead to the development of Friedreich Ataxia.*

May - June 2007: Stage at Pr. Rigoni's laboratory on " Heterologue expression, purification and activity essay of a Fe-Hydrogenase: Hyd-A" at the University of Padua (Italy).

22nd of April 2003: European computer driving licence achieved at "Primo Levi" high school

➢ CONFERENCES

10th – 12th September 2012: Oral presentation at the "Final Meeting" for the European Initial Training Network (ITN) at Brighton University (U.K.).

12th – 16th September 2011: Oral presentation at the "Report Meeting" for the European ITN at the University Bicocca in Milano (Italy).

25th – 29th July 2011: Poster presentation at FASEB "Genetic recombination and genome rearrangements" meeting in Steamboat Springs - Colorado (U.S.).

28th September 2010: Oral presentation at the "Midterm meeting" for the European ITN at the Institute Curie in Paris (France).

1st-2nd July 2010: Poster presentation at the "Marie Curie satellite Event" (ESOF 2010 Euroscience Open Forum) in Torino (Italy).

29th-30th October 2009: Partecipation at the kickoff meeting for the European ITN at the Brighton University (U.K.).

➢ FUNDINGS

- Marie Curie Initial Training Network (ITN) in FP7, PhD position for three years (2009-2012).

- Institut Curie Paris, UMR 3348, for one month (September 2012)

- Fondation ARC pour la recherché sur le cancer, for three months (October-December 2012).

➢ PUBLICATIONS

Iraqui I., Chekkal Y., Jmari N., Pietrobon V., Fréon K., Costes A., Lambert S. – **Recovery of arrested replication forks by homologous recombination is error prone** – 2012
PloS Genetics, in press.

➢ SKILLS

Ability to work in a team

Quick learning

Organization skills

Independent work

Cellular biology: cell cultures of fission yeast *S. Pombe* and mammalian cells

Molecular biology: Pulse Field Gel Electrophoresis (PFGE), DNA gel 2D, PCR, Western blot, Southern blot, Fluorescence Microscopy, Chromatin Immunoprecipitation (ChiP), Co-Immunoprecipitation and Quantitative PCR.

Informatics: Office pack, FIJI (Image J), Image Quant, Adobe Photoshop, Metamorph, Biorad CFX Manager, Internet programs (Blast, Primer3, NEBcutter, ClustalW).

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Recovery of Arrested Replication Forks by Homologous Recombination Is Error-Prone

Ismail Iraqui[1,2☯], Yasmina Chekkal[1,2☯], Nada Jmari[1,2☯], Violena Pietrobon[1,2], Karine Fréon[1,2], Audrey Costes[1,2], Sarah A. E. Lambert[1,2*]

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Abstract

Homologous recombination is a universal mechanism that allows repair of DNA and provides support for DNA replication. Homologous recombination is therefore a major pathway that suppresses non-homology-mediated genome instability. Here, we report that recovery of impeded replication forks by homologous recombination is error-prone. Using a fork-arrest-based assay in fission yeast, we demonstrate that a single collapsed fork can cause mutations and large-scale genomic changes, including deletions and translocations. Fork-arrest-induced gross chromosomal rearrangements are mediated by inappropriate ectopic recombination events at the site of collapsed forks. Inverted repeats near the site of fork collapse stimulate large-scale genomic changes up to 1,500 times over spontaneous events. We also show that the high accuracy of DNA replication during S-phase is impaired by impediments to fork progression, since fork-arrest-induced mutation is due to erroneous DNA synthesis during recovery of replication forks. The mutations caused are small insertions/duplications between short tandem repeats (micro-homology) indicative of replication slippage. Our data establish that collapsed forks, but not stalled forks, recovered by homologous recombination are prone to replication slippage. The inaccuracy of DNA synthesis does not rely on PCNA ubiquitination or trans-lesion-synthesis DNA polymerases, and it is not counteracted by mismatch repair. We propose that deletions/insertions, mediated by micro-homology, leading to copy number variations during replication stress may arise by progression of error-prone replication forks restarted by homologous recombination.

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Introduction

Maintenance of genome stability requires the faithful and accurate replication of the genetic material. Genome instability is a hallmark for most types of cancer and it is strongly associated with predisposition to cancer in many human syndromes (for a review, see [1,2]). Genome instability is manifest at two levels: at the nucleotide level, resulting in base-substitutions, frame-shifts or in micro-insertions/deletions (referred to herein as mutations); and at the chromosomal level, resulting in duplications, deletions, inversions and translocations (referred to herein as gross chromosomal rearrangements or GCRs).

Genome instability during cancer development and in other human genomic disorders may be consequences of failures in chromosome replication (for a review, see [3,4]). Origin spacing has recently been shown to cause chromosomal fragility at some human fragile sites [5,6]. Impediments to replication fork movements *per se* may also cause genome instability [7–9]. Indeed, both slowing down and blockages to fork progression can lead to chromosomal fragilities or GCRs in human cells and yeast models [10–14]. However, how a blocked replication fork leads to genetic instability remains poorly understood.

In eukaryotes, DNA replication is initiated at numerous origins along linear chromosomes, and impediments to fork progression appear unavoidable during each S-phase (for a review, see [9,15]). Impediments to fork progression can be caused by DNA lesions, by non-histone proteins tightly bound to DNA, by sequence-caused secondary structures such as cruciform structures and possibly G-quadruplexes, by nucleotide pool imbalance and by conflicts with transcription machinery (for a review, see [16,17]). In case of failures in fork progression, DNA replication can be completed either by the recovery of the arrested fork by fork-restart mechanisms, or as a result of the progression of a converging fork which can be ensured by activation of dormant origins [7,15,18]. Fork restart is presumably essential in unidirectional replication regions, such as the rDNA locus, in regions of low densities of origins, such as some human fragile sites, and when two converging forks are both impeded [5,19,20].

To ensure faithful and complete DNA replication, cells coordinate DNA synthesis restart with specific pathways, including DNA replication checkpoint and homologous recombination mechanisms [17]. The integrity of replication forks is guaranteed by the DNA replication checkpoint that maintains the replisome in a replication-competent state to keep DNA polymerases at the site

Author Summary

The appropriate transmission of genetic material during successive cell divisions requires the accurate duplication and segregation of parental DNA. The semi-conservative replication of chromosomes during S-phase is highly accurate and prevents accumulation of deleterious mutations. However, during each round of duplication, there are many impediments to the replication fork machinery that may hinder faithful chromosome duplication. Homologous recombination is a universal mechanism involved in the rescue of replication forks by rebuilding a replication apparatus at the fork (by mechanisms that are not yet understood). However, recombination can jeopardize genome stability because it allows genetic exchanges between homologous repeated sequences dispersed through the genome. In this study, we employ a fission yeast-based arrest of a single replication fork to investigate the consequences of replication fork arrest for genome stability. We report that a single blocked fork favours genomic deletions, translocations, and mutations; and this instability occurs during fork recovery by recombination. We also report that a single arrested fork that resumes its progression by recombination is prone to causing replication slippage mediated by micro-homology. We propose that deletions/duplications observed in human cancer cells suffering from replication stress can be viewed as scars left by error-prone replication forks restarted by recombination.

of nucleotide incorporation [21]. It remains unclear how the DNA replication checkpoint modulates replisome activities to maintain its function [21,22]. The DNA replication checkpoint also regulates nuclease activities (*e.g.* Exo1 or Mus81) which contribute to preserving the integrity of stalled forks [23,24]. If replisome function is lost or the replisome dissociates at broken replication forks, the resumption of DNA synthesis appears to require the replisome to be rebuilt. In *E.coli*, restart of a collapsed fork involves homologous recombination and the PriA helicase that allows replisome components to be loaded *de novo* on joint-molecule structures [25,26]. In eukaryotes, the restart of collapsed or broken replication forks is dependent upon homologous recombination, but the mechanism of origin-independent loading of the replisome remains to be described [20,27–30]. It has been proposed that the repair of a double-strand break (DSB) by recombination (break-induced replication, BIR) in budding yeast similarly involves the assembly of a replication fork (for a review, see [30–32]). When BIR occurs outside S-phase, recombination-dependent replication fork assembly can synthesise hundreds of kilobases (Kb). However, this DNA synthesis is highly inaccurate due to frequent template switching of nascent-strands and frame-shift mutations [33,34].

We previously reported a system that displays replication fork arrest at a specific locus in the fission yeast *S. pombe*. The system is a polar replication fork barrier (RFB) regulated by the Rtf1 protein binding to its *RTS1* binding site [35]. The *RTS1*-RFB causes fork arrest because of a non-histone protein complex binding to the DNA. As proposed for other polar RFBs, the *RTS1*-RFB is thought to block fork progression by directly (contact between proteins and the replisome) or indirectly (topological constraint) affecting the replicative helicase activity and thereby preventing DNA unwinding [36,37]. Recovery of the arrested fork occurs by a DSB-independent mechanism and involves the recruitment of recombination proteins at the *RTS1*-RFB site. We proposed that recombination proteins associate with unwound nascent strands that then anneal with the initial template to allow DNA synthesis to restart [11,20]. The causative protein barrier then has to be removed either by DNA helicase or by the recombination machinery itself to allow fork-progression to resume [38–40]. Occasionally, the unwound nascent strand can mistakenly anneal with a homologous template in the vicinity of the collapsed fork, resulting in the restart of DNA synthesis on non-contiguous template. This incorrect template switch of nascent strands results in inversions and iso-acentric and dicentric chromosomes in ~2–3% of cells/generation [11,20]. Error-free template switching between sister-chromatids provides an efficient mechanism for filling-in single-stranded gaps left behind damage-induced stalled forks [41]. Inverted chromosome fusions in yeast and rare-genome rearrangements in human genomic disorders, may both be consequences of template switching between ectopic repeats associated with impeded replication forks [8,14].

Here, we used the *RTS1*-RFB to investigate the consequences of fork collapse on genome instability. We report that recovery from a collapsed fork is associated with a high frequency of instability, with a single fork arrest increasing the rates of mutation, deletion and translocation by 10, 40 and 5 fold, respectively. We show that genetic instability associated with fork arrest is dependent on homologous recombination. Fork-arrest-induced GCRs (deletion and translocation) result from inappropriate ectopic recombination at the site of the collapsed fork. We also demonstrate that restoration of fork progression by homologous recombination results in error-prone DNA synthesis due to frequent replication slippage between short tandem repeats. We investigated the molecular mechanisms of this replication slippage and found that post-replication repair, including ubiquitination of PCNA or trans-lesions-synthesis (TLS) DNA polymerases, is not involved in fork-arrest-induced replication slippage. Micro-deletions/insertions flanked by micro-homology associated with copy number variations (CNVs) in cancer cells or in response to replication stress may therefore be scars left following the restoration of forks progression by homologous recombination.

Results

The conditional replication fork barrier *RTS1*

We generated fork arrest constructs by manipulating the polar *RTS1*-RFB (Figure 1A). We introduced the *RTS1* sequence on the centromere-proximal (*cen*-proximal) side of the *ura4* locus, 5 kb away from the strong replication origin (ori) 3006/7 on chromosome III. This created the *t-ura4<ori* locus, in which "*t*" and "*ori*" refer to the telomere and the origin 3006/7, respectively; and "<" and" >"refer to the *RTS1*-barrier and its polarity that is whether it blocks replication forks travelling from the ori 3006/7 towards the telomere or forks travelling from the telomere towards the ori 3006/7, respectively. We previously confirmed that forks moving from ori 3006/7 towards the telomere (*tel*) are efficiently blocked by the *RTS1*-RFB at the *t-ura4<ori* locus [35]. In this model system, fork arrest is activated by inducing the expression of $rtf1^+$ gene that is under control of the thiamine repressible promoter *nmt41*. Thus, the *RTS1*-RFB is inactivated by adding thiamine to the media and it is activated in thiamine-free media. Efficient induction of Rtf1 expression requires incubation for 12–16 hours in thiamine-free media. Replication intermediates were analysed by native 2-dimensional gel electrophoresis (2DGE). In conditions of Rtf1 expression, more than 95% of replication forks were blocked by the *RTS1*-RFB at the *t-ura4<ori* locus (see black arrow on Figure 1B, *t-ura4<ori* ON). Arrested forks were not detected without Rtf1 induction (Figure 1B, *t-ura4<ori* OFF) [20].

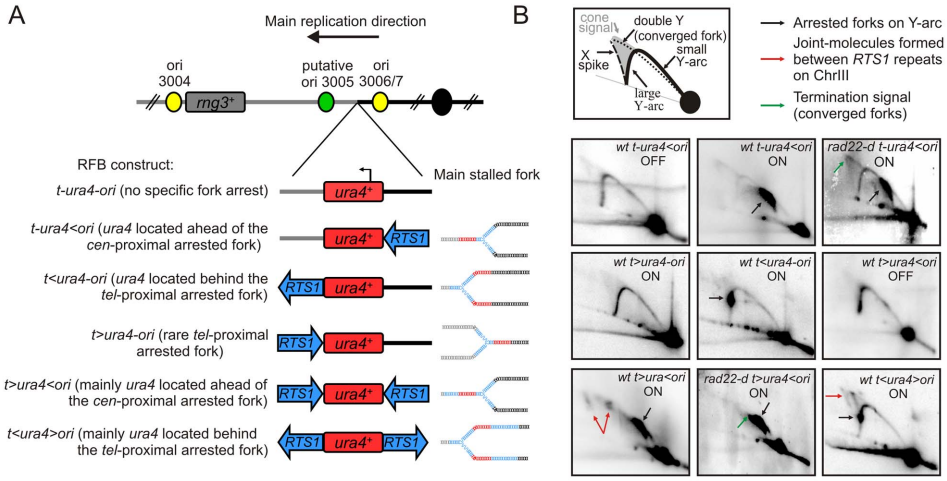

Figure 1. Conditional replication fork-arrest assays. A. Diagrams of fork-arrest constructs. *Centromere*-proximal and *telomere*-proximal regions are represented in black and grey, respectively. Strong or putative replication origins (ori) and the centromere are indicated by yellow, green and black circles, respectively. Blues arrows indicate the polarity of the *RTS1*-RFB. The *ura4*$^+$ gene is indicated in red and the arrow indicates its direction of transcription. Representations of the primary arrested fork structure are given for each construct. The name of each fork-arrest construct is given using the following nomenclature: "t" and "ori" refer to the telomere and the replication origin 3006/7, respectively; "<" and ">" indicate the *RTS1*-barrier and its polarity (< blocks replication forks moving from the ori3006/7 towards the telomere, and > blocks replication forks moving from the telomere towards the origin 3006/7. B. Diagrams of replication intermediates (RIs) within the AseI fragment analysed by 2DGE (top panel). Representative RIs analysed by 2DGE in indicated strains in OFF (Rtf1 being repressed) and ON (Rtf1 being expressed) conditions. Signal corresponding to arrested forks, joints-molecules (JMs) and termination structures are indicated by black, red and green arrows, respectively. Note that the *t>ura4-ori* construct does not result in a strong fork arrest as the *RTS1*-RFB is not orientated in the main direction of replication (see text for details).
doi:10.1371/journal.pgen.1002976.g001

Conditional fork-arrest constructs to investigate fork-arrest-induced genome instability

The *RTS1* sequence was inserted on the *tel*-proximal side of *ura4* creating the *t<ura4-ori* locus. 2DGE analysis of this construct revealed a strong fork arrest signal on the descending large Y arc (Figure 1A and 1B, *t<ura4-ori* ON). The *ura4*$^+$ gene, used in this system as a reporter to score genetic instability, is located behind the arrested fork when the *RTS1*-RFB is active at the *t<ura4-ori* locus and ahead of the arrested fork at the *t-ura4<ori* locus. This explains the distinct position of the arrested fork signal on the Y arc. Inversion of the *RTS1* sequence at the *tel*-proximal side of *ura4* created the *t>ura4-ori* locus and no fork arrest signal was detected for this construct by 2DGE when Rtf1 was expressed (Figure 1A and 1B, *t>ura4-ori* ON). Thus, *RTS1* behaves as a polar RFB at the *ura4* locus, and replication across this locus is strongly unidirectional due to the relative positions of the origins [42].

Introducing a second *RTS1* sequence, such that the two *RTS1* sequences are inverted repeats (IRs), created *t>ura4<ori* and *t<ura4>ori* loci (Figure 1A and 1B, *t>ura4<ori* and *t<ura4>ori* ON). Given the orientation of the polar *RTS1*-RFB in the *t<ura4>ori* strain, converging forks cannot be blocked. Whereas block of converging forks can virtually occur in the *t>ura4<ori* strain, 2DGE in this construct revealed that forks arrested on the *cen*-proximal side of *ura4* were efficiently recovered by recombination before forks are arrested on the *tel*-proximal side. Indeed, joint-molecules (JMs) resulting from recombination between *RTS1* repeats were detected by 2DGE (see red arrows on Figure 1B, *t>ura4<ori* and *t<ura4>ori* ON). Resolution of these JMs gives rise to chromosomal rearrangements [20]. In the absence of homologous recombination (*i.e.* in a *rad22-d* mutant), JMs were not detected and termination signals accumulated (see green arrow on Figure 1B, *t>ura4<ori rad22-d* strain). Similarly, termination signals accumulated in the *rad22-d t-ura4<ori* strain (see green arrow on Figure 1B, *t-ura4<ori rad22-d*), showing that, when arrested forks are not restarted by homologous recombination, the *RTS1*-RFB behaves as a hot spot for replication termination [20].

A single fork arrest induces genomic deletions

We investigated fork-arrest-induced genome instability by selecting for cell resistance to 5-FOA^R, the result of loss of *ura4*$^+$ function. Inducing fork-arrest at *t-ura4<ori* increased *ura4* loss 3 fold (Table 1). Rtf1 expression in the *t-ura4-ori* and *t>ura4-ori* strains did not cause site-specific fork-arrest at *ura4* as assessed by 2DGE and did not increase the rate of *ura4* loss. Thus, *ura4* loss results from the *RTS1*-RFB activity and not simply from the presence of *RTS1* and/or Rtf1 expression (Table 1). To investigate the nature of this genetic instability, primers were designed to amplify the *ura4* coding sequence and, as a control, the essential *rng3* gene, mapping 30 kb *tel*-proximal to *ura4*, that should not be rearranged (Figure 2A and 2B) [35]. The absence of *ura4* amplification was classified as a deletion event; sequencing of amplified *ura4* sequence was used to identify point mutation events (Figure 2B).

A single arrested fork at the *t-ura4<ori* locus was sufficient to increase the rate of genomic deletion up to 40 times over spontaneous events (*i.e.* in the *t-ura4-ori* strain, p = 0.006) (Figure 2C and Figure S1A). Fork-arrest-induced deletion was recombination-dependent. Spontaneously (*i.e.* when the *RTS1*-barrier was inactivated), the rate of genomic deletion in *rad22-d* or *rhp51-d* strains was higher than that in the wild-type strain (Figure S1B). Nonetheless, no further increase in the rate of genomic deletion was observed in the surviving *rad22-d* or *rhp51-d* cells upon activation of the *RTS1*-barrier (Figure S1B, *t-ura4<ori*). Frequent spontaneous genomic deletion in the *rad22-d* or *rhp51-d* strains is consistent with previous reports showing that mutations in recombination genes are associated with an increase level of GCRs [14,43,44]. Deleting the natural *RTS1* sequence from chromosome II abolished deletion events at collapsed forks,

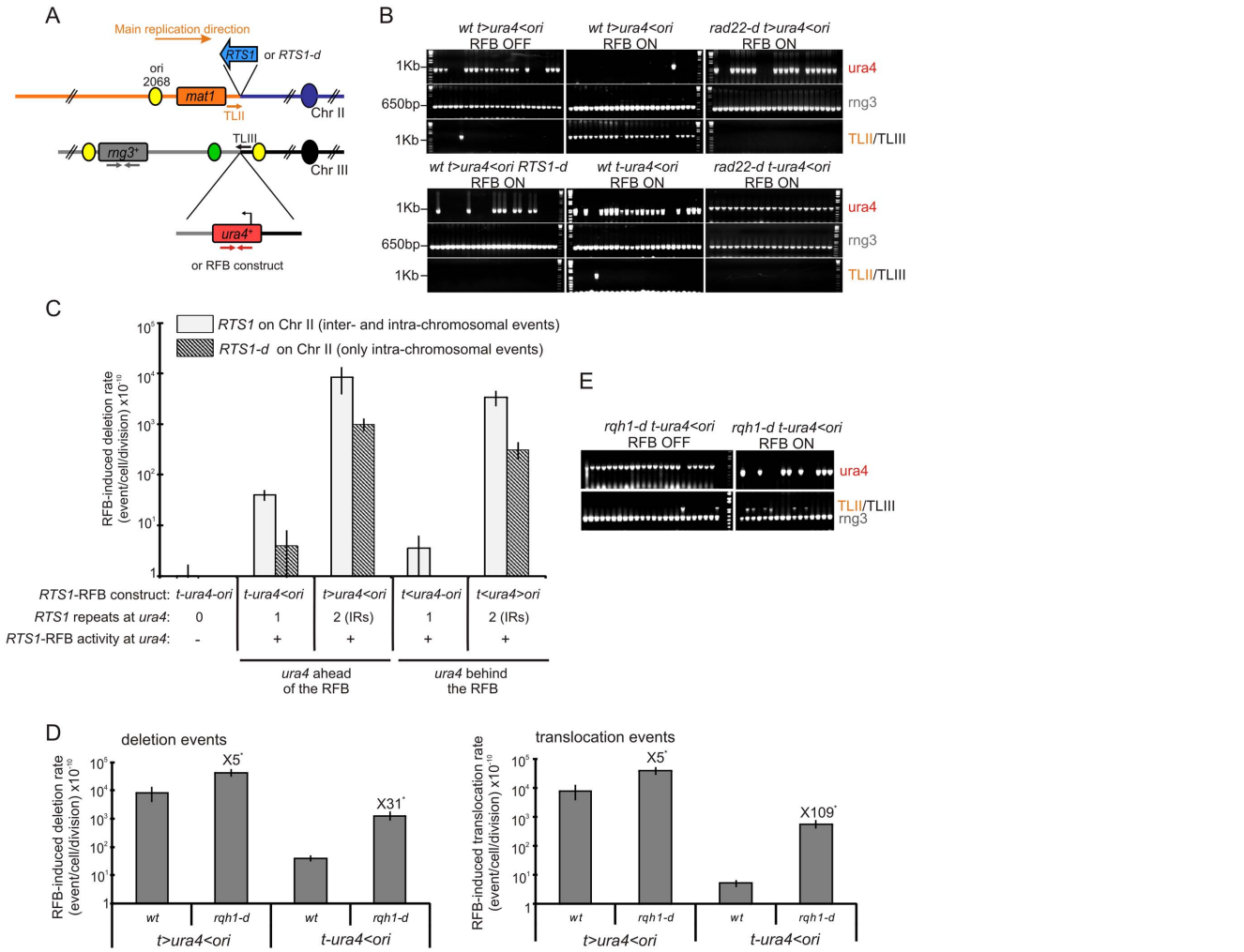

Figure 2. A single fork-arrest induces GCRs that are stimulated by inverted repeats near the site of fork arrest. A. Diagrams of chromosome II containing or not the *RTS1* sequence (blue arrow or *RTS1-d*) and of chromosome III containing *ura4*$^+$ alone or associated with *RTS1*-RFB constructs. The *RTS1* sequence maps near the *mat1* locus where it helps to ensure unidirectional replication [62]. Primers used for amplifying the 1 Kb *ura4* fragment or the 650 bp *rng3* fragment are depicted in red and grey, respectively. Primers used to amplify the translocation junction (1.2 kb) are represented in orange on chromosome II (TLII) and in black on chromosome III (TLIII). B. Representative PCR-amplifications from 5-FOA^R colonies of the indicated strains; ON and OFF refers to the *RTS1*-RFB being active or not, respectively. PCR products and their sizes are indicated on the figure. C. Effect of intra- and inter-chromosomal recombination between *RTS1* repeats on fork-arrest-induced genomic deletion. *RTS1*-RFB activity and *ura4* location with respect to the RFB are given for each construct. The % of deletion events, as determined by the PCR assay, was used to balance the rate of *ura4* loss. Then, the RFB-induced deletion rate was calculated by subtracting the rate obtained in the presence of thiamine (Rtf1 being repressed) from the rate obtained in the absence of thiamine (Rtf1 being expressed). The values reported are means of at least 3 independent median rates. Error bars correspond to the standard error (SE). D. Effect of Rqh1 on RFB-induced deletions (left) and translocations (right), as described for panel C. Error bars indicate SE. Statistically significant fold differences between the *rqh1-d* and the wild-type strains are indicated with an *. E. Representative PCR amplifications from 5-FOA^R colonies of the *rqh1-d t-ura4<ori* strain, as described for panel B. (Refer to Figure S1 for corresponding rates of deletion and translocation when Rtf1 is expressed or not).
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indicating that fork-arrest-induced deletion was also mediated by inter-chromosomal recombination (Figure 2C and *t-ura4<ori RTS1-d* on Figure S1A). Thus, these data are consistent with the view that homologous recombination makes a major contribution to suppressing genome instability, but can occasionally drive non allelic recombination events leading to GCRs [35,45].

We detected no fork-arrest-induced deletion in the *t<ura4-ori* strain, in contrast to the *t-ura4<ori* strain (Figure S1A and Figure 2C). The *ura4* marker is located behind and ahead of collapsed forks in the *t<ura4-ori* and *t-ura4<ori* strains, respectively (Figure 1A). Therefore, replicated regions, located behind collapsed forks, do not display instability, and fork-arrest-induced deletion occurs within unreplicated regions immediately in front of arrested forks. Overall, our data establish that genomic deletion at collapsed forks results from inappropriate recombination between ectopic sequences during the process of fork recovery by recombination proteins.

Inverted repeats stimulate fork-arrest-induced deletion by promoting inter- and intra-chromosomal recombination

Inverted repeats (IRs) are structural elements often associated with genome rearrangements [11,14,46,47]. We investigated the

Table 1. Rates of *ura4* loss (including genomic deletion, translocation, and mutation events), calculated using the method of the median.

strains[a]	Rate of *ura4* loss[b]		Fold induction by Rtf1 expression (−/+ thiamine)[c]	Fold induction by the RFB (relative to the *t-ura4-ori* construct)
	+ thiamine (Rtf1 repressed)	− thiamine (Rtf1 expressed)		
t-ura4-ori	4.6±1	4.3±1.8	0.9	
t-ura4<ori	4.1±0.9	13.9±2.8	3.4 (p = 0.002)	3.2
t-ura4<ori RTS1-d	4.9±1.4	11.4±4.1	2.3 (p = 0.03)	2.6
t<ura4-ori	3.9±1.3	4.8±2.5	1.2	1.1
t>ura4<ori	5.8±2.1	97.4±51.5	16.8 (p = 0.004)	22.6
t>ura4<ori RTS1-d	4.9±2.2	19.5±3.3	4 (p = 0.006)	4.5
t>ura4-ori	3.2±1.7	3.6±1.8	1.1	0.8
t<ura4>ori	6.6±1.7	50.3±14.2	7.6 (p = 0.014)	11.7

[a] the following nomenclature is used: "t" and "ori" refer to the telomere and the replication origin 3006/7, respectively; "<" and ">" indicate the *RTS1*-RFB and its polarity (< blocks replication forks moving from the ori3006/7 towards the telomere, and > blocks replication forks moving from the telomere towards the origin 3006/7).

[b] event/cell/division $\times 10^{-8}$ ± standard error. Values are means of at least 3 independent rates.

[c] statistical significance was determined using the nonparametric Mann-Whitney U test.

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effects of IRs in the vicinity of the *RTS1*-RFB on fork-arrest-induced genomic deletion. We first compared the *t>ura4<ori* strain (IRs flanking *ura4*) to the *t-ura4<ori* strain (no IRs near the *RTS1*-RFB). The rate of fork-arrest-induced genomic deletion was 200 times higher in the *t>ura4<ori* than that in the *t-ura4<ori* strain (p = 0.009, Figure 2C and Figure S1A). Thus, intra-chromosomal ectopic recombination permitted by the *RTS1* sequence on the *tel*-proximal side of *ura4* accounted for 99.5% of the genomic deletions observed in the *t>ura4<ori* strain (Figure 2C, compare with *t-ura4>ori*). Preventing inter-chromosomal recombination by deleting *RTS1* from the chromosome II (*t>ura4<ori RTS1-d*) abolished 90% of deletion events (Figure 2C and Figure S1A). Thus, genomic deletions induced by fork-arrest near IRs are due to inter- and intra-chromosomal recombination events. In support of this, stimulation of fork-arrest-induced deletion by IRs is mediated by homologous recombination. Indeed, the rate of genomic deletion was not increased upon induction of the *RTS1*-RFB in the surviving population of *t>ura4<ori rad22-d* and *rhp51-d* strains (Figure S1B). These data indicate that IRs favour genomic deletion at collapsed forks by promoting inappropriate inter- and intra-chromosomal recombination during fork recovery by recombination proteins.

We verified that our data were not influenced by the orientation of IRs or by rare blocking of converging forks in the *t>ura4<ori* strain. We analysed the *t<ura4>ori* construct in which *RTS1* repeats are in the opposite orientations relative to the *t>ura4<ori* construct, such that forks converging towards *ura4* cannot be blocked (Figure 1). The rate of fork-arrest-induced genomic deletion was 1,000 times higher in the *t<ura4>ori* than that in the *t<ura4-ori* strain, that does not contain IRs near the *RTS1*-RFB (p = 0.008, Figure 2C and Figure S1A). Thus, intra-chromosomal recombination, permitted by the *RTS1*-RFB sequence on the *cen*-proximal side of *ura4*, accounted for nearly 100% of the genomic deletions observed in the *t<ura4>ori* strain (Figure 2C, compare with *t<ura4-ori*). Preventing inter-chromosomal recombination by deleting *RTS1* from the chromosome II (*t<ura4>ori RTS1-d*) abolished 90% of deletion events (Figure 2C and Figure S1A). Importantly, the deletion rates for the *t<ura4>ori* and *t>ura4<ori* strains were not significantly different (Figure 2C), showing that IRs cause genomic deletion at collapsed forks irrespective of their orientations and independently of blockage of converging forks.

A single collapsed fork induces translocations that are stimulated by IRs

Fork-arrest at *t>ura4<ori* results in translocations between ectopic *RTS1* repeats on chromosomes II and III [35]. We investigated the influence of IRs on fork-arrest-induced translocation. We designed primers to amplify the predicted translocation junction between chromosomes II and III (TLII and TLIII on Figure 2A and 2B). A single arrested fork at the *t-ura4<ori* locus was sufficient to increase the translocation rate to 5 times higher than the spontaneous rate (p = 0.002, Figure 2D and Figure S1C). The translocation rate for the *t>ura4<ori* construct (containing IRs) was 1,500 fold higher than that for the *t-ura4<ori* strain that does not contain IRs near the *RTS1*-RFB (p = 0.009, Figure 2D and Figure S1C). Thus, intra-chromosomal recombination accounted for nearly 99% of translocation events observed in the *t>ura4<ori* construct (Figure 2D and Figure S1C, compare with *t-ura4<ori*). No translocation events were detected when inter-chromosomal recombination was prevented by deleting *RTS1* from the chromosome II (*t>ura4<ori RTS1-d* on Figure 2B). Therefore, as reported for genomic deletions, fork-arrest-induced translocation associated with IRs is due to inter- and intra-chromosomal ectopic recombination. No translocations were detected in the *t<ura4>ori* strain (data not shown), so we cannot formally exclude the possibility that fork-arrest-induced translocations in the *t>ura4<ori* strain was caused by blocking of converging forks. However, as no translocation event occurred in the absence of $Rad22^{Rad52}$ or $Rhp51^{Rad51}$, it is most likely that translocations occur during fork recovery by recombination (Figure 2B and [35]). Overall, our data indicate that recovery of a single collapsed fork causes translocations and IRs near the site of fork-arrest stimulate translocations by promoting inappropriate inter- and intra-chromosomal recombination.

Fork-arrest-induced GCRs are caused by inter- and intra-chromosomal recombination. We noticed a slightly greater contribution of intra- than inter-chromosomal recombination (Figure 2C). This is consistent with ectopic recombination preferentially occurring at the most proximal homologous sequence, as previously reported [48]. Nonetheless, the rate of fork-arrest-induced deletion in the *t>ura4<ori* strain ($8.4\ 10^{-7}$) was not simply the sum of the rates of intra-chromosomal recombination events ($9.9\ 10^{-8}$ in the *t>ura4<ori RTS1-d* strain) and inter-chromosomal recombination events ($4\ 10^{-9}$ in the *t-ura4>ori* strain). Similar reasoning can be applied for the *t<ura4>ori* strain. Thus, independent intra- and inter-recombination events cannot themselves explain high rate of GCRs induced by fork arrest near IRs. Therefore, we infer that there is interplay between inter- and intra-chromosomal recombination such that fork-arrest-induced GCRs may involve recombination between three homologous sequences (tri-parental recombination).

The RecQ helicase Rqh1 prevents GCRs at collapsed forks

To confirm that fork-arrest-induced GCRs are the result of inappropriate ectopic recombination during fork recovery, we analysed the involvement of the RecQ helicase Rqh1. We previously reported that Rqh1 limits inappropriate template switching of stalled nascent strands without affecting the efficiency of fork restart [20]. In the *t-ura4<ori* construct (in which only inter-chromosomal recombination is possible), fork-arrest-induced deletion and translocation rates were 31 and 109 times higher in the *rqh1-d* strain than that in the wild-type control, respectively ($p = 0.0003$, Figure 2D–2E and Figure S1C). For the *t>ura4<ori* construct (containing IRs near fork-arrest), fork-arrest-induced deletion and translocation rates were 5 times higher in the *rqh1-d* than that in the wild-type control ($p = 0.0007$, Figure 2D–2E and Figure S1C). Thus, Rqh1 limits GCRs at collapsed forks by preventing inappropriate ectopic recombination during the process of fork recovery by recombination proteins.

A single fork arrest induces mutations

We analysed the effects of collapsed forks on the mutation rate. We sequenced the *ura4* coding sequence from 5-FOA^R isolated cells and identified base-substitutions, frame-shifts and small insertions and duplications between short tandem repeats (Table 2). A single collapsed fork in the *t-ura4<ori* strain increased the overall mutation rate up to 10 times over spontaneous events (Figure 3A, $p = 0.003$). Similar increases in the overall mutation rate were found for the strains with IRs near the arrested fork and those with *RTS1* deleted from chromosome II (Figure 3A and Figure S2A). Thus, fork-arrest-induced mutation is not mediated by inappropriate ectopic recombination. Induction of the *RTS1*-RFB in the *t<ura4-ori* strain did not increase the mutation rate of the *ura4* gene. Thus, as for GCRs, replicated regions behind arrested forks are not prone to mutation. This observation rules out the hypothesis that fork-arrest-induced mutation is a consequence of the accumulation of damaged single-stranded DNA behind collapsed forks (see discussion). Our data suggest that recovery from collapsed forks results in error-prone DNA-synthesis.

Collapsed forks result specifically in replication slippage

We then analysed the spectra of mutations found in the *ura4* ORF by sequencing the PCR products. The rates of base-substitutions and frame-shifts were not significantly increased by the *RTS1*-RFB activity over spontaneous events (*i.e.* compare to *t-ura4-ori* strain, Figure 3C and Table 2). In contrast, the rate of deletions and duplications (Del/Dup) flanked by short homology was increased by 7 times over spontaneous events in the *t-ura4<ori* strain, but not in the *t<ura4-ori* strain (Figure 3C and Table 1). These data further confirm that fork-arrest does not promote mutation events behind collapsed forks.

We used reversed mutation assays to test if fork-arrest at the *RTS1*-RFB specifically induced Del/Dup mutations. We made use of strains harbouring a single mutation within the *ura4* ORF: either a single base-substitution or a −1 frame-shift in homo-nucleotide (Figure S2B). We also studied strains harbouring either a duplication of 20 or 22 nt flanked by 5 or 4 bp of micro-homology, respectively (defined as *ura4-dup20* and *ura4-dup22*, Figure S2B). These non-functional *ura4*$^-$ alleles were inserted in front of the *RTS1*-RFB in the *t-ura4<ori* configuration and we then tested whether fork arrest could restore a functional *ura4*$^+$ gene. Activation of the *RTS1*-RFB at *ura4* increased the frequency of Ura$^+$ revertants up to 15 and 7 times in strains harbouring *ura4-dup22* and *ura4-dup20*, respectively (Figure 3D and Figure S2B). Thirty Ura$^+$ colonies were studied by PCR and all gave a product of the same size as the wild-type *ura4*$^+$ gene: they had therefore lost the duplication (Figure 3E and data not shown). Sequencing the full *ura4* ORF confirmed that Ura$^+$ revertants contained an intact *ura4*$^+$ sequence, showing that the reversion of these alleles was due solely to the precise deletion of 20 or 22 nt (Figure 3F and data not shown). In contrast, activation of the *RTS1*-RFB did not increase the frequency of Ura$^+$ revertants of strains harbouring *ura4* alleles with a single base-substitution or a −1 frame-shift (Figure 3D and Figure S2B). Thus, collapsed forks tend to induce deletion events between short tandem repeats rather than base-substitution or frame-shift mutations.

Among Del/Dup events, deletions represented the two-third of events in the *t-ura4<ori* strain (Table 2). The median size of Del/Dup events was 24 and 22 nt respectively, and Del/Dup occurred between short direct repeats 1 to 10 nt long (Figure S3). Thus, the *ura4-dup20* and *ura4-dup22* alleles used in the reverse mutation assay were representative of the Del/Dup events observed. Del/Dup flanked by micro-homology result from intra-molecular template switching mechanisms in which nascent strands dissociate from the template and misalign with the template when restarting the elongation step. This leads to loop formation, either in the nascent strand or in the template, resulting in duplication or deletion events, respectively [49]. Consequently, we will hereafter refer to Del/Dup as replication slippage. Replication slippage was observed all along the *ura4* ORF and up to 1.2 kb ahead of the arrested fork, even if a hot spot of deletion was present 500 bp away from the *RTS1*-RFB (Figure 3G and Figure S3B). Thus, our data suggest that the DNA synthesis is prone to replication slippage at least for the first 1,200 nt synthetized during the recovery of collapsed forks. Inaccuracy of DNA synthesis on further distances was not directly addressed.

Replication slippage results from error-prone DNA synthesis during fork recovery

To confirm that replication slippage occurs as forks recover, and not behind the fork in the DNA already replicated, we inserted the *ura4-dup20* or the *ura4-dup22* allele either behind (in the *t<ura4-ori* configuration) or in front of the *RTS1*-RFB (in the *t-ura4<ori* configuration) (Figure 4). This allows the analysis of the same event of replication slippage behind and ahead of collapsed forks. In the *t-ura4<ori* configuration, induction of the *RTS1*-RFB resulted in a 8 and 16 fold increases in the replication slippage frequency for the *ura4-dup20* and *ura4-dup22* alleles, respectively (Figure 4A and 4B). Similar increases in the rate of replication slippages were observed (Figure 4C). In contrast, in the *t<ura4-ori* background, the frequency of replication slippage was induced by only 2–3 fold

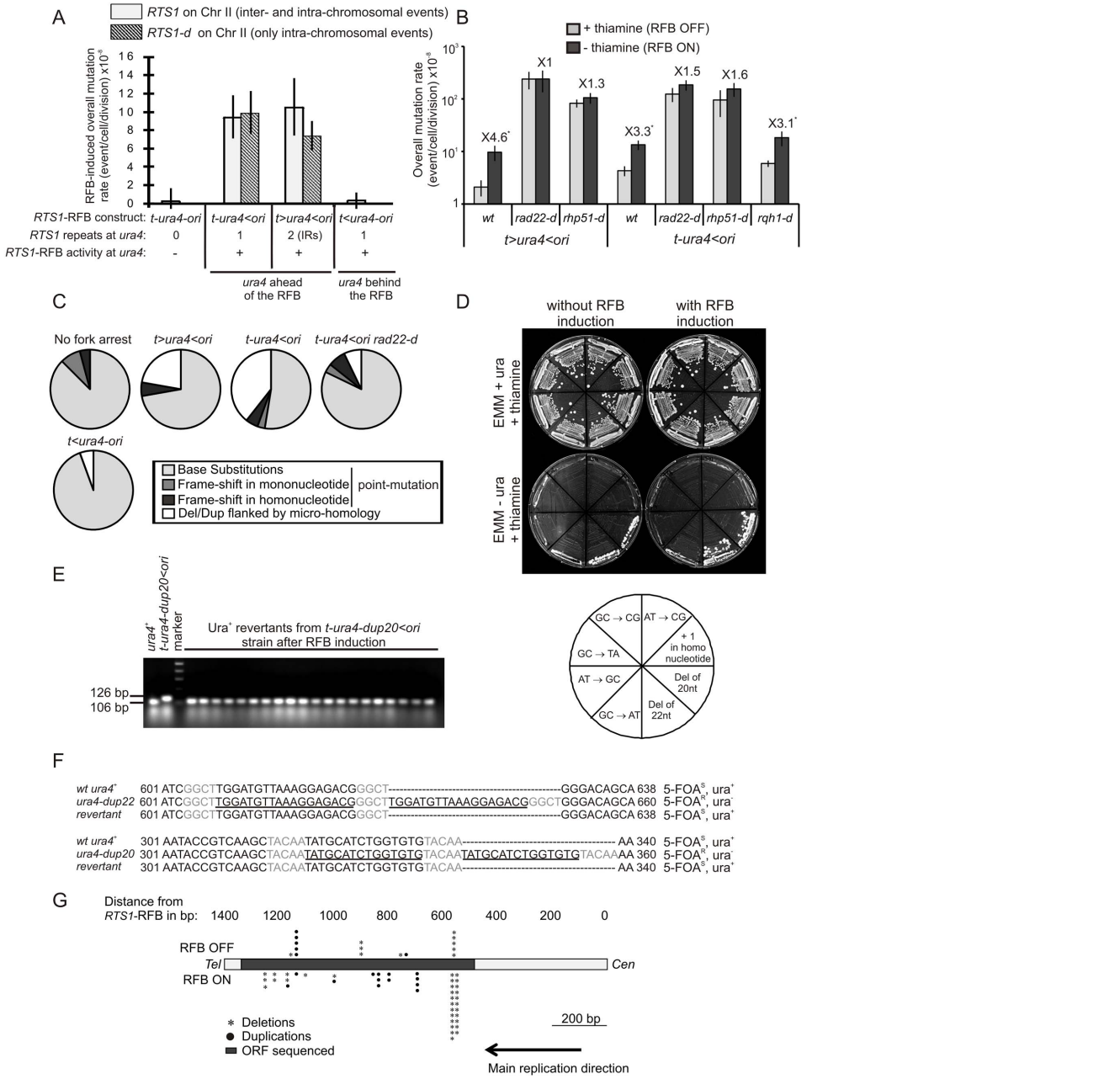

Figure 3. Fork-arrest induces mutations. A. Effect of intra- and inter-chromosomal recombination between *RTS1* repeats on fork-arrest-induced mutation rates (base-substitutions, frame-shifts and small insertions or deletions between short tandem repeats). *RTS1*-RFB activity and *ura4* location with respect to the RFB are given for each construct. The % of mutation events, as determined by the PCR assay and sequencing, was used to balance the rate of *ura4* loss. Then, the RFB-induced mutation rate was calculated by subtracting the rate obtained in the presence of thiamine (Rtf1 being repressed) from the rate obtained in the absence of thiamine (Rtf1 being expressed). The values reported are means of at least 3 independent median rates. Error bars correspond to SE. (Refer to Figure S2 for corresponding rates of mutation when Rtf1 is expressed or not). B. Rate of mutation for indicated strains; ON and OFF refers to the *RTS1*-RFB being active or not, respectively. The % of mutation events, as determined by the PCR assay and sequencing, was used to balance the rate of *ura4* loss. The values reported are means of at least 3 independent median rates. Error bars correspond to SE. Statistically significant fold differences in mutation rates between the ''OFF'' and ''ON'' conditions are indicated with an *. C. Spectra of mutation events in indicated strains upon RFB induction (refer to Table 2 for exact numbers and to Figure S3 for mapping of deletions/duplications and their features). D. Strains harbouring the *ura4* alleles with a single base-substitution or frame-shift or duplication of 20 or 22 nt, together with the *RTS1*-RFB in the *t-ura4<ori* configuration were streaked onto the indicated media after cell growth with (RFB ''OFF'') or without (RFB ''ON'') thiamine. The bottom diagram indicates strain positions and the mutation events required to obtain Ura^+ revertants. E. PCR analysis of Ura^+ revertants isolated from the *t-ura4-dup20<ori* strain (duplication of 20 nt in *ura4*) after RFB induction. With the primers used, a 106 bp fragment is amplified from the *ura4*$^+$ strain and a 126 bp fragment is amplified from the *t-ura4-dup20<ori* strain. F. Sequence alignments of *ura4-dup22*, *ura4-dup20*, *ura4*$^+$ alleles and corresponding Ura^+ revertants. Micro-homologies are indicated in grey and duplicated sequences are underlined in black. The phenotype of each allele is indicated on the figure. G. Map of deletion and duplication events within the *ura4* ORF.
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Table 2. Mutations spectra in the indicated strains.

	t-ura4-ori[a]		*t>ura4-ori*		*t-ura4<ori*		*t>ura4<ori*		*t<ura4-ori*		*t-ura4<ori rad22-d*	
Rtf1 expression	−	+	−	+	−	+	−	+	−	+	−	+
Fork arrest at *ura4*	−	−	−	−	−	+	−	+	−	+	−	+
Transitions[b]												
GC→AT	5			1	7	14	6	2		5	5	5
AT→GC	3		1	2	4	9	1	4	4	7	4	6
Transversions[b]												
GC→TA	3	5	1		45	20	5	2	4	2	3	4
GC→CG	1	3	4	2	2	3	2			1	8	5
AT→CG	3	3	1	1	6	4	2	2		1	4	
AT→TA		1	5	3	4	2	4	3	3	1	2	3
Rate of base substitutions[c]	4.6±1.1	3.7±1.5	3.3±1.6	4.2±1.3	3.4±0.9	6.4±1.2[d]	2.6±1.1	6.5±1.5[d]	2.5±1.3	3.3±1.7	115±34	152±33
+ Frame-shift[b]:												
in homo-nt		1							1			
in mono-nt		1	2	1	2	1	1		1		1	
- Frame-shift[b]:												
in homo-nt					1	4		1			1	2
in mono-nt					1	3			1			1
Rate of frame-shift[c]		0.6±0.25	0.5±0.3	0.5±0.1	0.2±0.06	1±0.2[d]	0.1±0.06	0.5±0.1	0.7±0.4		8.7±2.6	20.4±4.4
Del/Dupl[b]:												
Deletions					9	27		4		1		2
Duplications					5	12						
Rate of Del/Dupl[c]		<0.3		<0.5	0.7±0.2	4.9±1	<0.1	2.1±0.5	<0.2	0.2±0.1	<3.7	13±2.8
Other (complexe)					1							
Total	15	14	14	10	87	99	21	18	14	18	28	28

[a] the following nomenclature is used: "t" and "ori" refer to the telomere and the replication origin 3006/7, respectively; "<" and ">" indicate the *RTS1*-RFB and its polarity (< blocks replication forks moving from the ori3006/7 towards the telomere, and > blocks replication forks moving from the telomere towards the origin 3006/7).

[b] number of events.

[c] event/cell/division $\times 10^{-8}$ ± standard error. The % of each mutation events was used to balance the rate of mutation.

[d] value not significantly different from those for the *t-ura4-ori* strain (spontaneous events).

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by the *RTS1*-RFB (Figure 4B–4C). These data confirm that DNA located ahead of collapsed forks is more prone to replication slippage than replicated DNA adjacent to arrested forks, further evidence that replication slippage arises during fork recovery.

Replication slippage results from forks restarted by recombination

Replication slippage occurs in DNA in front of (and not behind) the arrested fork, this DNA being replicated only after restart of the fork. Thus, a defect preventing fork recovery would be expected to abolish the error-prone DNA synthesis during restart. We analyzed fork-arrest-induced mutation in recombination mutants in which collapsed forks at the *RTS1*-RFB cannot recover, resulting in cell death. Induction of the *RTS1*-RFB did not increase the overall mutation rate in the surviving populations of *t>ura4<ori* or *t-ura4<ori rad22-d* and *rhp51-d* strains (Figure 3B). In addition, only 7% of mutation events in the survivors of the *rad22-d t-ura4<ori* strain were Del/Dup mutations, compared to 40% in the wild-type strain (Figure 3C and Table 1). We currently cannot assess mutation events associated with defects in fork recovery because this appears to be lethal in the absence of recombination. Nevertheless, our data are consistent with fork-arrest-induced replication slippage being dependent on homologous recombination. The *rad22-d* and *rhp51-d* strains are themselves spontaneously mutagenic. Consequently, any small increase in the fork-arrest-induced mutation rate might be masked by the high frequency of spontaneous 5-FOA^R cells in *rad22-d* and *rhp51-d* strains. We therefore used a more specific mutation assay, based on the *ura4-dup20* allele, to determine the rate of replication slippage induced by the *RTS1*-RFB over spontaneous events.

Strains carrying mutations in recombination genes grow slowly, so replication slippage was scored as a function of the number of generations following thiamine removal (*i.e.* generations subject to fork arrest at *ura4*) (Figure 4D and 4E). In the wild-type strain, fork arrest at the *RTS1*-RFB resulted in a 10 fold-increase in the frequency of replication slippage, as expected. In recombination mutants (*rad50-d*, *rhp51-d* and *rad22-d*), fork-arrest at the *RTS1*-RFB increased the frequency of replication slippage by only 2 times over spontaneous events: therefore, replication slippage occurs less frequently in survivors from recombination mutants than those from the wild-type strain (Figure 4D–4F). Based on 2DGE analysis, fork-restart is severely impaired in the absence of

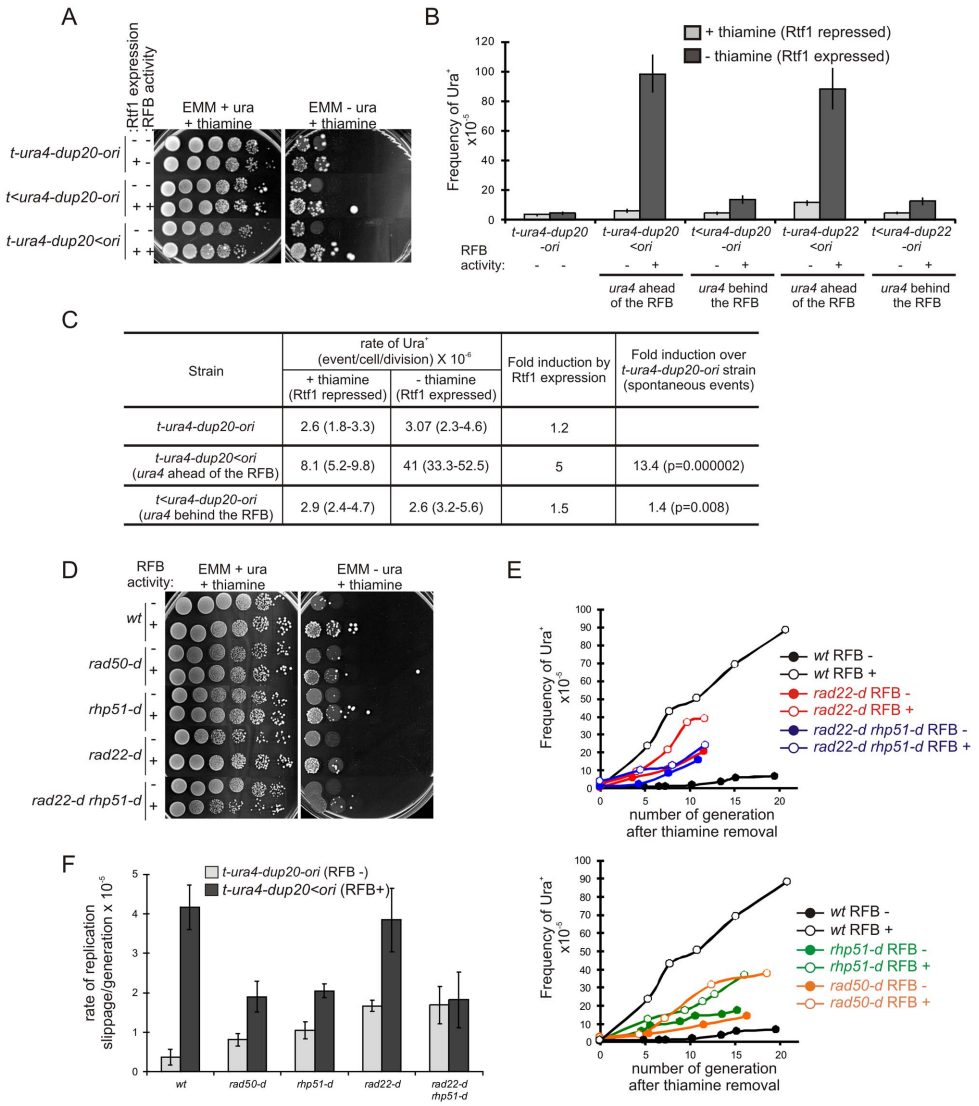

Figure 4. Fork recovery by homologous recombination results in replication slippage. A. Serial tenfold-dilutions from indicated strains (*t-ura4-dup20-ori* associated or not with the *RTS1*-RFB) were spotted onto the indicated media after cell growth with (Rtf1 -, repressed) or without (Rtf1 +, expressed) thiamine. *RTS1*-RFB activity is given for each construct and condition. B. Frequency of Ura$^+$ revertants in indicated strains after cell growth with (Rtf1 repressed) or without (Rtf1 expressed) thiamine. The *RTS1*-RFB activity is given for each construct and condition. Values correspond to the mean of at least three independent experiments and error bars correspond to the standard error of the mean (SEM). C. Rate of replication slippage in the indicated strains and conditions. The rate of Ura$^+$ revertants was calculated using the method of the median from at least 11 independent cultures. Values in brackets indicate the 95% confidence interval. Statistical significance was detected using the nonparametric Mann-Whitney U test. D. Serial tenfold-dilutions from the strains indicated spotted onto the media indicated after cell growth without thiamine. *RTS1*-RFB activity "–" refers to the strain *t-ura4-dup20-ori* and "+" refers to the strain *t-ura4-dup20<ori*. E. Kinetics of Ura$^+$ revertants frequency for the strains indicated as a function of the number of generations after thiamine removal. *RTS1*-RFB activity "–" refers to the strain *t-ura4-dup20-ori* and "+" refers to the strain *t-ura4-dup20<ori*. The values reported are the means of two experiments. F. The rate of replication slippage/generation for the strains indicated with (*t-ura4-dup20<ori*) or without (*t-ura4-dup20-ori*) the active *RTS1*-RFB. The rate was calculated from the slope of the curves presented in panel F. The values reported are means of three independent experiments and error bars correspond to SE.
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Rad22^{Rad52} (Figure 1B and [20]), such that even the two-fold induction in replication slippage by fork arrest in the *rad22-d* strain was surprising. The *rad22-d* strain accumulates suppressors involving the Fbh1 helicase that limits Rhp51^{Rad51}- dependent recombination at blocked replication forks [50,51]. Therefore, we analyzed replication slippage in the *rad22-d rhp51-d* double mutant in which no homologous recombination event occurs. In this background, there was no detectable fork-arrest-induced replication slippage (Figure 4D–4F). Thus, complete defect in fork restart results in a complete abolition of fork-arrest-induced replication slippage in the surviving population. Overall, our data establish that replication slippage results from inaccurate DNA synthesis during the restart of collapsed forks by recombination.

Replication stress leading to fork collapse induces replication slippage

We investigated the effects of replication stress, other than the replication block imposed by the *RTS1*-RFB, on replication slippage. Strains harbouring *ura4*$^-$ alleles (base-substitutions, −1 frame-shift, and *ura4-dup20*) were exposed to replication-blocking

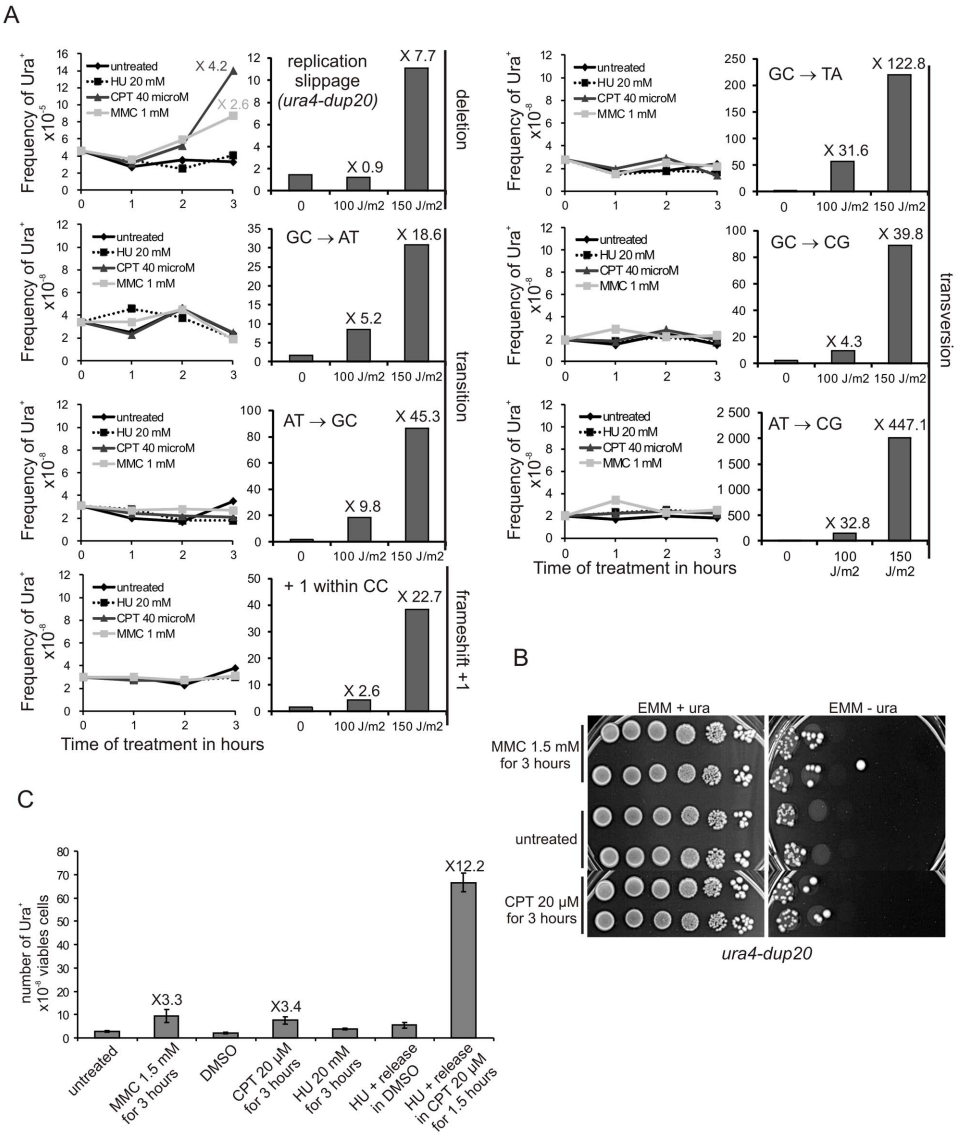

Figure 5. Collapsed forks, but not stalled forks, induce replication slippage. A. Left panel: the frequency of Ura^+ revertants as a function of time-contact with indicated drugs for the indicated *ura4* alleles (single base-substitution, frame-shift, duplication of 20 nt). Right panel: the frequency of Ura^+ revertants in response to UV-C irradiation as a function of dose for the indicated *ura4* alleles. The values reported are means of two independent experiments. Numbers indicate fold difference in the frequency of Ura^+ revertants between the treated and untreated control samples. For *ura4* alleles containing base-substitutions or frame-shifts, the mutation event required to obtain Ura^+ revertants is indicated on the figure. B. Serial tenfold-dilutions from *ura4-dup20* strain spotted onto the media indicated after treatment with MMC or CPT as indicated. C. Frequency of Ura^+ revertants after the indicated treatments in the *ura4-dup20* strain. DMSO (the vehicle) was used as control for CPT treatment. The values reported are means of at least three independent experiments. Error bars correspond to SEM.
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agents or UV-C-induced DNA damages and the frequency of Ura^+ revertants was scored. Three hours of treatment with either the topoisomerase I inhibitor camptothecin (CPT) or mitomycin C (MMC), an inter-strand cross-linking agent (ICls), increased the frequency of Ura^+ revertants by 3 to 4 fold in the *ura4-dup20* strain (Figure 5A and 5B). At equivalent survival (70–90%), DNA-damages induced by a dose of 100 J/m^2 of UV-C did not increase the frequency of Ura^+ revertants in the *ura4-dup20* strain. Increasing the UV-C dose (150 J/m^2) resulted in an increased reversion effect. The other *ura4* alleles exhibited an opposite behaviour pattern. As expected, UV-C-induced DNA damages, but not CPT or MMC treatment, increased the frequency of Ura^+ revertants of the base-substitution and the −1 frame-shift mutants (Figure 5A). Thus, replication slippage, unlike other point mutations, appears to be a mutation event specifically induced by replication stress.

Hydroxyurea (HU) that prevents the bulk of dNTP synthesis during S-phase by inhibiting the ribonucleotide reductase, results in a slow-down of fork progression which did not induce replication slippage (Figure 5A). In contrast, CPT and MMC treatments that lead to replication stress by causing fork collapse induced replication slippage. Homologous recombination is repressed during HU treatment and recombination proteins are recruited to collapsed but not stalled forks [52–54]. Consistent with this, we found that the *rad22-d* mutant is highly sensitive to acute exposure to CPT, but not to HU (Figure S4). Thus, acute exposure to HU results in stalled forks that recover without

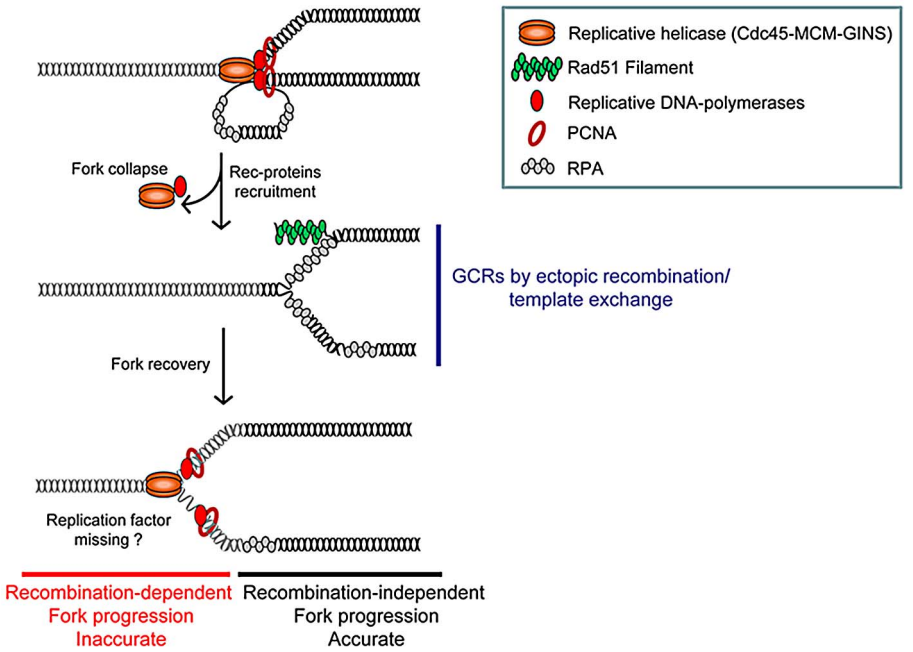

Figure 6. Model of replication-stress-induced genetic instability at collapsed forks. Collapsed forks might arise from torsional stress, fork breakage (*i.e.* at nick, ICLs), or proteins tightly-bound to DNA. Replisome disassembly at collapsed forks may favour the unwinding of the nascent strand on which Rad51 nucleates. At this initial stage of fork resumption by recombination, homology-driven template exchange can promote intra- or inter-recombination resulting in GCRs. Fork recovery by recombination overcomes the initial replication block and allows an inaccurate replisome to form (see text for details).
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recombination, whereas recombination may be required for restarting forks that have collapsed due to CPT or MMC treatment. We confirmed that CPT-induced replication slippage results from collapsed forks and was thus S-phase specific: the *ura4-dup20* strain was synchronized in early S-phase by HU treatment and released into S-phase with or without CPT. HU-synchronization and release into DMSO (used as vehicle for CPT) did not induce replication slippage. In contrast, the release of cells into S-phase in the presence of CPT stimulated replication slippage up to 12 fold (Figure 5C). These data indicate that CPT-induced fork collapse results in error-prone DNA synthesis characterized by replication slippage. These experiments further support the view that replication slippage results from recovery of collapsed forks by recombination and point out that the *RTS1*-barrier is representative of collapsed forks restarted by homologous recombination.

TLS–DNA polymerases are not involved in fork-arrest-induced replication slippage

To investigate the inaccuracy of the DNA synthesis occurring immediately following the restart of collapsed forks, we analysed the involvement of TLS-DNA polymerases. In fission yeast, TLS pathways require either mono- or poly-ubiquitination of the clamp loader PCNA on lysine 164 [55]. We found that mutating this lysine to arginine residue did not affect replication slippage induced by the *RTS1*-RFB. None of Rev1, Rev3 or DinB DNA polymerases were required for fork-arrest-induced replication slippage (Figure S5). Therefore, the error-prone DNA synthesis associated with fork recovery by recombination does not rely on TLS DNA polymerases activity.

The mismatch repair pathway does not counteract fork-arrest-induced replication slippage

The mismatch repair (MMR) pathway is temporally coupled to DNA replication, and MMR components are associated with replication centres [56]. The heterodimer Msh2/Msh6 recognises mispaired DNA and Msh2/Msh3 recognises small DNA loops up to 31 bases long, arising from replication slippage [57]. The failure to repair small DNA loops results in more frequent insertions and deletions [58]. Therefore, MMR activity could potentially lead to an underestimation of the extent of fork-arrest-induced replication slippage. However, replication slippage induced by the *RTS1*-RFB activity was as frequent in *msh2-d*, *msh6-d* and *msh3-d* strains as in wild-type control. Also, spontaneous replication slippage at *ura4* (without *RTS1*-RFB) was unaffected by the absence of MMR proteins (Figure S5). Therefore, there is no evidence that MMR repairs small DNA loops (20 nt) in fission yeast and fork-arrest-induced replication slippage is not counteracted by MMR in our model system.

Discussion

Using conditional fork arrest constructs, we studied the consequences for genome instability of impediments to replication forks progression. A single fork arrest results in large-scale genomic changes and mutations that occur during recombination-mediated fork recovery (Figure 6). Inappropriate ectopic recombination at arrested forks results in GCRs, whereas appropriate restarting of the fork on the initial template results in error-prone DNA synthesis. GCRs and mutations at collapsed forks are genetically separable: Rqh1 limits fork-arrest-induced GCRs but not mutations (Figure 2D and Figure 3B). We demonstrate here that collapsed forks whose progression resumes by recombination lose accuracy during DNA synthesis, resulting in frequent intra-template switches. Thus, homologous recombination contributes to completion of DNA replication when forks progression is impeded but also fuels genome modifications both at the chromosomal and nucleotide level.

Non allelic homologous recombination (NAHR) between low copy number repeats (LCR) contributes to recombination-mediated GCRs in mitosis and meiosis. NAHR is responsible for translocations, deletions, inversions and loss of heterozygosity [59]. Ou *et al.* predicted 1143 LCR pairs in the human genome liable to mediate recurrent translocations [60]. In budding yeast, a single DSB is sufficient to mediate recombination-dependent translocation [61]. Here, we report that a single collapsed fork increases the rate of genomic deletion 40 fold, and that of translocation 5 fold. Fork-arrest-induced GCRs are mediated by NAHR between heterologous chromosomes. It is not clear whether fork arrest on both homologous repeats contributes to fork-arrest-induced GCRs. We could not address this question in our model system, because the *RTS1* sequence close to the *mat1* locus on chromosome II has a low RFB activity [62]. Also, the recruitment of recombination proteins at the *RTS1* sequence near the *mat1* locus is not regulated by the level of Rtf1 expression, showing that regulating Rtf1 expression was in itself insufficient to regulate the *RTS1*-RFB activity at the *mat1* locus [35].

Inverted repeats (IRs) are structural elements that contribute to genome instability. Impediments to replication forks progressing through IRs favour their fusion to generate acentric and dicentric inverted chromosomes [11,14]. IRs in humans can also trigger the formation of inverted genomic segments and complex triplication rearrangements by a replication-based mechanism [47]. Here, we report that IRs near collapsed forks can increase the rate of GCRs by up to 1,500 fold. This high level of GCRs cannot be explained merely by the addition of independent inter- and intra-chromosomal recombination events. Rather, our analyses suggest that IRs may stimulate tri-parental recombination events induced by template switching of nascent strands at collapsed forks, such that three homologous sequences are involved. Similarly, recombination-dependent translocations induced by a single DSB in budding yeast is proposed to be the consequence of tri-parental recombination [63,64]. One possible mechanism is that IRs-induced GCRs result from successive template switches initiated by nascent strands at collapsed forks, reminiscent of the multiple template switches during break-induced-replication (BIR) in budding yeast [33]. Interestingly, Rqh1 prevents fork-arrest-induced GCRs by limiting both inter- and intra-chromosomal recombination without affecting fork restart efficiency. Thus, tri-parental recombination might correspond to multiple and successive template switches between homologous repeats.

The high accuracy of DNA replication is compromised by impediments to fork progression, and recombination-mediated fork recovery results in decreased processivity of DNA synthesis (Figure 6). Recombination-induced mutations associated with DSBs or impeding DNA replication have been described previously. The formation of damaged single-stranded DNA during the resection of DSBs favours base-substitutions [65]. We detected the recruitment of the single-strand binding protein RPA up to 1.4 kb behind, but not ahead of the *RTS1*-arrested fork (data to be published), showing that fork-arrest-induced mutation is not correlated with damaged single-stranded DNA exposed behind collapsed forks. Nevertheless, there were rare replication slippage occurred behind *RTS1*-arrested forks (in the *t<ura4-ori* construct), suggesting that resumption of DNA synthesis can in some cases occur at a position behind the site of the collapsed fork. Recombination-dependent DNA synthesis occurring outside S-phase is also highly inaccurate during gene conversion and BIR, resulting in either template switches, base-substitutions or frame-shifts [33,34,66]. Elevated dNTP pools, due to activation of the DNA-damage checkpoint in G2 cells, contributes to the generation of mutations when hundreds of kbs are synthesised during BIR [34]. Here, we demonstrate that during normal S-phase progression, a single collapsed fork, restored by recombination, results in replication slippage up to 1.2 Kb away from the initial restarting point.

Recombination-induced replication slippage has been reported previously.

In fission yeast, a defect in pol alpha (*swi7-H4* mutant) is associated with a recombination-mediated mutator phenotype characterized by an increased frequency of base-substitutions and Del/Dup between short direct repeats [67]. DNA-polymerase kappa (DinB) and zeta (Rev3) are responsible for the increased base-substitution rate, but the DNA-polymerases involved in Del/Dup mutations were not identified [68]. In budding yeast, a defect in polymerase delta (*pol3-t* mutant) results in an increased level of replication slippage, mediated by homologous recombination [49]. In contrast, the increased rate of replication slippage in the absence of the accessory subunit of polymerase delta, Pol32, does not depend on a functional recombination pathway [69]. Here, we report that recovery of collapsed forks by recombination is specifically associated with replication slippage. Nonetheless, spontaneous replication slippage events are also increased in strains mutated for recombination genes (Figure 4D–4F). Pol32 is required for BIR and replication-induced template switches leading to segmental duplication [70,71]. Recombination is responsible for only half of these segmental duplications. Thus, it is possible that fork-restart mechanisms dependent on Pol32 and homologous recombination are prone to replication slippage and that in the absence of these pathways alternative micro-homology mediated mechanisms are revealed.

We suggest that at least two steps of the recombination-based fork recovery mechanism can compromise genome stability (Figure 6). At an initial stage, recruitment of recombination proteins on stalled nascent strands favours both fork recovery and ectopic template switches leading to GCRs. At a later stage, once the replisome has been reconstituted and the fork has resumed its progression, the nascent strands are prone to intra-template switching leading to replication slippage. The reasons for the inaccuracy of DNA synthesis associated with restarted forks during scheduled DNA replication (*i.e.* in S-phase) remain to be determined. One possibility is that one or more factors are missing in the rebuilt replisome during recovery by recombination. Oncogene-induced replication stress results from unbalanced DNA replication that contributes to genome instability in precancerous cells [12]. Completion of DNA replication in such stress conditions is likely to rely on recombination-mediated fork recovery that in turn generates genome instability. Insertions/deletions flanked by micro-homology, responsible for copy number variations (CNVs), have been identified both in cancer cells and also in response to replication inhibition [72,73]; their reported sizes are between 1 Kb and several tens of mega bases, but the analysis of these features has been limited by the resolution of array-based genomics approaches. Sub-microscopic insertions/deletions flanked by micro-homology have been also described at loci in which replication origins are scarce, including the human fragile site FRA3B, the instability of which is a consequence of replication stress [74]. Interestingly, homologous recombination contributes to the stability of fragile sites by facilitating complete replication or by repairing gaps and breaks at these sites. Thus, we propose that micro-homology-mediated CNVs could be viewed as scars left by error-prone replication forks restarted by recombination.

Materials and Methods

Standard genetic and molecular biology

Strains used were constructed by standard genetic techniques and are listed in Table S1. 2DGE was performed as previously

described [20]. To create *ura4-dup20* and *ura4-dup22* alleles associated or not with the *RTS1*-RFB, genomic DNA was isolated from selected 5-FOA^R cells containing duplications identified by sequencing. A PCR fragment containing duplications within the *ura4* ORF was amplified using the following primers: TTC-TGTTCCAACA-CCAATGTTT and TCACGTTTATTTTCA-AACATCCA. The PCR products were purified and used to transform strains SL206 (*ura4*$^+$), SL350 (*t-ura4<ori*) and SL504 (*t<ura4-ori*). Transformants were selected on 5-FOA-containing plates. Appropriate replacement of *ura4+* by *ura4-dup20* or *ura4-dup22* was verified by PCR and sequencing.

ura4 loss assay

A minimum of 11 independent single colonies from appropriate strains growing with or without thiamine were inoculated in 10 ml of non-selective media (with or without thiamine) and grown to stationary phase. Appropriate dilutions were plated on supplemented YEA to determine plating efficiency and on 5-FOA-containing plates. Colonies were counted after 5–7 days of incubation at 30°C. The rate of *ura4* loss was determined with the method of the median and data are presented on Table 1. Statistical significance was detected using the nonparametric Mann-Whitney U test.

PCR assays and sequencing to determine the rates of genomic deletion, translocation, and mutation

At least 200 5-FOA^R colonies per strain and condition were subjected to PCR analysis with the following primers: AAAA-CAAACGCAAACAAGGC and GTTTAACTATGCTTCGT-CGG to amplify *ura4* ORF, TGAATCCTCCGTTCAGTAGG and AAGGACTGCGTTCTTCTAGC to amplify *rng3* and TTTCCTTTCACGGCTAACCC (TLII) and TGTACCCAT-GAGCAAACTGC (TLIII) to amplify the translocation junction. The amplified *ura4* fragments were then sequenced on both strands, with the primers used to amplify the *ura4* ORF. Only mutations present on both strands were considered to determine mutation spectra. Deletions, mutations and translocations were scored as percentages of all events and these values were used to balance the rates of *ura4* loss to determine the respective rates of deletion, mutation and translocation (see Figure S1 for deletion and translocation rates and Figure S2 for mutation rates).

The fork-arrest-induced deletion, translocation and mutation rates (Figure 2C–2E and Figure 3A) were calculated by subtracting the rate obtained in presence of thiamine (Rtf1 being repressed, OFF) from the rate obtained in the absence of thiamine (Rtf1 being expressed, ON). This method allows the spontaneous instability of IRs and the leakiness of the *RTS1*-RFB activity to be disregarded to determine strictly the rate of events induced by fork-arrest. The nonparametric Mann-Whitney U test was used to test for statistically significant differences.

Reverse mutation assay

Exponentially growing cells were treated with 20 mM of HU, 40 µM of CPT or 1 mM of MMC. At indicated times, samples were taken and appropriate dilutions were plated on supplemented minimal media to determine plating efficiency and on uracil-free plates. Colonies were counted after incubation at 30°C for 5–7 days and the frequency of Ura$^+$ colonies was determined.

Replication slippage assay using *ura4-dup20* and *ura4-dup22* strains

For strains showing a slow growth phenotype (recombination mutants), the frequency of Ura$^+$ revertants was determined as a function of the number of generations experiencing fork arrest at *ura4*. Exponentially growing 5-FOA^R cells were washed twice in water and used to inoculate uracil-containing media without thiamine. Every 24 hours, cells were counted to determine the number of generations, and appropriate dilutions were plated on supplemented minimal media and on uracil-free plates. Colonies were counted after incubation at 30°C for 5–7 days and the frequency of Ura$^+$ colonies was determined. The slope of the curves presented on Figure 4F corresponds to the rate of replication slippage/generation. For strains showing similar growth to wild-type cells, a single 5-FOA^R colony was grown on uracil-containing plates with or without thiamine for 2–3 days, and then grown in uracil-containing media with or without thiamine for 2 days at 30°C. Appropriate dilutions were plated on supplemented minimal media and on uracil-free plates. Colonies were counted after incubation at 30°C for 5–7 days and the frequency of Ura$^+$ colonies was determined.

Supporting Information

Figure S1 Fork-arrest results in GCRs in a recombination-dependent manner. A. The rate of deletion for indicated strains, in the presence (Rtf1 repressed) and in the absence (Rtf1 expressed) of thiamine. Numbers of *RTS1* repeats present in the *S. pombe* genome and the presence of a visible fork arrest (based on 2DGE presented on Figure 1B) are given for each strain. The % of deletion events, as determined by the PCR assay, was used to balance the rate of *ura4* loss. The values reported are means of at least 3 independent median rates. Error bars correspond to the standard error (SE). Statistically significant fold differences in the rate of deletion events between the Rtf1 "repressed" and "expressed" conditions are indicated with an *. B and C. Rate of deletion (B) and translocation (C) for the strains indicated; ON and OFF refers to the *RTS1*-RFB being active or not, respectively. The % of deletion and translocation events, as determined by the PCR assay, was used to balance the rate of *ura4* loss. The values reported are means at least 3 independent median rates. Error bars correspond to SE. Statistically significant fold differences in the rates of deletion or translocation events between the "OFF" and "ON" conditions are indicated with an *. Translocation events (based on the detection of the TLII/TLIII PCR product) were not detected in *rad22-d* or *rhp51-d* strains, whatever the conditional fork arrest construct.
(TIF)

Figure S2 Fork-arrest induces replication slippage. A. The rate of mutation for indicated strains, in the presence (Rtf1 repressed) and in the absence (Rtf1 expressed) of thiamine. Numbers of *RTS1* repeats present in the *S. pombe* genome and the presence of a visible fork arrest (based on 2DGE presented on Figure 1) are given for each strain. The % of mutation events, as determined by the PCR assay and sequencing, was used to balance the rate of *ura4* loss. The reported values are means of at least 3 independent median rates. Error bars correspond to SE. Statistically significant fold differences in the rate of mutation events between the Rtf1 "repressed" and "expressed" conditions are indicated with an *. B. The frequency of Ura$^+$ revertants for the indicated strains and conditions. All strains harbour a non-functional *ura4* allele due to a single base-substitution or a frame-shift or a duplication of 20 or 22 nt, together with the *RTS1*-RFB in the *t-ura4<ori* context. The initial mutations and expected reverted mutations are indicated in the table. #1 and #2 correspond to two independent mutated strains for each type of mutation.
(TIF)

Figure S3 Features of replication slippage induced by fork arrest. A. Table of deletion/duplication and micro-homology features. B. Map of deletion and duplication events observed within the *ura4* ORF in the *t-ura4<ori* construct upon fork arrest. Del and Dup stand for deletion and duplication, respectively.
(TIF)

Figure S4 Sensitivity of *rad22-d* strain to acute exposure to 20 mM of HU or 20 μM of CPT. The values reported are means of two to four independent experiments. Error bars indicate the standard error of the mean (SEM).
(TIF)

Figure S5 Fork-arrest-induced replication slippage is independent of the post-replication repair and mismatch repair. A–C. Left panels: Serial tenfold-dilutions of indicated strains cultured in thiamine-free medium spotted onto the medium indicated. *RTS1*-RFB activity "–" refers to the strain *t-ura4-dup20-ori* and "+" refers to the strain *t-ura4-dup20<ori*. Right panels: The frequency of Ura^+ revertants from the strains indicated (*t-ura4-dup20-ori* associated or not with the *RTS1*-RFB) in the conditions indicated. The values reported are means of at least three independent experiments and error bars correspond to SEM.
(TIF)

Table S1 Strains used in this study.
(DOCX)

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Author Contributions

Conceived and designed the experiments: II AC SAEL. Performed the experiments: II VP YC NJ. Analyzed the data: II YC NJ KF AC SAEL. Wrote the paper: SAEL.

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Abstract

The replication of chromosomes can be challenged by endogenous and environmental factors, interfering with the progression of replication forks. Therefore, cells have to coordinate DNA synthesis with mechanisms ensuring the stability and the recovery of halted forks. Homologous recombination (HR) is a universal mechanism that supports DNA repair and the robustness of DNA replication. Nonetheless, mechanisms regulating HR pathways, such as ectopic versus allelic recombination, remain poorly understood. Another essential pathway for genome stability is the wrapping of newly replicated DNA around nucleosomes, leading to the constitution of a chromatin fibre, which allows the structural organization of the genetic material. In *Saccharomyces cerevisiae*, deficiencies in chromatin assembly pathways lead to replication forks instability and consequent increase in the rate of HR. Histone chaperones play a crucial role during chromatin assembly, thus I decided to focus on the H3-H4 histone chaperone Chromatin Assembly Factor 1 (CAF-1), to study its role in HR processes in *Schizosaccharomyces pombe*. Indeed, HR includes a DNA synthesis step and little is known about the associated chromatin assembly. My data excluded a role for CAF-1 in allelic recombination and in the maintenance of forks stability. However, CAF-1 was found to play an important role during ectopic recombination, in promoting chromosomal rearrangements induced by halted replication forks. My data support a model according to which CAF-1 allows the stabilization of early recombination intermediates (D-loop), via nucleosome deposition during the elongation of these intermediates. Doing so, CAF-1 counteracts the dissociation of early recombination intermediates by the helicase Rqh1. Therefore, CAF-1 appears to be part of an equilibrium that regulates stability/dissociation of early steps of recombination events. Importantly, I found that the role of CAF-1 in this equilibrium is of particular importance during non-allelic recombination, revealing a novel regulation level of HR mechanisms and outcomes by chromatin assembly.

Key words: homologous recombination, Chromatin Assembly Factor 1 (CAF-1), chromatin assembly, D- loop stabilization, ectopic recombination.

Résumé

La réplication des chromosomes est altérée par les facteurs endogènes et/ou exogènes qui perturbent la progression des fourches de réplication. Les cellules doivent donc coordonner la synthèse d'ADN avec des mécanismes assurant la stabilité et le rétablissement des fourches bloquées. La recombinaison homologue (RH) est un mécanisme universel qui permet la réparation de l'ADN et participe au maintien de la réplication des chromosomes. Néanmoins, les mécanismes qui régulent la RH, notamment la RH ectopique versus la RH allélique, restent mal compris. Un autre mécanisme essentiel assurant la stabilité des génomes est l'assemblage de l'ADN néo-synthétisé autour de nucléosomes, conduisant à la constitution de fibres chromatiniennes nécessaires à l'organisation structurale du matériel génétique. Chez *Saccharomyces cerevisiae*, des défauts d'assemblage de la chromatine conduisent à une instabilité des fourches de réplication et augmentent le taux de RH.

Sachant que les chaperonnes d'histones jouent un rôle crucial durant l'assemblage de la chromatine, j'ai décidé de me concentrer sur le rôle de la chaperonne d'histones H3-H4 appelé *Chromatin Assembly Factor 1* (CAF-1) dans les mécanismes de RH, chez *Schizosaccharomyces pombe*. En effet, la RH est associée à une étape de synthèse de l'ADN, et peu de choses sont connues sur l'assemblage de la chromatine au cours de cette synthèse. Mes résultats ont exclu un rôle de CAF-1 dans la recombinaison allelique et le maintien de la stabilité des fourches de réplication. Par contre, CAF-1 joue un rôle important dans les mécanismes de recombinaisons ectopique et dans la formation de réarrangements chromosomiques induits par des blocages de fourches. Mes données suggèrent un modèle selon lequel CAF-1 permet la stabilisation d'intermédiaires de recombinaison précoces (D-loop), *via* le dépôt de nucleosomes au cours de l'extension par polymérisation de ces intermédiaires. Ainsi CAF-1 neutralise la dissociation des intermédiaires de recombinaison précoces par l'ADN helicase Rqh1. CAF-1 ferait partie d'un équilibre qui règle la stabilité/dissociation des intermédiaires de recombinaison précoces. J'ai montré que le rôle de CAF-1 dans cet équilibre a une importance toute particulière pendant la recombinaison non-allelique, révélant ainsi un nouveau niveau de régulation des mécanismes de RH par l'assemblage de la chromatine.

Mots clés: Recombinaison homologue, Chromatin Assembly Factor 1 (CAF-1), assemblage de la chromatine, stabilisation D-loop, recombinaison ectopique.