UNIVERSITÀ DEGLI STUDI
DI MILANO

Functional Analysis of the Cell Cycle Protein Dpb11 in Response to DNA Damage and Replicative Stress

Fabio Puddu

XXII Cycle ∞ Academic Years 2006 - 2009

Supervisor: prof. Marco Muzi-Falconi

Coordinator: prof. Roberto Mantovani

Doctorate School in Genetics and Biomolecular Sciences

Department of Biomolecular Sciences and Biotechnology

It is a miracle

that curiosity

survives formal education.

(Albert Einstein)

Abstract

DNA molecule is complex, fragile and can suffer different damages. Specific DNA repair mechanisms were evolved to respond to these challenges, and to allow a faithful transmission of genetic information throughout generations. If the damaging conditions are extensive, a mechanism called DNA damage checkpoint takes care of arresting the progression of the cell division cycle to allow the cell to repair the damage before proceeding further. Genes involved in the DNA damage checkpoint are conserved throughout evolution and mutations in the human genes are known to produce severe illnesses — like Ataxia Telangiectasia — and genomic instability, which is usually considered as the onset of cancer: indeed checkpoint genes, like *BRCA1,* were found to be mutated in different types of cancers.

The yeast *Saccharomyces cerevisiae* has been widely used to study the DNA damage checkpoint because, despite its evolutionary distance, the easiness in generating knockout and mutant strains has facilitated the understanding of the underlying mechanisms. In this yeast, as in humans, the signal that activates the checkpoint is represented by the ssDNA covered by RPA, to which many different checkpoint and repair factors are recruited.

ssDNA signals are responsible for the activation of Mec1 (hATR), the apical kinase of the checkpoint pathway, but in humans two other factors are required for this signalling to occur: a ring-like heterotrimer — the PCNA-like complex — which is loaded onto DNA in response to damage and which recruits the second factor, TopBP1. Once active, Mec1 kinase phosphorylates a series of substrates, among which there is the Ddc1 subunit of the PCNA-like complex, and the Rad9 protein; phosphorylated Rad9 allows the recruitment of Rad53, the central kinase of the checkpoint whose Mec1-dependent activation contributes to cell survival after DNA damage and replication stress.

To be phosphorylated by DNA-bound Mec1, the Rad9 protein must be recruited to chromatin: this process involves the binding of a Rad9 domain — the Tudor domain — to a methylated lysine on histone H3. Indeed, cells mutated in the conserved H3 lysine, in the Tudor domain or in the histone methyl-transferase Dot1 are defective in Rad9 and Rad53 phosphorylation when DNA is damaged in the G1 phase of the cell cycle. Surprisingly, when these mutants receive a DNA damage

in mitosis, they are still able to phosphorylate Rad9 and Rad53, suggesting the presence of a second pathway that, in M phase, provides an alternative way for Rad9 to be phosphorylated.

In this thesis evidences regarding this alternative pathway for Rad9 recruitment and phosphorylation are provided. This pathway depends upon the C-terminal tail of Dpb11, the yeast homologue of human TopBP1, and on the Mec1-dependent phosphorylation of threonine 602 of the Ddc1 subunit of the PCNA-like complex. We show that Dpb11 itself is phosphorylated after DNA damage and that this phosphorylation is reduced in the presence of a non-phosphorylatable 602-residue on Ddc1, suggesting that in these conditions Dpb11 cannot be functionally recruited. Supporting this idea the two-hybrid interaction between Ddc1 and Dpb11 requires the presence of a functional Mec1 kinase.

Although being capable of *in vitro* stimulation of Mec1 kinase activity, after UV irradiation in M phase, Dpb11 is not required for Mec1 to phosphorylate its binding partner Ddc2. On the other hand, we provide evidences that Dpb11 performs its Mec1 activation task during the response to global replication stress; indeed Dpb11 and the PCNA-like complex are independently required to obtain a proper phosphorylation of histone H2A — here used as a marker of Mec1 kinase activity — and a full Rad53 activation. Consistent with this observation *ddc1Δdpb11-1* mutants are extremely sensitive to chronic exposition to hydroxyurea, a commonly used chemotherapeutic drug that generates replication stress by reducing the concentration of dNTPs in the cell. We also provide evidence that this lethality is not due to classical checkpoint functions like the stabilisation of stalled replication forks or the ability to delay entrance in M phase. We suggest also that other proteins known to be involved in checkpoint activation after hydroxyurea treatment are working in the pathway in which Dpb11 is involved.

Contents

Part I

Introduction

1

The Cell Division Cycle and its Control

General mechanisms of the eukaryotic cell cycle

The cell division cycle is a genetically controlled process in which a series of coordinated events takes place and allow the correct proliferation of eukaryotic cells. These events must provide the exact duplication and division of the genetic material and its correct segregation into the daughter cells.

In each cell cycle two main phases can be defined: the S phase (for Synthesis) in which genomic DNA is faithfully replicated and the M phase (for Mitosis) in which the cell segregates its chromosomes and divides its cytoplasm, giving birth to two daughter cells.

Two other phases, called G phases (for Gap) separate these two moments. In these periods of time the cell evaluates the environment and its own metabolic conditions and prepares to the following phases growing in mass and synthesising the required proteins. In particular the gap phase occurring between the end of M and the beginning of the next S phase is called G1 whereas G2 is called the phase occurring between S and M phase. (See Figure 1.1 on the following page).

The duration of the cell cycle can vary a lot, and it is influenced by growth conditions, the cell type and the species that is considered. In particular G1 and G2 phases normally have variable lengths and in some cases can be completely skipped, whereas the S and M phases represent key events of the cell cycle and the correct alternation between them is essential for cell viability. Most of the controls that the cell uses to establish the correct progression of the cell cycle are executed in

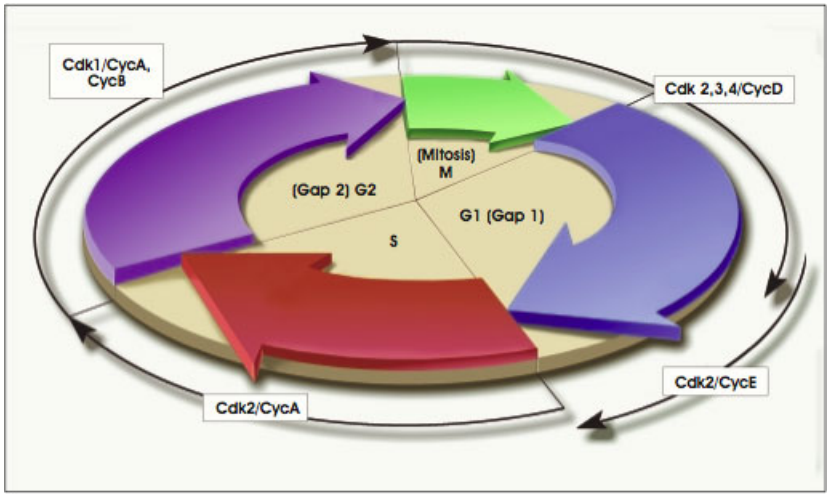

Figure 1.1: The Cell Division Cycle and the CdK-cyclin complexes involved. (from "Stem Cells: Scientific Progress and Future Research Directions, USA Department of Health and Human Services, June 2001.")

the G phases (Hartwell and Weinert, 1989). For example cells verify that a certain phase has correctly ended before starting the next one. It is also essential that any damage to the genome, which carries the genetic information, is recognised and corrected before DNA replication or mitosis can start. For this reason different factors participate to the fine tuning of cell cycle progression.

Many of the genes involved in the control of cell cycle were originally discovered at the beginning of the seventies by pioneeristic studies conducted by Lee Hartwell using *Saccharomyces cerevisiae* as a model organism. Hartwell and colleagues isolated different conditional mutants that exhibited alterations in different stages of the cell cycle. The correspondent genes were called for this reason *CDC* genes as the acronym for Cell Division Cycle (Hartwell *et al.*, 1974).

One of the most important advances obtained from the characterisation of *cdc* mutants, as well as from biochemical studies carried out using sea urchins egg extracts (Evans *et al.*, 1983), has been the demonstration that the motor that drives cell cycle progression is the activation and inactivation of a class of special protein kinases called CdK, for Cyclin-dependent Kinases. Indeed the kinase activity of this proteins is strictly regulated by association of the catalytic subunit (CdK) with regulative subunits called cyclins. Cyclins are unstable proteins which are periodically synthesised and degraded and are generally allowed to accumulate only in the cell cycle phase in which they are required. The binding of cyclins to CdK is not only necessary for CdK activation, but it also provides

substrate specificity to CdK. In higher eukaryotes, the need to cope with a greater amount of both external and internal stimuli, led to the evolution of different CdKs: the CdK-cyclin complexes that are formed in such organisms differ for both the regulatory and the catalytic subunit (see figure 1.1 on the preceding page), generating the required combinatorial complexity. In each phase of the cell cycle only specific CdK-cyclin complexes are catalytically active and, depending on the nature of the complex, different target molecules are phosphorylated.

A further regulatory level is represented by CKI or CdK-Inhibitors (Mendenhall, 1993). These are usually proteins that bind the catalytic subunit, inactivating it, whether bound or not to cyclin. Covalent modifications of the different subunits, in particular phosphorylations and dephosphorylations, represent another level for regulating and fine tuning this extraordinary intricate machinery (Mendenhall and Hodge, 1998).

Cell cycle progression in *Saccharomyces cerevisiae*

The yeast *Saccharomyces cerevisiae* is one of the most important model organisms to study, at the genetic level, the control of cell cycle progression. In addition to the easiness in creating knockout strains by gene targeting and in creating conditional mutants by random mutagenesis, *S. cerevisiae* can be propagated in both haploid and diploid form, allowing the study of the effect of both dominant and recessive mutations. *S. cerevisiae* is commonly known as budding yeast because the daughter cell is generated by budding: this mechanism is very useful for the study of cell cycle because yeast cells displays morphological characters typical of the cell cycle phase in which they are. In more details bud emergence mark the beginning of the entry into S phase; the bud then grows during all the S and G2 phase reaching a volume equal to the mother cell before mitosis (See figure 1.2 on the following page).

The control of cell cycle entry occurs mainly in late G1, in a specific moment called START, after which cells are committed to complete the newly initiated cycle. The existence of this control is due to the fact that cells must reach a critical mass before entering a new cell cycle to avoid, at each division, a progressive reduction in cellular dimensions.

In budding yeast the product of the *CDC28* gene is of capital importance for cell cycle progression (Lörincz and Reed, 1984). This gene encodes for a 34 kDa protein with serine/threonine kinase

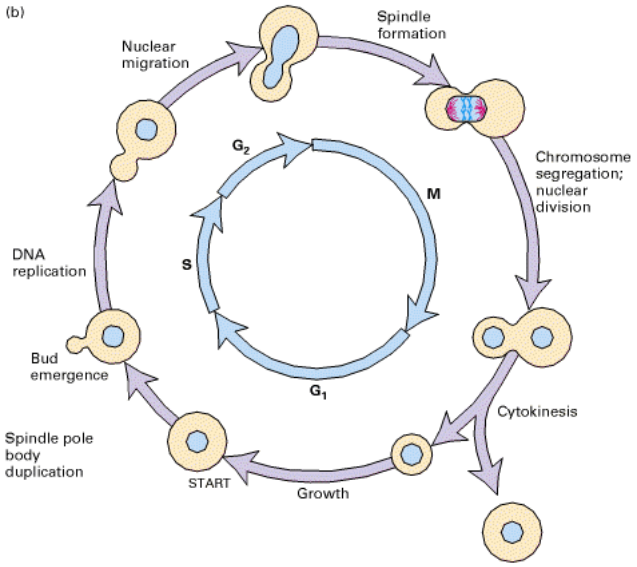

Figure 1.2: Morphological events during budding yeast cell cycle, adapted from Lodish *et al.* (1999).

activity required for both G1/S and G2/M transitions and it is the only essential CdK present in this organism (Piggott *et al.*, 1982; Reed and Wittenberg, 1990). The cyclins that Cdc28 is able to bind are at least nine and belong to two subclasses: G1 cyclins (Cln1, Cln2 and Cln3) and B type cyclins (Clb1, Clb2, Clb3, Clb4, Clb5, Clb6). In each subclass some cyclins seem to be at least partially redundant with others as it is demonstrated by the fact that none of the genes coding for cyclins is essential for cell viability (Nasmyth, 1996).

The contemporary absence of the three G1 cyclins is lethal and arrests yeast cells in G1 phase (Richardson *et al.*, 1989), while their overexpression entails the initiation of a new cell cycle before reaching the critical mass (Futcher, 1996). The complexes between Clb5/Clb6 and CdK are important for DNA replication, but double deletion *clb5Δclb6Δ* cause only a slowing down of S phase, as a demonstration of cyclin redundancy. B type cyclins Clb1, Clb2, Clb3 and Clb4 are important in promoting M phase and their complete absence causes cells to arrest in G2 with budded cells, spindle pole bodies duplicated and a fully replicated genome (Andrews and Measday, 1998). With the exception of Cln3 the level of all cyclins fluctuates during cell cycle: *CLN1–2* and *CLB5–6* are expressed at G1/S transition; *CLB3–4* in late S phase and *CLB1–2* in late G2 phase. On this periodic accumulation of different cyclins depends the oscillation of Cdc28 kinase activity which, in physiological conditions, is low in G1, increases at the G1/S transition and reaches its peak in M

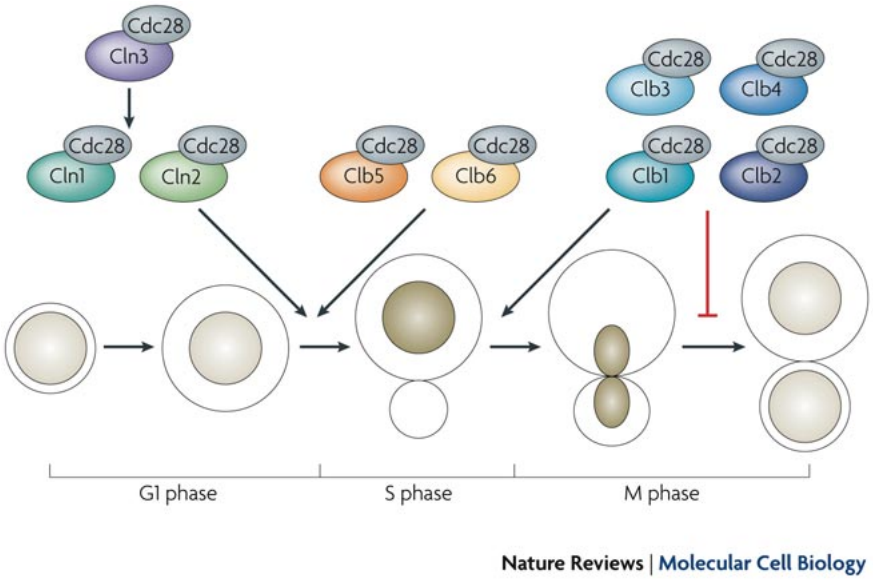

Figure 1.3: Role of cyclins in controlling cell cycle progression (from Bloom and Cross, 2007)

phase at the metaphase/anaphase transition: after that point the rapid degradation of all cyclins leads to a drop in CdK kinase activity.

Low CdK Activity and Replication Origin Licensing

The Cln3 protein has been suggested to act as a sensor of cell dimensions; indeed when the cell has reached its critical mass Cln3 concentration increases and it associates with Cdc28. The formation of this complex allows the activation of a wide transcriptional program characteristic of S phase, commonly known as *CLN2*-cluster. This program initiates the transcription of many genes, among which there are those coding for cyclins Cln1, Cln2, Clb5 and Clb6, which immediately associate with Cdc28. This group of genes is controlled by two transcriptional factors, SBF and MBF, which bind to specific promoters and are constituted of a regulatory subunit, Swi6, and a DNA binding subunit, Swi4 in SBF and Mbp1 in MBF (Breeden, 1996).

The Cln1–2/Cdc28 complex is the one required for passing the START point and it is responsible for bud emergence and spindle pole body duplication. The Clb5–6/Cdc28 complex is formed but kept temporarily inactive by the binding of the inhibitory factor Sic1 (see figure 1.3).

As mentioned before, the main event during S phase is DNA replication: a complex process which requires the organised work of a great number of factors. Eukaryotic chromosomes, due to

their huge dimensions, are replicated starting from many origins of replication, called Autonomously Replicating Sequences (ARS) in yeast. In order to preserve genome stability it is of great importance that each replication origin is activated only once per cell cycle. This control is achieved dividing the activation of ARS in two subsequent steps that cannot take place at the same time. The formation of pre replication complexes (pre-RC), known as origin licensing, can occur only in conditions of low CdK activity, from late M phase to G1. On the other hand origin firing, which also converts the pre-RC into post-RC, is allowed only in conditions of high CdK activity during S phase (Diffley, 2004).

The pre-RC assembly reaction consists in the loading of the putative replicative helicase — the Mcm2–7 complex — onto ARS, which are marked by the hexameric origin recognition complex (ORC). This reaction requires the presence of two essential factors: Cdc6 and Cdt1.

CdK activity prevents origin licensing in S, G2 and M phase by directly inhibiting different pre-RC component: Cdc6 is phosphorylated and targeted to ubiquitin-dependent degradation, mediated by the product of the gene *CDC4 (*Piatti et al.*, 1995;* Drury et al.*, 1997)*. Both the Mcm2–7 complex and Cdt1 appears to be regulated in localisation so that they are nuclear during G1 and cytoplasmic during the rest of the cell cycle (Labib *et al.*, 2001; Tanaka and Diffley, 2002).

Once loaded the MCM complex is stably associated with chromatin and removal of Cdc6, Cdt1 or ORC does not affect its binding (Donovan *et al.*, 1997). Another MCM protein, Mcm10, is part of the pre-RC and it is required for both loading and maintaining the MCM helicase on the replication origin (Homesley *et al.*, 2000).

DNA Replication and the Function of Dpb11

Cln1–2/Cdc28 complexes are being accumulated starting from late G1 together with Sic1-bound Clb5–6/Cdc28. The first complex is able to phosphorylate Sic1 at least on six sites, targeting it to Cdc4-dependent degradation. When Cln1–2/Cdc28 reach a high level all the Sic1 present in the cell gets degraded in a short period of time, freeing the Clb5–6/Cdc28 complex, which directly activates DNA replication (Schwob and Nasmyth, 1993).

A second protein kinase activity, which is independent of Cln1–2/Cdc28 and which is required for S phase entry, is Cdc7. When *cdc7* thermosensitive mutants are shifted to the restrictive temperature

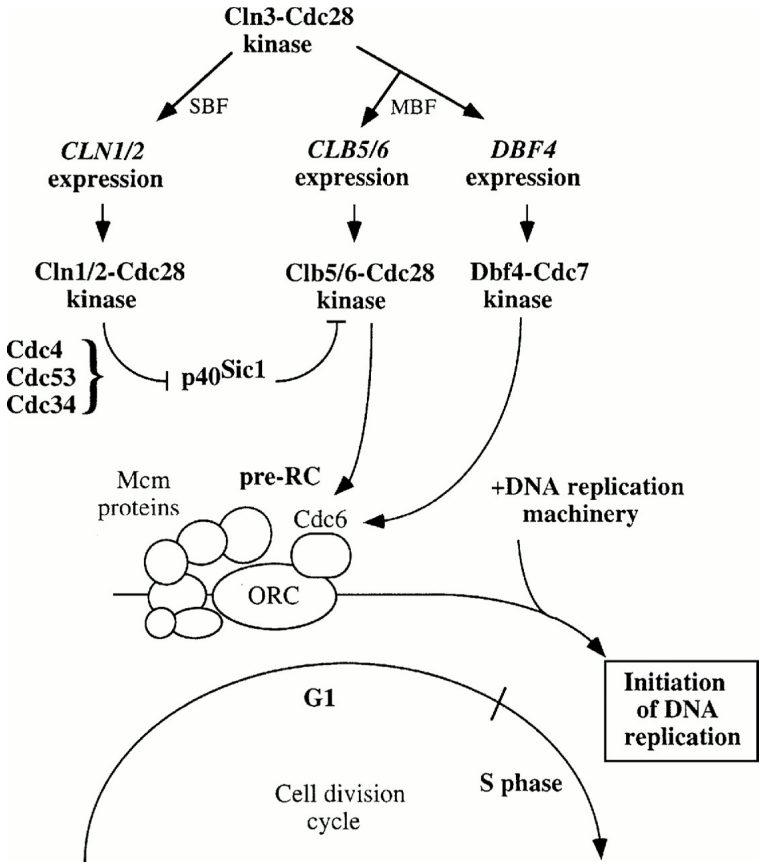

Figure 1.4: Triggering initiation of DNA replication in S phase requires not only the formation of the pre-RC, but it also depends on signals resulting from passage through START (from Toone *et al.*, 1997).

they arrest in late G1, after START but prior to S phase and once returned to permissive conditions they are able to start S phase, even without protein synthesis, suggesting that Cdc7 represents the last regulatory step for DNA replication initiation (Hollingsworth and Sclafani, 1990). Cdc7 protein is present throughout the cell cycle, but its kinase activity peaks at the G1/S transition because its activity requires the association with a regulatory subunit called Dbf4 (Jackson *et al.*, 1993; Kitada *et al.*, 1992).

How this kinases promote replication fork assembly is not completely understood, but recent insights shed light on this event (see figure 1.4). The activity of both kinases is required for loading the Cdc45 replicative protein onto chromatin. This factor, in turn, is required for the recruitment of all the replicative apparatus, consisting of DNA polymerase α and ε, Replication Protein A (RPA), the processivity factor PCNA and the GINS complex (Takisawa *et al.*, 2000; Zou and Stillman,

2000). Whereas the CdK functions as a global S-phase promoting factor, Cdc7 acts locally to implement initiation at individual replication origins (Pasero *et al.*, 1999).

The mechanism of action of Cdc7 has been known for a long time thanks to a mutation in a subunit of the MCM complex, the mutation *mcm5-bob1*, which is able to bypass the essentiality of *CDC7* (Hardy *et al.*, 1997). This effect and the observation that Cdc7 is able to phosphorylate *in vitro* Mcm2, suggest that the essential function performed by this kinase is the activation of the MCM helicase (Lei *et al.*, 1997).

The minimal set of CdK-dependent substrates required for replication initiation has been identified only recently. The two essential targets that need to be phosphorylated by CdK in order to fire origins are Sld2/Drc1 and Sld3. Phosphomimicking mutations in these proteins can bypass the requirement for CdK in promoting DNA replication, which becomes dependent only upon Cdc7 (Zegerman and Diffley, 2007). The function of this phosphosites is to promote the formation of a complex between Sld2, Sld3 and Dpb11. This interaction is thought to be required for the association of the pre-loading complex (Tak *et al.*, 2006), formed by DNA polymerase ε, Dpb11, Sld2 and GINS with the origin associated Cdc45-Sld3 complex. The formation of this bridge helps the recruitment of Pol ε at the origins and the assembly of the replication machinery.

Once all the factors are recruited at the level of the ARS, the DNA replication process can start. This consists in the opening of the parental DNA and in the polymerisation of the new filaments. After each origin has fired the pre-RC is converted into the post-RC, which will be maintained until the next cell cycle by Cdc28 activity, to avoid the re-use of the same origin and thus rereplication (Noton and Diffley, 2000).

High CdK Activity and Mitosis

At the end of S phase the level of Clb3 and Clb4 associated with Cdc28 peak and this promotes the assembly of the mitotic spindle. Then, in G2 phase, a second set of genes is transcribed, including *CLB1* and *CLB2*, whose products, in complex with Cdc28 are required for entry into mitosis, spindle elongation and transcriptional repression of the *CLN2* cluster (See figure 1.3 on page 13). In this phase Clb3–4/Cdc28 activates also the transcription of *ACE2*, *SWI5* and *APC1*. The products of the first two genes are transcription factors momentarily confined in the cytoplasm, while the latter

codes for the largest subunit of the Anaphase Promoting Complex / Cyclosome (APC/C).

APC/C is a large multimeric complex with ubiquitin ligase activity, whose substrate specificity is conferred by the association of two activating proteins: Cdc20 and Cdh1. APC^{Cdc20} plays a double role in the first phases of mitosis: it mediates the degradation of the securin Pds1, a regulator of sister chromatid cohesion, and it mediates the degradation of Clb5 which could inhibit the action of APC^{Cdh1}, required for the complete degradation of all B type cyclins and thus for the exit from mitosis (Zachariae and Nasmyth, 1999).

Chromatid cohesion is established by a multiprotein complex called cohesin in S phase and it is maintained until metaphase, when an endoprotease called Esp1 or separase became active thanks to the degradation of its inhibitory partner, the securin Pds1. This event marks the passage to anaphase. At this point Ace2 and Swi5 enter the nucleus and activate the transcription of different genes including *CTS1*, whose product is required for cytokinesis, and *SIC1* that produces the CdK inhibitor mentioned above.

In order to activate the APC^{Cdh1} complex, which will remain active during all the next G1 phase, the protein Cdh1, that was been phosphorylated and inactivated in S/G2 by Clb/Cdc28, has to be dephosphorylated. The Cdc14 phosphatase takes care of this, promoting also the dephosphorylation of the Sic1 inhibitor, making it able to bind and inactivate the future Clb5–6/Cdc28 complexes (Visintin *et al.*, 1998).

2

DNA Damage and DNA Repair

The DNA molecule is as complex as fragile: it can be damaged by a plethora of chemical and physical agents, either from exogenous sources or generated by the cell metabolism itself (see figure 2.1 for a summary).

During the DNA replication process, for instance, errors in the correct base pairing can introduce mutations in the newly synthesised DNA strand. The frequency of such errors is relatively low, thanks to the 3'→5' exonuclease activity associated with replicative DNA polymerases which can remove, before proceeding further, the misincorporated nucleotide. DNA replication itself is a very delicate process: the double helix structure is modified and very vulnerable regions constituted of ssDNA are generated. Nitrogen bases can also be damaged by different spontaneous reactions, like hydrolysis, methylation, deamination and oxidation; moreover DNA could be damaged by products of the oxidative metabolism, like free radicals.

Many different chemicals can damage DNA and introduce mutations: some are compounds structurally similar to nitrogen bases, that once incorporated into DNA, induce misincorporation of nucleotides at the next replication; another class of DNA damaging agents are direct modifiers of nitrogen bases, like MMS, an alkylating agent that adds alkyl groups to the bases.

DNA is also subject to damages induced by high energy radiations, such as γ- or X-rays, that generate different kind of lesions, among which are Single Strand Breaks (SSB), Double Strand Breaks (DSB), damages to nitrogen bases, and covalent adduct between protein and DNA. Finally

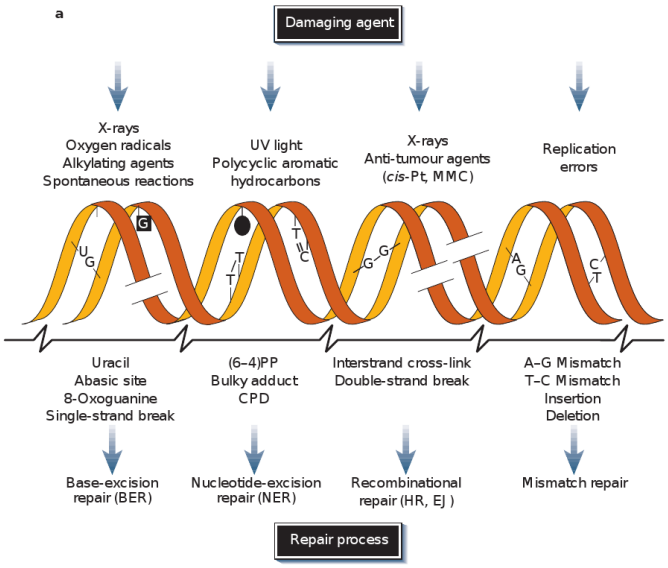

Figure 2.1: Main DNA damaging agents and the correspondent DNA repair pathways (Hoeijmakers, 2001)

ultraviolet radiations (UV), induce two different kind of lesions when two consecutive pyrimidines are present on DNA: Cyclobutane-Pyrimidine Dimers (CPD) and 6-4 photoproducts (6-4PP).

To maintain genome integrity, eukaryotic and prokaryotic cells evolved many specific systems that are able to recognise and repair all the damages DNA can suffer. The molecular mechanisms of the main DNA repair pathways has been, in fact, highly conserved during evolution (Critchlow and Jackson, 1998; Li, 2008; Hoeijmakers, 1993b,a).

DNA repair systems can be classified in five great categories: Direct Damage Reversal systems (DDR), Base Excision Repair (BER), Nucleotide Excision Repair (NER), Mismatch Repair (MMR) and Double Strand Breaks Repair (DSBR).

Direct Damage Reversal (DDR)

The direct reversion of a damaged nucleotide to an undamaged one, is the simplest repair mechanism that cells can use to maintain the informational integrity of their DNA. This system involves only one enzyme, that usually catalyse a one-step reaction. For this reason, and for its extremely low demand of energy, DDR is very useful to cells, but its limit reside in the fact that it can repair only very specific and particular damages (Eker *et al.*, 2009).

A classical example of this repair system is the photoreactivation of pyrimidine dimers. Through this process, dimers are converted back to their original structure, thanks to the exposition to visible light in a range of wavelength between 320 and 370 nm. The enzyme responsible for this reaction is photolyase: a flavoprotein which, once activated by a light photon, is able to convert the pyrimidine dimer back to its original state (Eker *et al.*, 2009).

Base Excision Repair (BER)

Base Excision Repair acts on DNA lesions represented by the formation of molecular adducts, depurination of nucleotides and deamination of nitrogen bases. In this repair mechanism the single damaged base is removed by a DNA N-glycosylase, which hydrolyses the N-glycosidic bond anchoring the base itself to the deoxyribose scaffold.

In *S. cerevisiae* three proteins with partially redundant roles take care of this step: Ntg1, Ntg2 and Ogg1 (Girard and Boiteux, 1997). The reaction catalysed by these proteins generates an apurinic-apyrimidinic (AP) site in DNA which can also originate from the spontaneous loss of a nitrogen base. In any case, this structure is normally recognised by an AP-endonuclease, which cuts the phosphodiester bond on the side of the abasic site. In yeast, the main pathway involves Apn1 and Apn2 which carry out this endonucleolytic reaction (figure 2.2, left). In their absence a secondary pathway is unmasked: the three N-glycosylases mentioned above are able to perform a similar cutting reaction, thanks to their AP-lyase activity (right pathway in figure 2.2) (Girard and Boiteux, 1997).

Processing of the lesion by AP endonucleases generates a single strand break with a 5´-deoxyribose phosphate (5´-dRP) end that has to be removed. The resulting gap, made of one or few nucleotides, has a 3'-OH end which is engaged by a DNA polymerase and, finally, the action of a DNA ligase restores the continuity of the DNA strand.

In yeast genetic and biochemical data suggest a major role for Rad27 in the removal of the 5´-dRP (Wu and Wang, 1999), for Pol2 (Polε) in the DNA repair synthesis (Wang *et al.*, 1993) and Cdc9 in the ligation step.

In mammals the situation is a little different: the 5´-dRP is released by the 5´-dRPase activity of DNA polymerase β in a pathway called short-patch BER. Alternatively, the 5´-dRP can be excised

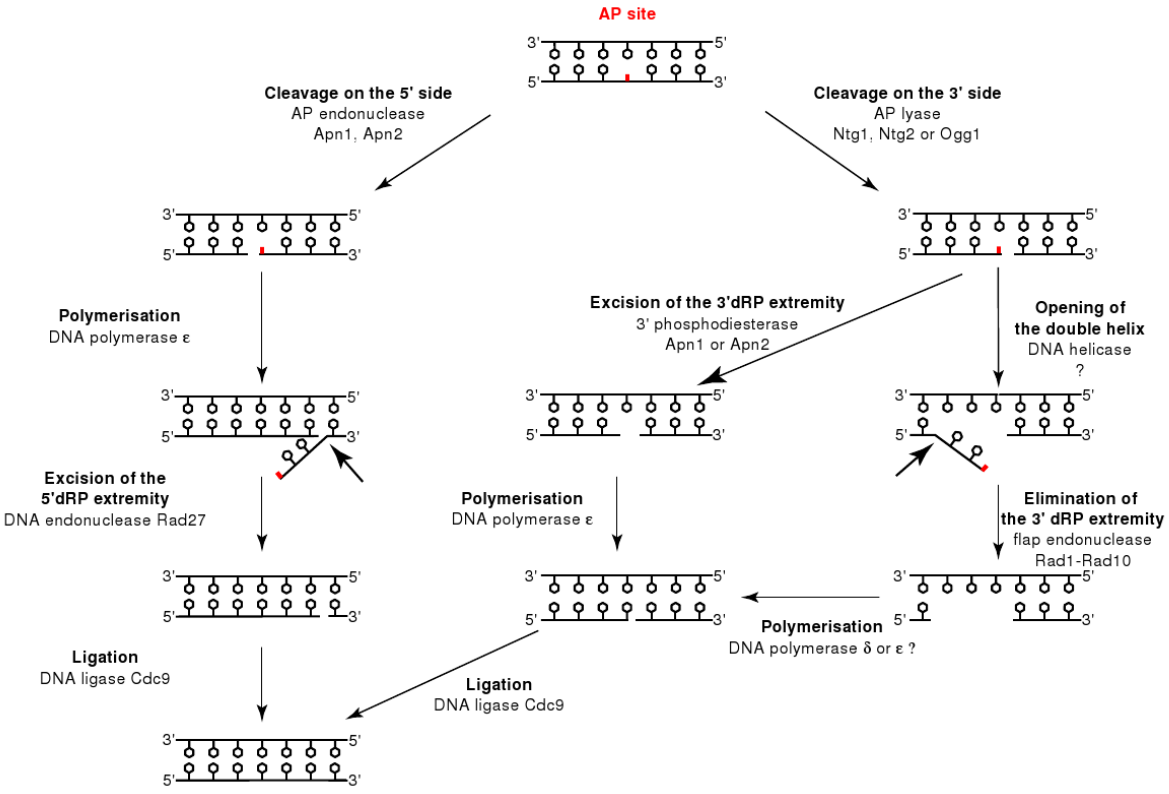

Figure 2.2: Model for Base Excision Repair in yeast (Boiteux and Guillet, 2004)

by the 5´-flap endonuclease Fen1 which generates a more extended ssDNA gap (long-patch BER) (Kelley *et al.*, 2003).

Nucleotide Excision Repair (NER)

Nucleotide Excision Repair is the main repair system for lesion induced by UV light and also for many other lesions that introduce a distortion in the double helix, such as DNA-protein covalent adducts. Considering the wide range of lesions that NER is able to repair, it is likely that this repair pathway is able to recognise, rather than specific damaged nucleotides, the distortion of the double helix itself. The fundamental steps of this repair system are showed in figure 2.3 and include the recognition of the lesion (b), the formation of a repair bubble (c-d), a double incision, upstream and downstream of the lesion (e), the removal of a 24–32 nucleotide fragment of DNA containing the lesion, the repair synthesis (f) and finally the sealing of the DNA ends (g), which reconstitutes the integrity of the molecule.

In *S. cerevisiae* the complex Rad4-Rad23 (XPC-Rad23 in mammals), in cooperation with RPA and the protein Rad14 (XPA), is able to recognise distortions of the double helix caused by UV induced pyrimidine dimers (Jansen *et al.*, 1998; Guzder *et al.*, 1998, 2006). Afterwards, Rad3 (XPD) and Rad25 (XPB), two helicases with opposite polarity belonging to the general transcription factor TFIIH, unwind the DNA, making the filament containing the lesion accessible. Then the two endonucleases Rad1-Rad10 (XPF-ERCC1) and Rad2 (XPG) cut the DNA respectively 5' and 3' of the lesion, generating a fragment that has a length between 24 and 27 nucleotides (Guzder *et al.*, 1995). The ssDNA gap originated in this way is then filled thanks to the replicative factors RPA, RFC, PCNA and to DNA polymerase δ and ε. The newly synthesised oligonucleotide is then sealed with the rest of the molecule by Cdc9 DNA ligase (Wu *et al.*, 1999a).

This repair mechanism has been extensively studied because mutations in the correspondent human genes are the cause of severe genetic diseases which include Xeroderma Pigmentosum (XP), Cockayne Syndrome (CS) and Trichothiodystrophy (TTD) (Schärer, 2008).

Evidences coming from genetic and biochemical studies suggest the existence of two parallel pathways for nucleotide excision repair: Global Genome repair (GG-NER), involved in the repair of lesions that occur in the non-transcribed strand of DNA and in non-coding regions of the genome;

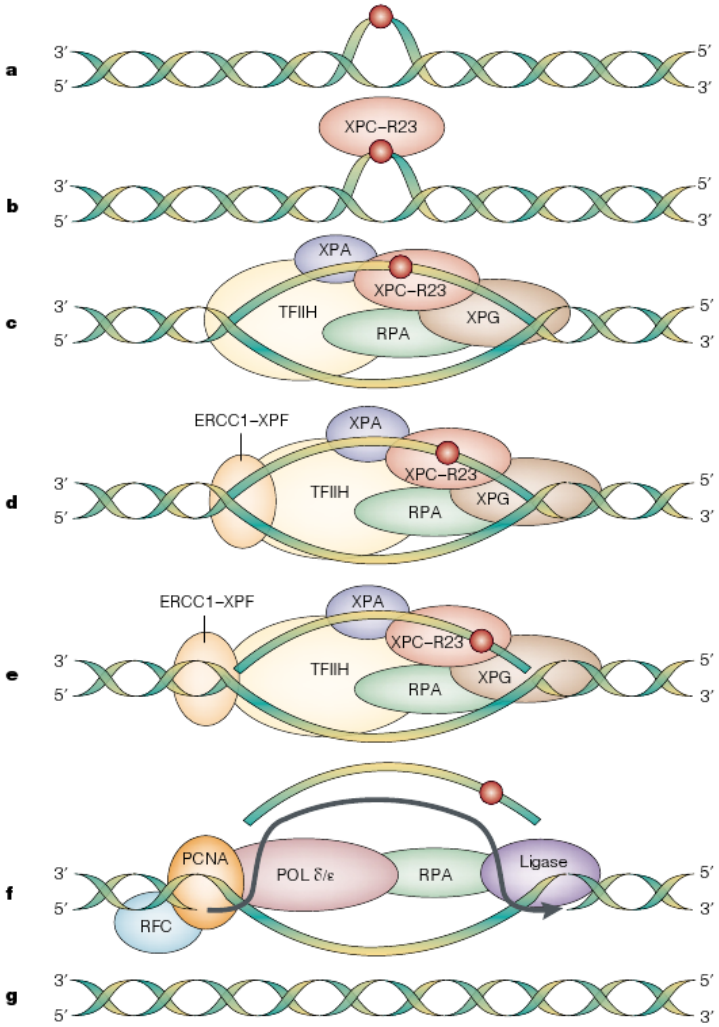

Figure 2.3: Fundamental steps of Nucleotide Excision Repair (Friedberg, 2001).

Transcription Coupled repair (TC-NER), that acts more rapidly on lesions that are present on the transcribed filament of genes (Shuck *et al.*, 2008). GGR depends upon the Rad7-Rad16 complex, which has ATPase and helicase activity and it is able to bind specifically non-transcribed DNA in a ATP-dependent manner. Rad26 (hCSB), instead, plays a key role in TCR, thanks to physical and functional interactions with transcription complexes (Fousteri and Mullenders, 2008).

Numerous models have been proposed to explain the order of assembly of proteins at the site of DNA damage and the mechanism of the NER pathway. One of the first theories suggested that a pre-assembled complex was able to carry out the entire excision reaction. Several lines of investigation, however, argue against the existence of a complex capable of carrying out all the necessary steps of NER. The precise order of assembly of these core recognition factors on the site of DNA damage has been the subject of many studies, with early *in vitro* data supporting models of either XPC-Rad23 or XPA-RPA as the first complex that binds to a UV-induced DNA lesion (Sugasawa *et al.*, 1998; Wakasugi and Sancar, 1999). While initial evidence suggests that RPA or an RPA-XPA complex binds first to the damage and subsequently recruits XPC-Rad23 with TFIIH, more recent analyses support the hypothesis that XPC-Rad23 is the primary damage recognition factor (Volker *et al.*, 2001).

A cause for inconsistent findings in regard to the order of assembly can be attributed either to the different experimental assays employed or to the fact that no specific order is in fact necessary. Given the absence of a preformed complex, the assembly of NER factors at the site of a UV-induced DNA lesion must occur in either a sequential, ordered process or a random addition process. Our understanding of the assembly process has been greatly aided by cell-based immunofluorescence analysis using fluorescent tagged NER factors which have enabled a subset of interactions to be better characterised. For example, analyses revealed that XPC-Rad23 is required for TFIIH assembly at the sites of DNA damage (Volker *et al.*, 2001). These results and analysis of various XP cell lines led to the conclusion that XPC-Rad23 represents the first factor bound at the sites of DNA damage. Analysis of the XPA dynamics revealed that XPA rapidly accumulates at the sites of DNA damage in an XPC-dependent manner (Rademakers *et al.*, 2003). These results are consistent with the hypothesis that XPC represents the first molecule bound at the site of a UV-induced DNA lesion. For example, transient XPA binding to a UV-damage site with a fast rate of dissociation would

result in the inability to localise XPA at the lesion in the absence of XPC. If XPC-Rad23 decreased the rate of XPA dissociation, the accumulation of XPA at the sites of UV damage would then only be observed in XPC-positive cells.

Mismatch Repair (MMR)

Mismatch Repair corrects the base pairing errors which were not corrected by the proofreading activity of DNA polymerases during DNA replication. These kind of damages are divided in mispairings and IDLs (Insertion, Deletion, Loop) which, if not rapidly repaired, can induce point mutations or frame-shift mutations, respectively, in the following round of replication.

The mechanism of mismatch repair has been first described in *E. coli*, where the protein MutS can recognise and bind this kind of lesions. Then the newly synthesised filament is identified because it lacks methylated GATC sites. Subsequently the proteins MutL and MutH associate with MutS: the endonucleolytic activity of MutH cuts the daughter filament and UvrD, a 5' $\rightarrow$ 3' helicase loaded by MutL, removes it. This filament is then degraded by different exonucleases, and DNA polymerase III fills the gap.

In *Saccharomyces cerevisiae* six homologues of MutS (*MSH1-6*) and four of MutL (*MLH1-3* and *PMS1*) has been identified. The products of the genes *MSH2/3/6* form the heterodimeric complexes MutSα (Msh2-Msh6) and MutSβ (Msh2-Msh3) that specifically recognise mispairs and IDLs, respectively. The remaining MutS homologues seem not to be involved in MMR (Sia and Kirkpatrick, 2005; Hollingsworth *et al.*, 1995; Ross-Macdonald and Roeder, 1994). Mlh1 with Pms1 forms the MutLα complex, which interacts with both MutSα and MutSβ. The endonucleolytic activity of the bacterial protein MutH, whose eukaryotic homologs have not been identified yet, is currently attributed to MutLα.

The demonstration of a physical interaction between PCNA and the Mut factors has suggested a model in which the MMR process is associated with replication. In this view the presence of a double helix that is not properly paired would induce the association of MutSα with PCNA, forming an heterotrimeric complex that could slide along the DNA looking for the mispairing (Kleczkowska *et al.*, 2001; Lee and Alani, 2006). Once this has been identified, PCNA is excluded from the complex and MutSα is allowed to bind the DNA molecule. The Msh2 subunit, which has ATPase

activity, would then induce a conformational change in the complex, through the hydrolysis of an ATP molecule and would trigger two key events in the MMR process: the binding of MutLα to DNA and the sliding of this factor along the molecule, on the search for a signal that allows the discrimination between the template and the newly synthesised strand. The nature of this signal, which in prokaryotes is represented by the methylation of the maternal filament, is still under discussion, but the most favoured theory suggests that it may be the discontinuity of the newly synthesised strand that is recognised by this repair system, probably due to the intrinsically slow ligation of all the nicks, at the origin of replication on the leading strand and between each Okazaki fragment on the lagging strand.

Once the daughter molecule has been recognised, MutLα generates an incision at the level of the mismatch. The degradation of the fragment containing the misincorporated nucleotide is then carried out by exonucleases with 3'→5' polarity on the leading strand and 5'→3' on the lagging strand. The evidences collected in *Saccharomyces cerevisiae* indicates that the only exonuclease certainly involved in MMR is Exo1, which seems to participate in both the 3'→5' and 5'→3' degradation. Exo1 interacts with both Msh2 and Mlh1 (Tishkoff *et al.*, 1998; Tran *et al.*, 2001) and this interaction seems to increase the processivity of Exo1. The last phase consists in the resynthesis of the DNA and requires many proteins among which there are RPA, RFC, PCNA and Pol δ.

Double Strand Break Repair (DSBR)

The disruption of the physical continuity of the DNA molecule, Double Strand Break, is one of the most dangerous lesions the genome can experience, because it can cause the loss of genetic material and also chromosomal translocations. To prevent these events, eukaryotic cells evolved three different mechanisms to repair DSBs: the repair by homologous recombination (HRR), the repair by direct ligation of the two DNA ends, named Non-Homologous End Joining (NHEJ) and the repair by Single Strand Annealing (SSA).

NHEJ The first step of this repair pathway (figure 2.4/A) is the recognition of the DNA ends mediated by the heterodimeric complex Ku70-Ku80, which forms a ring-like structure that binds DNA and, as revealed by atom force microscopy, bridges the two ends (Pang *et al.*, 1997). Once

present, the KU complex facilitates the recruitment of other factors like XRCC4 (Lif1 in yeast) and DNA ligase IV (Dnl4), which stimulate the direct ligation reaction (Chen *et al.*, 2001).

Many DSBs generated by IR cannot be ligated in this way, but a partial degradation of the DNA and a repolymerization is required for an efficient repair. These reactions requires the flap endonuclease Rad27, the polymerase Pol4 and the MRX complex (Wu *et al.*, 1999b; Lobachev *et al.*, 2004). This last complex is made of three subunits, Mre11, Rad50 and Xrs2, each one with a specific function.

Mre11 has both endo- and 5'→3' exo-nucleolytic activity and it is probably involved in the degradation of the DNA ends, even if its nuclease activities are not required for this function (Moreau *et al.*, 1999). Rad50 belong to the Structural Maintenance of Chromosome protein family, whose members are implicated in sister chromatid cohesion and, for this reason, it is probably involved, like KU, in the bridging of DSB end. Finally Xrs2 seems to be involved in the recruitment of the MRX complex to DSB (D'Amours and Jackson, 2002).

The role of MRX in NHEJ is supported by the fact that it is able to bridge DNA ends *in vitro* and to stimulate the activity of KU and Lig4 (Chen *et al.*, 2001); moreover deletion of any of the genes coding for MRX significantly reduces NHEJ efficiency (Boulton and Jackson, 1996; Moreau *et al.*, 1999).

HRR In HRR the damaged DNA molecule pairs to an intact DNA molecule with a significant homology, which is usually the homolog chromosome or, in G2, the sister chromatid. This last molecule is then used as a template for the elongation of the 3' ends of the DSB, which is required to resynthesize the region containing the break. Three different types of HRR exist, but the first events are common to all the pathways. DSB ends are initially resected by specific nucleases in 5'→3' direction: this process leads to the exposition of long 3' ssDNA tails which are recognised by the recombination promoting factor Rad51. One of the two tails, in a Rad51-dependent process called strand invasion, pairs with the complementary sequence present on the intact homolog, displacing its counterpart. DNA polymerases then elongate the filament containing the break using the paired homolog filament as a template. The destiny of this structure is then different in the different pathways (reviewed in Pâques and Haber (1999)).

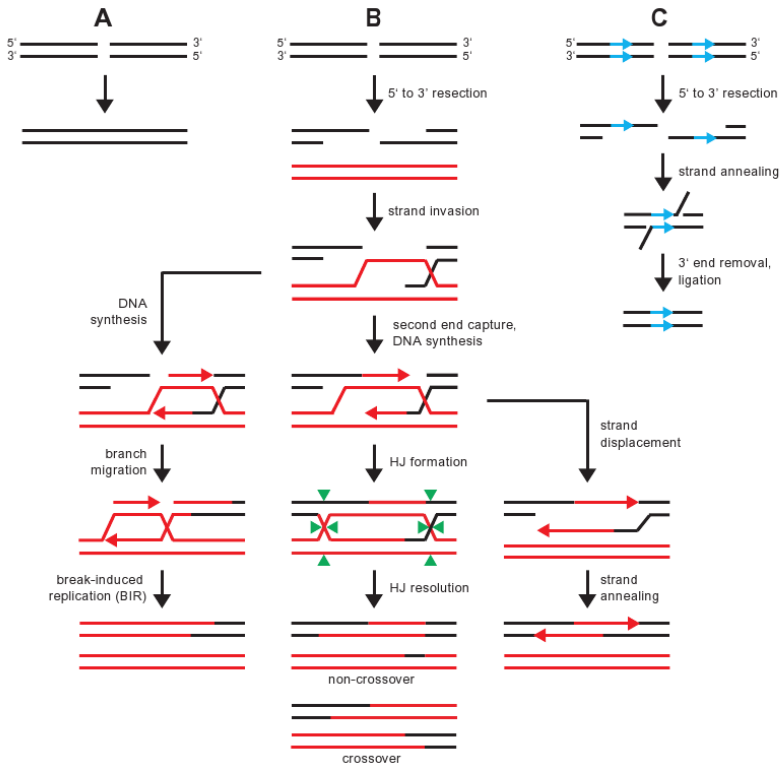

Figure 2.4: Different mechanisms repair Double Strand Breaks (LONGHESE *et al.*, 2006)

During Break Induced Replication (BIR) (figure 2.4/B, left), which occurs generally at telomeres, primed DNA synthesis starts at the level of the displaced sequence. This structure then moves towards the end of the molecule in a process called branch migration and DNA synthesis proceeds until the polymerase reaches the end of the telomere.

In the second repair pathway, which is properly DSBR (figure 2.4/B, centre), after strand invasion the displaced molecule captures the second end of the break, which is then extended using the displaced strand as a template. This generates a DNA molecule containing two Holliday Junctions which can move in opposite directions and which are finally resolved by a HJ-resolvase that can lead to both crossover or non-crossover events.

The last possibility is Synthesis Dependent Strand Annealing (figure 2.4/B, right), in which the invading molecule is displaced as soon as the region containing the break has been resynthesized. It is then this displaced filament then captures the second DNA end and another synthesis event can occur to fill the gap and seal the break.

In more detail HRR begins with the 5'→3' degradation of DNA ends in which many actors are

involved: MRX (Haber, 1998), Exo1 (Moreau *et al.*, 2001), Sae2 (Rattray *et al.*, 2001; Clerici *et al.*, 2006) and possibly other unknown proteins. The role of MRX is still confused: deletion of one of its subunit delays but not eliminates the processing of an HO-induced DSB (Ivanov *et al.*, 1994). Moreover the exonuclease activity of Mre11 is opposite to that of the resection and mutants in this activity are only partially sensitive to IR and do not show any delay in DSB processing (Moreau *et al.*, 1999; Llorente and Symington, 2004). A commonly accepted explanation is that MRX only maintain the association between the ends of the broken chromosome, as it is suggested by the fact that in its absence DSB ends cohesion is lost (Unal *et al.*, 2004).

Sae2 belongs to the same epistasis group as MRX with regard to DNA resection and also in *sae2Δ* cells DNA resection is delayed and DSB end cohesion is lost (Clerici *et al.*, 2005, 2006). Moreover in the absence of Sae2, MRX complex cannot dissociate from unprocessed meiotic DSBs (Borde *et al.*, 2004).

The resection observed in the absence of MRX is almost completely dependent on Exo1, an exonuclease with 5'→3' polarity that has a role in different DNA repair processes (Clerici *et al.*, 2006). A role for Exo1 in DSB resection is also suggested by the fact that *mre11Δexo1Δ* strains exhibit growth defects and are more sensitive to IR than the single mutants, even though *EXO1* deletion alone does not induce any defect in the resection process (Tsubouchi and Ogawa, 2000; Nakada *et al.*, 2004).

3' ssDNA tails, formed as a consequence of the resection process, are immediately covered by RPA, whose role is to protect the DNA from further processing and to prevent the formation of secondary structures in the DNA, which would inhibit the binding of HR factors (Alani *et al.*, 1992; Sugiyama *et al.*, 1997). RPA is then removed and substituted by Rad51 in a process that is promoted by the presence of Rad52, which can interact with both proteins. The assembly of Rad51 coated nucleoprotein filament is also favoured by the presence of its paralogues Rad55 and Rad57, which *in vitro* form a heterodimer with DNA binding activity (Sung, 1997; Sugiyama and Kowalczykowski, 2002).

The Rad51 nucleoprotein filament then locates a homologous sequence in collaboration with the Rad54 ATPase, and enter in synapsis with it. The 3'-OH is then extended by a DNA polymerase, allowing the cell to recover the genetic information lost in the break; the repair process is concluded

by a DNA ligase sealing the nick.

SSA If a DSB falls into a DNA region containing direct repeats cells have another opportunity to repair the break. In this repair system 5' DNA ends of the break are processed with the same mechanism of HR, generating 3' ssDNA tails (figure 2.4/C). When the resection uncovers the ssDNA containing the repeats, these two can pair together and the 3' tails in excess are removed by the endonuclease Rad1/Rad10. The remaining nicks are then sealed by DNA ligases. Other genes required for SSA are *RAD52*, *MSH2*, *MSH3* and *SRS2*.

Differently from HR, which is an error free repair system and differently from NHEJ, whose fidelity depends upon the end processing and ligation reactions, SSA is always an error-prone repair system because it causes the deletion of the region in between the direct repeats (Pâques and Haber, 1999).

3

Checkpoints: Places to Control Cell Cycle Progression

To maintain cell viability and genome integrity, the different phases of the cell cycle must follow one another in a precise order. For this reason the control of cell cycle progression is of capital importance for sustaining life and eukaryotic cells have evolved genetically determined control mechanisms, called checkpoints, extremely important for genome integrity. Alterations in these surveillance mechanisms, which superintend the coordination between DNA metabolic processes and the alternation of cell cycle phases, can lead to uncontrolled cell proliferation and/or cell death (Weinert and Lydall, 1993).

The term "checkpoint" has been used for the first time in a study by Ted Weinert and Lee Hartwell when, using a *S.cerevisiae rad9* mutant, they demonstrated that checkpoint proteins were the product of genes which negatively regulate cell cycle progression in response to damage. Indeed, *rad9* mutants loose the ability to arrest in G2 phase if irradiated with UV light and were thus sensitive to this damaging agent (Weinert and Hartwell, 1988).

There are two distinct class of checkpoints: intrinsic checkpoints, which work in physiological conditions in an unperturbed cell cycle and extrinsic checkpoints that are activated only in pathological conditions.

Some examples of checkpoints are:

- morphogenesis checkpoint, which restricts mitosis if the cell has a damaged actin cytoskeleton (Lew and Reed, 1995);

- spindle assembly checkpoint, which restricts mitosis until the mitotic spindle is formed and controls the bipolar attachment of each chromosome to a microtubule fibre (Hoyt *et al.*, 1991);
- spindle orientation checkpoint which delays mitotic exit until one of the spindle pole bodies has moved into the daughter cell (Pereira *et al.*, 2000);
- S/M checkpoint which prevents entry into mitosis if the DNA has not been completely replicated (Allen *et al.*, 1994; Weinert *et al.*, 1994).
- DNA damage checkpoint which is activated in response to damages in the genome (described later in further details).

Checkpoint malfunctioning can lead to events that increase the mutation rate and genomic instability; defects in DNA damage checkpoint are likely to play an important role in cancer development, allowing rapid accumulation of oncogenic mutations (Hartwell and Kastan, 1994). For example, in humans, mutations in checkpoint genes ATM and p53, involved in the response to DNA damage, are associated with chromosomal aberrations and predisposition to cancer.

The DNA damage checkpoint

DNA damage checkpoint mechanisms are highly conserved between different eukaryotic species. Studies performed on simple model organisms like *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* allowed the isolation and characterisation of many checkpoint factors, a great number of which have functional homologues in *Homo sapiens*. The DNA damage checkpoint is an extrinsic surveillance mechanism that is activated in the presence of lesions in the DNA molecule and which give rise to a series of complex cellular responses, the most evident of which is cell cycle arrest.

Depending on the phase in which the cell receives the damage, three different checkpoints can be activated: G1/S checkpoint, which prevents DNA replication in the presence of a damaged template (Siede *et al.*, 1993, 1994); intra-S checkpoint, which slows the speed of S phase progression and promotes alternative DNA replication mechanisms (Paulovich and Hartwell, 1995); G2/M checkpoint which prevents segregation of damaged chromatids, blocking, in budding yeast, the transition from metaphase to anaphase (Weinert and Hartwell, 1988).

Class	Function	*S.cerevisiae*	*S.pombe*	*H.sapiens*
Sensors	Checkpoint clamp	Ddc1	Rad9	Rad9
		Mec3	Hus1	Hus1
		Rad17	Rad1	Rad1
	Clamp loader	Rad24	Rad17	Rad17
	Apical Kinase	Mec1	Rad3	ATR
	& its binding partner	Ddc2	Rad26	ATRIP
	Apical Kinase	Tel1	Tel1	ATM
	Adaptor ? / Activator?	Dpb11	Rad4/Cut5	TopBP1
Adaptors	DNA Damage Adaptor	Rad9	Crb2	53BP1, MDC1, ?
	Replication Stress Adaptor	Mrc1	Mrc1	Claspin
Transducers	Kinase	Rad53	Cds1	Chk2
	Kinase	Chk1	Chk1	Chk1

Table 3.1: Conservation between eukaryotes of the different proteins involved in DNA damage checkpoint activation.

The DNA damage checkpoint is organised as a signal transduction cascade, which involves different classes of proteins (summarised in table 3.1), highly conserved throughout evolution.

In the first stages of activation of the cascade, sensor proteins recognise the presence of a damage on DNA and generate a phosphorylation mediated signal to adaptor proteins. Adaptors allow the transmission and propagation of the initial signal to transducer proteins, which are required for signal amplification and transmission to the effector proteins, most of which are still unknown. Effectors are the ultimate responsible for the different strategies that cells adopt to survive DNA damage and maintain genome integrity, which include the arrest of cell cycle progression, the transcription of DNA repair genes, the delay of late replication origin firing, the regulation and coordination of complex processes such as DNA recombination, translesion synthesis and apoptosis (see figure 3.1).

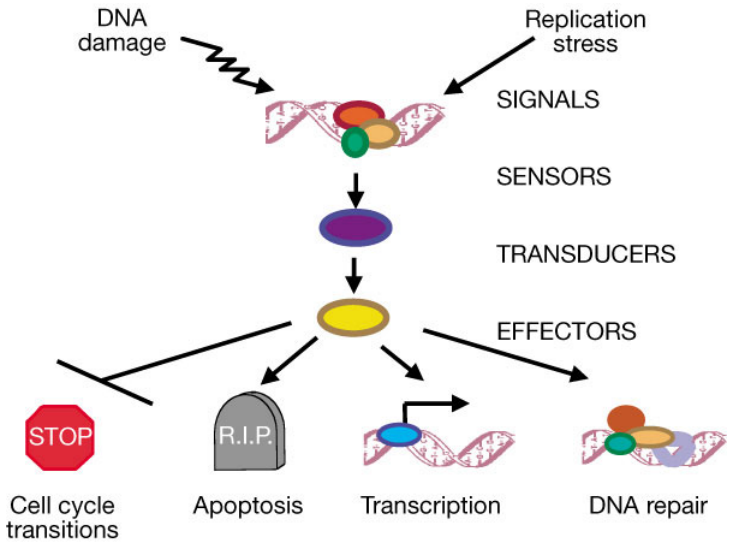

Figure 3.1: Outline of the DNA damage checkpoint signal transduction cascade (Zhou and Elledge, 2000)

The signal responsible for checkpoint activation

A lot of work has been done to understand how cells become aware of the presence of a damage in their genome and how such event triggers the activation of the DNA damage checkpoint. The understanding of these mechanisms has been particularly difficult especially in the light of the variety of damages that can occur on DNA. In recent years different theories suggested that either different sensor proteins are able to recognise different kind of damages, or that each damage is converted, during its own repair process, into a common molecular intermediate that is able to activate the DNA damage response.

Many experimental evidences support this last hypothesis and, in particular, the common intermediate that has been identified as necessary to trigger checkpoint cascade is single stranded DNA (ssDNA) (Garvik *et al.*, 1995): each DNA lesion is likely converted into this structure that allows the recruitment of checkpoint proteins, increasing their local concentration.

In vitro and *in vivo* studies showed that in human cells exposure to ionising radiations (IR) promotes the formation of IR Induced Foci (IRIF) of RPA, indicative of the presence of ssDNA. Moreover ATR-ATRIP apical complex co-localise with these foci, and RPA is necessary for both the localisation of this complex and for its functional activation (Zou and Elledge, 2003). It has been also demonstrated that RPA is required for the proper damage induced localisation of human Rad9

and its yeast homologue Ddc1 (Zou *et al.*, 2003).

The mechanism of ssDNA generation is different, depending on the original lesion but, in general, endonuclease and exonuclease activities are required for this first step. Proteins belonging to the different DNA repair pathways (described in chapter 2) have a role in checkpoint activation either recruiting checkpoint factors or generating the ssDNA recognised by checkpoint proteins, suggesting thus a tight connection between DNA repair and DNA damage checkpoint activation. In this direction many evidences were produced: the MRX complex, which is involved in the first steps of double strand breaks (DSB) repair, is also required for checkpoint activation after treatment with drugs that induce DSBs (Nakada *et al.*, 2004); NER processing of UV lesions is necessary for UV induced checkpoint activation and the NER protein Rad14 functionally and physically interact with the checkpoint protein Ddc1 (Giannattasio *et al.*, 2004). Exonucleases, in particular, are important for signal generation because, by resecting DNA ends, are able to generate the great amounts of ssDNA required for checkpoint activation. In fact, Exo1, an exonuclease involved in many repair processes, is also required for checkpoint activation after DSB induction and UV irradiation (Nakada *et al.*, 2004; Giannattasio *et al.*, Ms. in preparation).

In contrast with the poor knowledge of the exact dynamics of the events, the factors involved in DNA damage checkpoint have been described in detail in many different model organisms and in humans. In *S. cerevisiae* these upstream proteins were found from different screening: for radiation sensitivity, Rad9, Rad17 and Rad24, for Mitosis Entry Checkpoint defects, Mec1 and Mec3, for synthetic lethality with a damage-inducing allele of DNA primase Ddc1 and Ddc2 proteins (DNA Damage Checkpoint).

Rad17, Mec3 and Ddc1 proteins are associated in an heterotrimeric complex that has a ring-structure similar to PCNA (Doré *et al.*, 2009), the clamp that in eukaryotes confers processivity to DNA polymerases encircling the DNA strand; for this reason this complex has been named PCNA-like or 9-1-1 complex, from the name of human subunits.

Rad24 is a protein that share some homology with Rfc1, the major subunit of Replication Factor C (RFC) which is the protein complex that, during replication, loads PCNA onto DNA. Rad24 has been co-purified with the minor subunits Rfc2-5, demonstrating the presence of a hybrid complex, called RFC-like, which has been demonstrated to load the PCNA-like complex onto DNA during

the DNA damage response (Green *et al.*, 2000; Majka and Burgers, 2003). *In vitro* studies demonstrated that 9-1-1 is preferentially loaded on primer-template junctions and that it can slide along the dsDNA (Majka and Burgers, 2003). A possible function for this complex is to recruit different substrates for the Mec1 kinase in the proximity of DNA (Melo *et al.*, 2001; Giannattasio *et al.*, 2002) and the fact that the Ddc1 subunit is subjected to damage- and cell cycle-dependent phosphorylation (Longhese *et al.*, 1997) suggests possibly a fine regulation for the interactions of this complex with other proteins.

Mec1 is a protein kinase associated with the product of the gene *DDC2/LCD1/PIE1*, which mediates its binding to DNA. It has been demonstrated that after DNA damage the two apical complexes Mec1/Ddc2 and 9-1-1 are recruited independently of each other, at least on lesions induced by HO endonuclease overexpression (Melo *et al.*, 2001).

Early events in checkpoint activation

Mec1 (hATR) and Tel1 (hATM) were identified as the two apical kinases responsible for the activation of the signal transduction pathway: each phosphorylation event of the DNA damage induced checkpoint cascade depends upon them. As protein kinases, they belong to the PIKK family (Phosphatidyl Inositol(3) Kinase-like Kinase) and they are also involved in pathways different from DNA damage checkpoint: Mec1 controls the levels of the dNTPs pools during an unperturbed S phase and is essential for the completion of DNA replication (Cha and Kleckner, 2002); Tel1 is involved in the maintenance of telomeres.

In higher eukaryotes ATR and ATM have clearly different roles, ATM signals mainly in the presence of DSB and ATR signals in the presence of ssDNA due to replication stress or other kind of DNA damage. Differently, in budding yeast, Mec1 is the most important kinase and Tel1 has only a minor role in DNA damage signalling: *mec1* mutants are far more sensitive than *tel1Δ* to any kind of DNA damage. On the other hand, the fact that the double mutant *mec1Δtel1Δ* is more sensitive than the single ones and that the overexpression of *TEL1* partially rescues the phenotype of a *mec1Δ* strain suggest that Tel1 is also able to perform some of the functions normally carried out by Mec1 (Morrow *et al.*, 1995). This is also confirmed by the fact that, in the absence of Mec1, Tel1 can promote the phosphorylation of the transducer Rad53, although less efficiently (Usui *et al.*,

2001).

Mec1 interacts physically with the protein Ddc2, which is required for its binding to ssDNA and *in vitro* can localise on this structure independently of Mec1 (Zou and Elledge, 2003). Differently, in living cells, Ddc2 cannot be efficiently recruited to sites of DNA damage in the absence of Mec1 (Melo *et al.*, 2001), suggesting that for a stable association of Ddc2 with the RPA-ssDNA complex the presence of Mec1 is essential.

Ddc2 is phosphorylated in an unperturbed cell cycle during S phase and in response to DNA damage in a Mec1-dependent manner (Paciotti *et al.*, 2000). Since Ddc2 phosphorylation does not depends on any other checkpoint factor, with the exception of Mec1, this protein has been widely used as an *in vivo* marker of Mec1 kinase activation.

Recent *in vitro* works, using the Xenopus cell free system, demonstrated clearly that the presence of single stranded DNA alone is not sufficient for checkpoint activation, but a 5' primer-template junction is required (Macdougall *et al.*, 2007). This evidence and the observation that, if the DNA is covered by RPA, the PCNA-like complex is preferentially loaded onto 5' recessed DNA ends (Majka *et al.*, 2006a), indicates that the second essential step for checkpoint activation is 9-1-1 loading occurring onto a specific DNA structure.

Intriguingly recent experiments have suggested that the only function of ssDNA and the 5' primer-template junction is to act as a scaffold to increase the local concentration of checkpoint factors: indeed, the induction of artificial co-localisation of the sensors, 9-1-1 and Mec1/Ddc2, appears to be sufficient to activate the checkpoint even in the absence of any damage (Bonilla *et al.*, 2008).

Once the PCNA-like complex has been loaded, the Ddc1 subunit, which is normally phosphorylated in S phase in at least one of the three Cdc28 consensus sites, became hyper-phosphorylated in a Mec1-dependent manner on at least one of the eight consensus sites for PIKK (Longhese *et al.*, 1997). The function of all these phosphorylation events is currently unknown because they are not required for complex formation but, on the contrary, it seems that the presence of a loaded complex is required for the damage induced phosphorylation of Ddc1, as this modification appears to be greatly reduced in *rad24Δ* mutant Paciotti *et al.* (1998).

Why the contemporary presence of these two complexes is required for Mec1 activation? And how

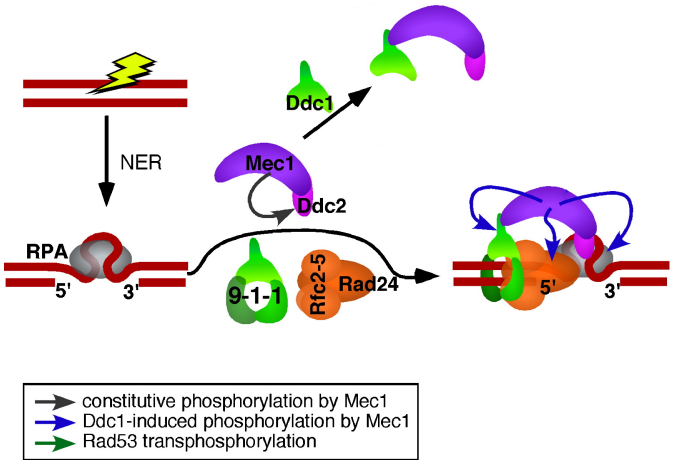

Figure 3.2: Model for Mec1 activation following DNA damage (adapted from Navadgi-Patil and Burgers, 2009)

this activation take place? The answer to these questions was found from an apparently unrelated protein.

A new player

TopBP1, for Topoisomerase Binding Protein 1, is BRCT protein found in higher eukaryotes, homologous to yeast Dpb11. Apart from its well established role in the initiation of DNA replication (described in chapter 1 on page 14), it has been recently demonstrated, using *Xenopus leavis* egg extracts, that TopBP1 can stimulate *in vitro* ATR kinase activity and that this function can be reduced to a small region of the protein termed AAD, for ATR Activation Domain (Kumagai *et al.*, 2006). This domain, even if not conserved in sequence was found also in Dpb11, where it has been demonstrated that the carboxy-terminus of Dpb11 is able to stimulate *in vitro* Mec1 kinase activity (Mordes *et al.*, 2008b). Moreover, these authors observed that Mec1-dependent phosphorylation of Dpb11 on Thr 731 further enhances the ability of Dpb11 to stimulate Mec1 kinase activity.

Data obtained from *S. pombe* and human cells demonstrated that Rad9 (corresponding to scDdc1) recruits TopBP1 (Rad4/Cut5 in *pombe*) via an interaction between one of its phosphorylated residues and a BRCT of TopBP1, explaining thus the requirement of the loading of the PCNA-like complex for full ATR activation (Furuya *et al.*, 2004; Delacroix *et al.*, 2007; Lee *et al.*, 2007).

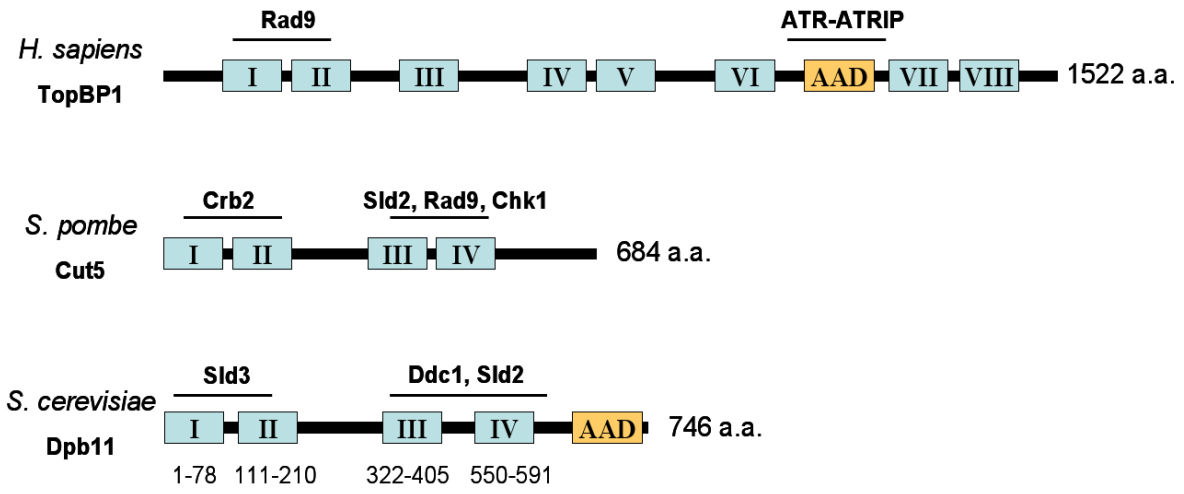

Figure 3.3: Conservation of BRCT domains (blue) and AAD domain (yellow) of Dpb11. The names of proteins interacting with each couple of BRCTs are written in boldface.

In budding yeast the situation is somehow more complicated: Dpb11, as said before, is able to stimulate Mec1 kinase activity, but it has been reported that also the PCNA-like complex, and in particular its Ddc1 subunit, is competent for this function in condition of low ionic strength (Majka *et al.*, 2006b; Navadgi-Patil and Burgers, 2008). Moreover, differently from *S.pombe*, Rad53 phosphorylation depends upon Ddc1 after DNA damage, but not after replication stress (Pellicioli *et al.*, 1999).

Whether this additional function of Ddc1 is dependent or independent of Dpb11 it is still under investigation, but the fact that Dpb11 and Ddc1 display a synergic effect on Mec1 activity suggest that there is at least a cooperation between these two proteins (Navadgi-Patil and Burgers, 2008).

In vivo data are still controversial: Dpb11 is an essential protein and thus the thermosensitive mutant *dpb11-1* has been used for this kind of studies. Strains carrying this mutation are reported to be unable to establish a normal response to replication stress at restrictive temperature (Wang and Elledge, 1999), but are unexpectedly only mildly sensitive to the treatment with hydroxyurea, a chemical compound that induces replication stress (Araki *et al.*, 1995); moreover their survival to this treatment depends upon the PCNA-like complex, suggesting that 9-1-1 and Dpb11 could act redundantly in promoting survival to replication stress (Wang and Elledge, 2002).

It is thus auspicable a better understanding of the *in vivo* mechanisms that result in Mec1 activation and this is one of the aims of this work.

Rad9 and the chromatin context

RAD9 has been the first checkpoint gene to be isolated and initially classified as a damage sensor required in G1 and G2 phases and only partially in S phase (Weinert and Hartwell, 1988). Subsequent studies demonstrated that, after DNA damage, Rad9 becomes hyper-phosphorylated (step 1 in figure 3.4 on the facing page) in a manner that depends on Mec1, Tel1 and the Rad24 epistasis group. It is generally thought that this phosphorylation (2) generates a docking site for Rad53 which could bind (3) to the phospho-sites near to the Serine Cluster domain (SCD) of Rad9, using its FHA (Fork-Head Associated) domains (Gilbert *et al.*, 2001). This binding facilitates a Mec1 dependent phosphorylation of Rad53 (4), which is required for the activation of Rad53 kinase activity. Indeed it has been observed *in vitro* that the presence of Rad9 facilitates Mec1 dependent Rad53 phosphorylation (Sweeney *et al.*, 2005). Moreover the oligomerization of Rad9, mediated by the binding of its BRCTs domains to phosphorylated residues on the same protein, generates a protein scaffold (Soulier and Lowndes, 1999; Usui *et al.*, 2009), which allows an increase in the local concentration of Rad53 that facilitates auto-phosphorylation events (5–6). Rad9 is required also for the activation of Chk1, which is a transducer in a pathway parallel to that of Rad53 (Sanchez *et al.*, 1999). Rad9 domains that are required for Rad53 activation are functionally different from the one that are required for Chk1 regulation, indeed a Chk1 Activation Domain (CAD in figure 3.5 on page 44) is required for Chk1 activation, but is dispensable for Rad53 phosphorylation (Blankley and Lydall, 2004).

The response of the cell to DNA damage, as any other event in DNA metabolism, is influenced by the structure of chromatin. Histones are substrates of many post translational modifications such as acetylations, methylations, phosphorylations and ubiquitinations, which change their structure and as a consequence the structure of chromatin itself. It has been demonstrated that in yeast, serine 129 of histone H2A is phosphorylated in response to DSBs, and a similar event take place also on histone H2AX in mammalian cells (Downs *et al.*, 2000). The phosphorylated form of histone H2AX, called γ-H2AX, has been shown to contribute to DNA repair and to be required, both in

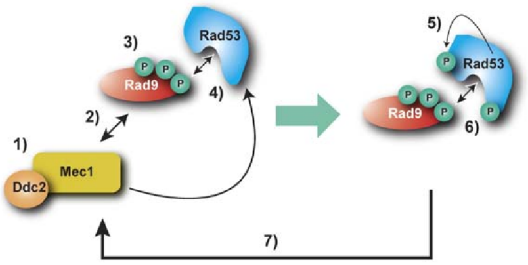

Figure 3.4: Mechanism for Rad9 function in Rad53 activation (Sweeney *et al.*, 2005).

yeast and animal cells, for survival to DNA damage treatments. γ-H2AX plays also a conserved role in the maintenance of the checkpoint, because in its absence this signal extinguishes prematurely (Nakamura *et al.*, 2004).

On the other hand, an important histone post-translational modification, required for a correct checkpoint establishment is ubiquitination of histone H2B on lysine 123. This event is promoted by the ubiquitin-conjugating enzyme Rad6, in association with the ubiquitin ligase Bre1, which gives substrate specificity. As a consequence of this event, Dot1 methyltransferase methylates histone H3 on lysine 79. This last modification is required for Rad9 phosphorylation, and also to transfer the signal from the apical kinase Mec1 to the adaptor kinase Rad53 (Giannattasio *et al.*, 2005). It has been also demonstrated that H3-^{me}K79 is necessary for the physical recruitment to the chromatin of 53BP1, the human orthologue of Rad9, through a direct interaction between the modified residue of the histone and the conserved Tudor domain (see figure 3.5 on the following page) of 53BP1, which constitutes an hydrophobic pocket that can host the methylated lysine (Huyen *et al.*, 2004). This H3-K79 mediated chromatin binding of Rad9 is not only required for maintaining the integrity of the signalling cascade, but it controls also the amount of resection, that generates ssDNA, which acts as the first signal of DNA damage, suggesting that a Rad9-mediated negative feedback loop could regulate this important process (Lazzaro *et al.*, 2008).

Unexpectedly, in the absence of Dot1, lack of checkpoint activation is observed only in G1 arrested cells, while M phase arrested cells are only partially defective in Rad53 phosphorylation (Giannattasio *et al.*, 2005). It has been demonstrated that, in *Schizosaccharomyces pombe*, the alternative pathway for the recruitment of Rad9 homolog Crb2, relies on the presence of the protein

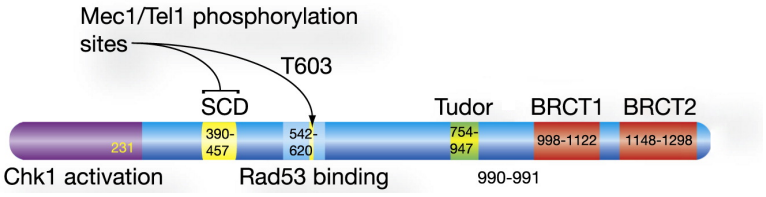

Figure 3.5: Model of Rad9 protein and its domains (adapted from Usui *et al.*, 2009)

Cut5, homolog of budding yeast Dpb11 (Du *et al.*, 2006), therefore another aim of this work is to better define the histone-modification independent pathway for checkpoint activation in budding yeast.

Rad53, Chk1 and the effectors

Rad53 is protein kinase, whose essential activity is required to complete a normal S phase. Rad53 is also required for cell cycle arrest of cells experiencing DNA damage or replication stress. Rad53 phosphorylation can be observed as an electrophoretic shift, when cells are treated with DNA toxins, and it is currently used as a molecular marker of checkpoint activation (Pellicioli *et al.*, 1999). Rad53 has two FHA domains, which are responsible for interactions with phospho-proteins: Rad53 binding to the hyperphosphorylated form of Rad9, mediated by these domains, leads to the phosphorylation of Rad53 by Mec1. Once phosphorylated, Rad53 becomes active as an autokinase, promoting a series of *in trans* auto-phosphorylation events that generate the fully phosphorylated form. It has been suggested that Rad9 acts like a solid-phase catalyst that allows a local increase in Rad53 concentration, which is essential for these reactions. The autophosphorylation of Rad53 mediates its release from Rad9, allowing the former to phosphorylate and activate the final effectors (Gilbert *et al.*, 2001).

Chk1 is the second transducer protein in this pathway but its functions, in budding yeast, are partially hidden by Rad53 activity: *chk1Δ* differently from *rad53Δ* strains are only mildly sensitive to DNA damaging agents. Whereas Rad53, but not Chk1, is required for the activation of the checkpoint during S phase, at the G2/M transition these proteins are partially redundant in signalling the presence of a DNA damage and the deletion of one of them induces only a partial defect in this phase of the cell cycle (Gardner *et al.*, 1999; Sanchez *et al.*, 1999).

Even if the most part of the targets of checkpoint proteins is still unknown, it has been clearly demonstrated that checkpoint activation induces a delay or a block of the cell cycle, accompanied by the transcriptional induction of genes involved in different aspects of DNA repair. It is thus likely that there should be some negatively induced effectors which regulate cell cycle progression and positively induced effectors which modulate the repair processes.

In G1 phase the protein Swi6 is one of the putative effectors, negatively regulated by the checkpoint. Swi6 form a complex with Swi4 and it is required for the transcription of G1 cyclins *CLN1* and *CLN2* and this complex is inactivated by a Rad53 dependent phosphorylation of Swi6 (Sidorova and Breeden, 1997).

In G2/M checkpoint the effectors Pds1 and Cdc5 are implicated. Following a damage in M phase arrested cells, a delay in the transition from metaphase to anaphase can observed. This effect is due to the Rad53 and Chk1 dependent phosphorylation of the securine Pds1: the former phosphorylation inhibits the binding of APC/Cdc20 to Pds1, whereas the latter inhibits the ubiquitin ligase reaction itself, preventing chromosome segregation (Cohen-Fix and Koshland, 1997; Agarwal *et al.*, 2003). Moreover Rad53 phosphorylates Cdc5, stabilising it. The persistence of Cdc5, which normally blocks mitotic exit by preventing the activation of the APC/Cdh1, prevents the exit from mitosis, cyclin degradation and the fall in Cdc28 kinase activity (Sanchez *et al.*, 1999).

During S phase, checkpoint proteins may have targets also in the DNA replication machinery: it has been demonstrated that *PRI1*, encoding a subunit of the Polα-primase complex, and replication protein A (RPA) are implicated in the DNA damage response. In both cases, mutants that are defective in the responses to genotoxic agents have been identified (Longhese *et al.*, 1996). The *pri1-M4* mutant is indeed incapable of delaying S phase even if Rad53 is active (Marini *et al.*, 1997) and mutations in the *RFA1* gene, coding for one of the three subunits of RPA, are defective in delaying cell cycle after DNA damage in G1 and S phase (Brush *et al.*, 1996; Brush and Kelly, 2000).

There are evidences of the involvement of DNA damage checkpoint also in the regulation of the firing of late replication origins: in wild type cells, in the presence of genotoxic agents, late replication origins are inactivated, and this phenotype depends on the presence of functional Mec1 and Rad53 protein kinases (Santocanale and Diffley, 1998).

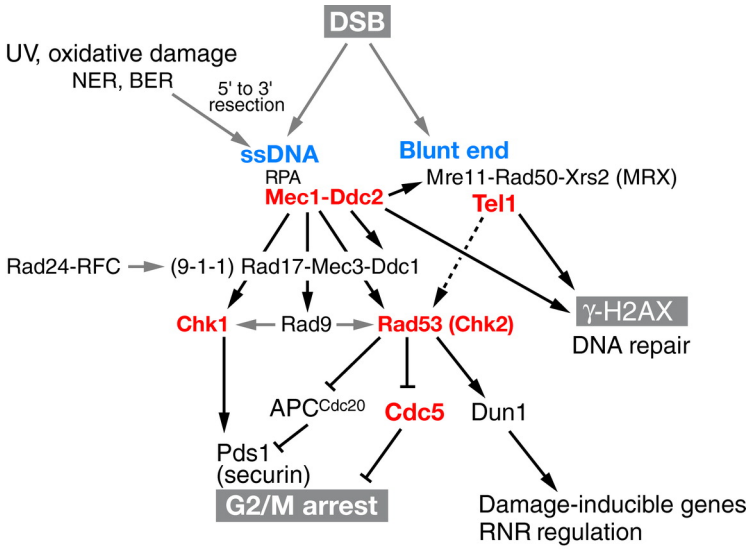

Figure 3.6: The DNA damage checkpoint in *S.cerevisiae* (Harrison and Haber, 2006)

Checkpoint activation controls also the transcription of some genes correlated with DNA repair and the regulation of the concentration of dNTPs in the cell. In one of these pathways the kinase Dun1 is involved. Dun1 is phosphorylated and activated in a Mec1- and Rad53-dependent manner and once active it phosphorylates the repressor Crt1, inducing its detachment from DNA. This event allows the increase in transcription of genes like *RAD2* and *RAD7*, involved in NER (see on page 23) or *RAD51* and *RAD54*, involved in DSBR (see on page 27). Dun1 induces also the transcription of genes involved in the regulation the dNTP pool, such as *RNR1*, *RNR2* and *RNR3*, whose products are the three subunits of ribonucleotide reductase.

The response to replication stress

During S phase, in many occasions, cells can experience situations that generate stress on the replicative machinery. This can happen if the MCM helicase encounters lesions that prevent the separation of the two template filaments, such as interstrand crosslinks or some covalent adducts generated by chemotherapeutics like etoposide or camptothecin, which block the topoisomerase covalently bound to DNA (Hsiang *et al.*, 1985).

Replication stress is also observed when cells encounter damaged nucleotides that cannot function

as a template for Polδ or Polε and when cells are treated with chemotherapeutics like hydroxyurea, which reduce the level of dNTPs by inhibiting RNR. Finally, in an unperturbed S phase, cells can experience replication stress when the replisome encounters regions of the genome particularly difficult to replicate, RSZ — Replication Slow Zones — (Cha and Kleckner, 2002) or when it encounters replication fork barriers (RFB), such as in ribosomal DNA (Brewer and Fangman, 1988).

Sensing the replication stress

In budding yeast, replication stress is sensed through the same proteins that normally work in DNA damage checkpoint pathway and that lead to Mec1 activation: RPA, Mec1-Ddc2, PCNA-like, RFC-like, Dpb11. Although the structure that elicits checkpoint activation is likely ssDNA covered by RPA, as in G1 and in G2, the reason why the ssDNA exposed in an unperturbed replication does not activate this response is still not known and the precise mechanisms that trigger replication checkpoint activation have not been defined yet. The fact that there could be something else beyond this pathway is suggested by the involvement of other factors, which seems not to be required in DNA damage checkpoint activation.

As previously described, the 9-1-1 checkpoint clamp is loaded onto 5' primer-template junctions adjacent to RPA-coated ssDNA. In humans, 9-1-1 then recruits the Dpb11 homolog TopBP1, that binds ATRIP and contributes to ATR activation (Delacroix *et al.*, 2007; Lee *et al.*, 2007). By contrast, it has been reported that S. cerevisiae 9-1-1 can activate Mec1-Ddc2 directly *in vitro* (Majka *et al.*, 2006b), and the fact that *ddc1Δ* strains still displays Rad53 phosphorylation after HU (Pellicioli *et al.*, 1999), suggests that Dpb11 can work either in the absence of a functional PCNA-like complex.

In addition to proteins that function specifically in checkpoint signalling, several proteins, essential for a proper DNA replication, are also implicated in the activation of the S-phase checkpoint. In budding yeast, besides the small RFC subunits that complex with Rad24, DNA polymerase ε and the initiation factor Drc1/Sld2 are also required for efficient checkpoint activation (Navas *et al.*, 1995; Wang and Elledge, 1999). Notably, the checkpoint functions of these proteins seems partially linked to their replication functions, suggesting that they might contribute to damage detection, at

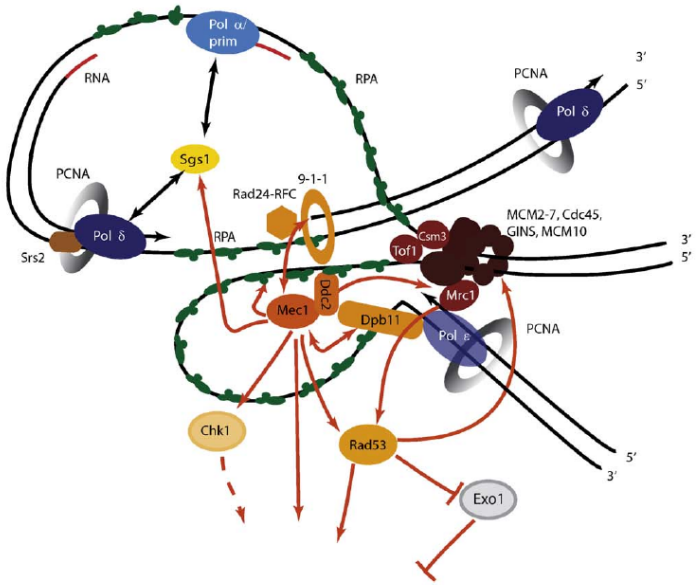

Figure 3.7: Possible structure of a stalled replication fork (adapted from FRIEDEL *et al.* 2009)

least in part, by supporting efficient DNA replication. Moreover, deletion of the RFC- or PCNA-like proteins from *dpb11-1* mutant renders them more sensitive to HU, suggesting that these proteins might collaborate to monitor the progression of replication forks (WANG and ELLEDGE, 2002).

Also the Ctf18-Dcc1-Ctf8 trimer, which has a role in the maintenance of chromatid cohesion during DNA replication (HANNA *et al.*, 2001), appears to be involved in checkpoint signalling. This trimer complexes with the small RFC subunits and generates and alternative RFC-like, that seems to work in a pathway parallel to that of Rad24 as the double mutant *ctf18Δrad24Δ* appears to be hypersensitive to hydroxyurea (NAIKI *et al.*, 2001) and almost completely unable to phosphorylate Rad53 (BELLAOUI *et al.*, 2003). *In vitro* evidences suggested that it could perform this function by unloading PCNA in particular circumstances (BYLUND and BURGERS, 2005).

Additional research is needed to confirm exactly how Dpb11 is recruited to damage sites since it appears to co-localise with Pol ε during initiation, but not during elongation (MASUMOTO *et al.*, 2000). In one model, 9-1-1 and Mec1-Ddc2 are recruited independently of RPA-ssDNA, and Mec1 subsequently phosphorylates the Ddc1 subunit of 9-1-1. In an alternative model, 9-1-1 and Dpb11 act in parallel to activate Mec1-Ddc2. In this case Dpb11 could be recruited to RPA-ssDNA via its interaction with other proteins, for example, Pol ε or Sld2 and Sld3 (ZEGERMAN and DIFFLEY, 2007; TANAKA *et al.*, 2007).

What structure do these proteins recognise? The RNA portion of the primer synthesised by primase was initially thought to be the activator of the checkpoint, partly because actinomycin D, an inhibitor of primase, blocks the checkpoint response to aphidicolin (Michael *et al.*, 2000). However, this interpretation has been re-evaluated in the light of the fact that actinomycin D has been found to prevent the chromatin binding of RPA and Polα (You *et al.*, 2002). Whether the RNA primer is directly involved in checkpoint activation is still unclear, but it is suggestive the fact that it could provide the 5' end that is required for 9-1-1 loading, otherwise absent in the replicon structure. Nonetheless, recombinant wild-type human primase, but not a primase mutant, can restore the checkpoint response in primase-depleted *Xenopus* extract (Michael *et al.*, 2000), suggesting that primase could be indeed involved in checkpoint activation.

Mediators of the replication checkpoint

The mediator of the DNA damage checkpoint, Rad9, although being required for Rad53 phosphorylation after DNA damage is not required to phosphorylate Rad53 when cells are treated with the RNR inhibitor hydroxyurea (HU) (Pellicioli *et al.*, 1999): for this reason it was believed that another protein could perform its functions. This protein has been identified as Mrc1 (Alcasabas *et al.*, 2001). *mrc1Δ* strains, as well as *rad9Δ* ones, have only a minor defect in Rad53 phosphorylation after HU treatment because, in the absence of Mrc1, DNA damage likely occur at stalled forks, promoting Rad9-dependent Rad53 activation. In agreement with this *mrc1Δrad9Δ* strains are completely unable to hyperphosphorylate Rad53 after HU.

Several observations suggest that Mrc1 mediates Rad53 activation in a fashion similar to that of Rad9. First, like Rad9, Mrc1 is hyperphosphorylated in response to replication blocks and contains many [S/T]Q residues, which are putative Mec1 phosphorylation sites (Kim *et al.*, 1999). Second, Mrc1 addition to an *in vitro* kinase assay greatly stimulate the ability of Mec1 to phosphorylate and activate Rad53 (Chen and Zhou, 2009). Moreover, the mammalian counterpart of Mrc1, Claspin, interacts with the effector kinase Chk1, the functional orthologue of Rad53, and it is essential for its phosphorylation (Kumagai and Dunphy, 2000).

Mrc1 is also an integral part of the replication complex: it is loaded onto replication origins and it travels with forks; *mrc1Δ* strains displays a slow S phase during which the DNA damage

response is spontaneously activated. Replicative and checkpoint functions of Mrc1 can be separated as, the *mrc1-AQ* mutant, in which all [S/T]Q are mutated to alanine, is unable to carry out Rad53 phosphorylation without having any defect in an unperturbed replication (Osborn and Elledge, 2003).

Effects of replication checkpoint activation

The most striking S phase phenotype of *rad53* and *mec1* mutants is their total inability to complete DNA replication if transiently exposed to global replication stress (Desany *et al.*, 1998), suggesting that checkpoint activation is essential to maintain the integrity of replication forks and to promote fork restart after replisome arrest/pausing. In fact, chromatin immunoprecipitation experiments have demonstrated that in *mec1* and *rad53* mutants replicative DNA polymerases dissociates more easily than wild type from replication forks when replication is inhibited by HU, indicating their inability to stabilise replisomes (Lucca *et al.*, 2004). Indeed it has been observed that, in the absence of Rad53, cells experiencing HU-induced stress accumulate two class of aberrant DNA structures: long ssDNA stretches and four-branched structures, likely generated by the reversal of replication forks (Lopes *et al.*, 2001; Sogo *et al.*, 2002).

Among the enzymatic activities that can process these pathological structures, Exo1 plays a prominent role: it has been shown that the presence of Exo1 counteracts reversed fork formation, as in *rad53exo1* mutants a much larger accumulation of reversed forks is observed compared to the one of of *rad53* (Cotta-Ramusino *et al.*, 2005). Moreover *EXO1* deletion can suppress DNA damage sensitivity and replication defects of *rad53* mutants, suggesting that Exo1 might be a primary target of Rad53 and that Exo1 phosphorylation could act to restrain Exo1-dependent replication fork breakdown (Segurado and Diffley, 2008). Consistently with this, a recent study indicated that Rad53 phosphorylation of Exo1 could act to limit ssDNA accumulation and act as a feedback loop to restrain checkpoint activation (Morin *et al.*, 2008).

Inability to activate the replication checkpoint correlates also with the inability to prevent the firing of late replication origins (Santocanale and Diffley, 1998): this could be also reflected in the fact that, when replication is hindered by MMS-induced lesions, checkpoint mutants replicate their genome faster than wild type.

Part II

Results

4

Dpb11 acts as an adaptor during the DNA damage response

Dpb11 is required for Dot1-independent checkpoint activation

It has been previously shown that ubiquitylation of histone H2B by the Rad6/Bre1 complex and methylation of histone H3 on the K79 residue, mediated by Dot1, are prerequisites for a functional response to DNA damage in the G1 phase of the *Saccharomyces cerevisiae* cell cycle (Giannattasio *et al.*, 2005). This requirement seems to be ascribed to the capacity of the Rad9 checkpoint protein to bind methylated H3-K79 through its Tudor domain. In fact, in the absence of H3-K79 methylation or if the Rad9 Tudor domain is mutated, yeast cells damaged in G1 do not exhibit Rad9 loading onto DNA and are deficient in transmitting the checkpoint signal from the ATR-like kinase Mec1 to the Chk2-like kinase Rad53 (Wysocki *et al.*, 2005; Hammet *et al.*, 2007). Surprisingly, if *dot1Δ* mutant cells are treated with Zeocin or UV light in the M phase of the cell cycle, residual phosphorylation of Rad53 can be observed and the G2/M checkpoint response is partially proficient, allowing *dot1Δ* mutant cells to survive the treatment (Giannattasio *et al.*, 2005). This finding suggests that a different mechanism of Rad9 recruitment can compensate for the loss of H3-K79 methylation in M cells.

To define the nature of this second pathway, active in the M phase of the cell cycle, we first

verified whether the activation of Rad53 observed in the absence of H3-K79 methylation (i.e., *dot1Δ* mutant cells) was due to the unscheduled activation of a pathway dependent upon the apical kinase Tel1 and/or Chk1. *dot1Δ*, *dot1Δtel1Δ*, *dot1Δchk1Δ*, and *dot1Δmec1-1* mutant cells were arrested with nocodazole and UV irradiated to trigger the DNA damage checkpoint. Phosphorylation of Rad53 was evaluated as a mobility shift of Rad53 on SDS-PAGE. Cells with a *DOT1* deletion still exhibit significant Rad53 phosphorylation when irradiated in the M phase of the cell cycle; deletion of *TEL1* or *CHK1* does not affect this residual Rad53 phosphorylation, which is instead abolished in a *mec1-1* background (FIGURE 1/A AND DATA NOT SHOWN).

In *S. pombe*, Crb2 can be recruited to chromatin through an interaction with Cut5/Rad4 to fulfil its function in the checkpoint response (DU *et al.*, 2006). For this reason we decided to analysed whether Dpb11, the budding yeast orthologue of Cut5/Rad4, might be involved in recruiting Rad9 to chromatin and possibly be responsible for the activation of Rad53 observed in UV-irradiated *dot1Δ* mutant M-phase cells. In order to address this question, we generated strains carrying a temperature-sensitive *dpb11-1* mutation — which encodes for a truncated protein, lacking the last 182 amino acids — in a *dot1Δ* mutant background and monitored the cellular response to UV. The *dpb11-1* mutant at permissive temperature has only a mild defect in S phase entry (ARAKI *et al.*, 1995).

Under our experimental conditions, when exposed to different levels of UV light, the *dpb11-1* and *dot1Δ* mutant strains are slightly more sensitive than WT cells. Interestingly, the *dot1Δ* and *dpb11-1* mutations exhibit synergistic effects on sensitivity to UV; indeed, the *dot1Δdpb11-1* double mutant is noticeably more sensitive than either one of the single mutants and closely resembles a *rad9Δ* mutant strain (FIGURE 1/B).

In order to test their capacity to delay cell cycle progression following UV irradiation, the WT and mutant strains were arrested with nocodazole, treated with UV light, and released into the cell cycle. Nuclear division was monitored by DAPI staining and microscopic analysis. As shown in FIGURE 1/C, UV-treated *dpb11-1* and *dot1Δ* mutant cells exhibit a nuclear division profile which is very similar to the profile of a WT strain, suggesting an almost normal checkpoint response after UV damage. On the other hand, the double mutant completely loses the delay and behaves almost identically to *mec3Δrad9Δ* checkpoint-null control cells.

We then analysed the phosphorylation cascade that is triggered by UV, monitoring the phosphorylation state of the Ddc2, Rad9, Rad53 and Chk1 factors, which act sequentially in the checkpoint cascade. FIGURE 1/D shows that in M phase, *dot1Δ* mutant cells partially maintain the capacity to activate the checkpoint after UV irradiation and to significantly phosphorylate both Rad9 and Rad53. This residual response to UV damage, observed in the absence of H3-K79 methylation, is dependent upon *DPB11*. Indeed, Rad9 and Rad53 do not exhibit any DNA damage-induced modification and Chk1 phosphorylation appears to be greatly defective in the *dot1Δdpb11-1* double mutant, while Mec1 activity, as measured by Ddc2 phosphorylation, does not seem to be significantly reduced.

Our results on Ddc2 phosphorylation seem to be in contrast with the reports demonstrating the function of Dpb11 and its orthologues in ATR/Mec1 activation (KUMAGAI *et al.*, 2006; MORDES *et al.*, 2008b; NAVADGI-PATIL and BURGERS, 2008). For this reason we decided to monitor Ddc2 phosphorylation at 37°C in order to maximise any possible defect that the *dpb11-1* ts allele could have. Again we were clearly able to see the band corresponding to hyperphosphorylated Ddc2, indicating that in our conditions — after UV irradiation in M phase — Dpb11 C-terminus is dispensable for Mec1 activation (FIGURE 1/D, LAST ROW). This result exclude also the possibility that the synthetic defect on Rad53 and Rad9 phosphorylation could arise from a combination of low Mec1 activity and defective Rad9 recruitment.

The data described so far indicate that the role of *DPB11* in this pathway is to facilitate Rad9 phosphorylation, possibly by providing an alternative way for its recruitment to chromatin, suggesting that *DPB11* and *DOT1* may be working in two parallel pathways leading to Rad9 and Rad53 phosphorylation.

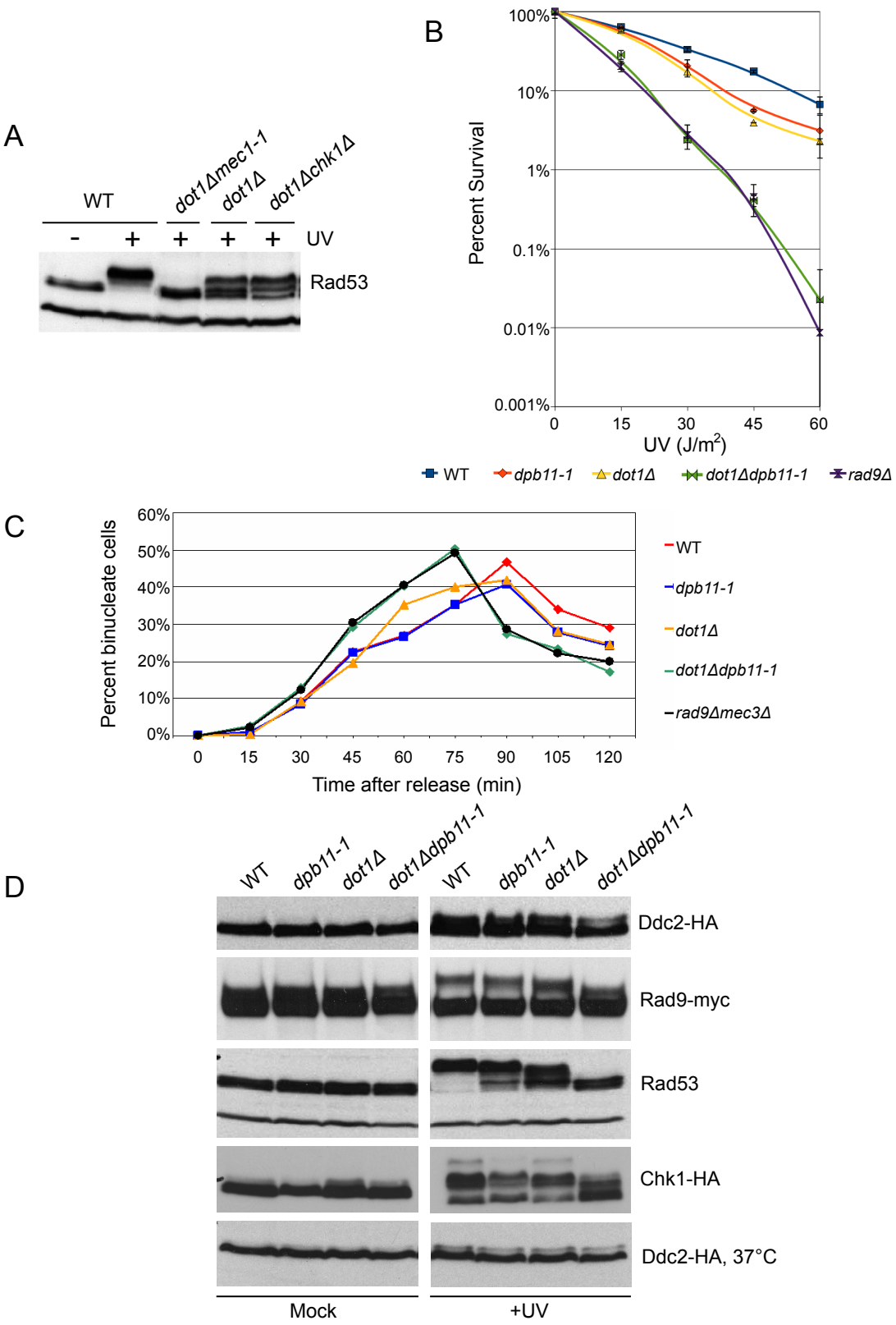

Figure 1. Dpb11 function is required for the Dot1-independent checkpoint activation pathway after UV irradiation.
(A) K699 (WT), YFL234 (*dot1Δ*), YFL499/3d (*dot1Δchk1Δ*) and YFL438 (*dot1Δmec1-1*) cells were arrested in M phase with nocodazole and either mock or UV irradiated (75 J/m2). Analysis of Rad53 phosphorylation, 30 minutes after UV irradiation, was performed by monitoring the mobility shift in SDS-PAGE. **(B)** UV survival assay. Strains K699 (WT), YMIC4E8 (*rad9Δ*), YFP20 (*dpb11-1*), YFL234 (*dot1Δ*), and YMAG6 (*dot1Δdpb11-1*) were grown overnight to stationary phase, diluted and plated on YPD plates, which were irradiated with the indicated UV doses. Survival was assayed by determining the number of colonies formed after 3 days. **(C)** UV checkpoint assay. Yeast strains K699 (WT), YFP20 (*dpb11-1*),YFL234 (*dot1Δ*), YMAG6 (*dot1Δdpb11-1*), and YMIC4F6 (*mec3Δrad9Δ*) were synchronized in M phase with nocodazole, UV irradiated (40J/m2), and released in YPD plus α-factor. Every 15 min, samples were taken and scored for the presence of binucleated cells. **(D)** Analysis of the phosphorylation of checkpoint factors. WT, *dpb11-1*, *dot1Δ*, and *dot1Δdpb11-1* mutant cells carrying Ddc2-HA, Rad9-myc or Chk1-HA were arrested with nocodazole and either mock or UV irradiated (75 J/m2); 30 min after irradiation, Ddc2, Rad9, Rad53 and Chk1 phosphorylations were analyzed by SDS-PAGE and Western blotting.

Dpb11 and H3-^{me}K79 promote Rad9 binding to chromatin after UV damage

The function of Rad9 in checkpoint activation, in the G1 phase of the cell cycle, is dependent upon the presence of both H3-^{me}K79 and the phosphorylation of histone H2A on S129; it has been suggested that these two histone modifications constitutes two different but interdependent pathway for Rad9 recruitment to the damaged chromatin (Javaheri *et al.*, 2006; Hammet *et al.*, 2007). Since in M phase, after UV irradiation, two different pathways for Rad9 activation exists, one dependent upon H3-^{me}K79 and the other upon Dpb11, we decided to test if also phosphorylation of S129 of H2A was helping Rad9 and Rad53 phosphorylation in the absence of H3-K79 methylation.

To assess this, we generated an H2A mutant in which S129 cannot be phosphorylated because this residue has been mutated to alanine. WT, *hta-S129A*, *dot1Δ* and *dot1Δhta-S129A* yeast cells were arrested in M phase and either mock treated or UV irradiated. As it is shown in figure 2/A, mutation of serine 129 to alanine does not reduce Rad9 or Rad53 phosphorylation in the presence of Dot1. These data indicates that H2A phosphorylation does not participate in promoting Rad9 phosphorylation in the absence of Dot1. Unexpectedly, when the methylation of H3-K79 is impaired, we observed that the H2A phosphorylation defect seems to rescue the mild Rad53 phosphorylation defect exhibited by the *dot1Δ* strain.

After a DNA damage in the G1 phase of the cell cycle, Rad9 is recruited to the chromatin via its Tudor and BRCT domains and it has been demonstrated that this binding is required for the checkpoint activation function of Rad9 (Wysocki *et al.*, 2005; Hammet *et al.*, 2007). Since the double mutant *dot1Δdpb11-1* lacks completely Rad53 phosphorylation and checkpoint activation after UV irradiation, we decided to check whether this is due to the lack of Rad9 recruitment to chromatin.

For this reason WT, *dot1Δ*, *dpb11-1* and *dot1Δdpb11-1* cells, harbouring a myc-tagged version of Rad9, were arrested in M phase and either mock treated or UV irradiated. After 30 minutes native whole cell extracts were prepared and the chromatin enriched fraction was separated by centrifugation. In these conditions, in a WT strain, the majority of Rad9 protein is found in the supernatant and only a minor fraction of Rad9 is bound to chromatin (Figure 2/B).

In untreated conditions this fraction of Rad9 seems to be retained onto chromatin in all the

mutants that were analysed: in fact a clear band corresponding to Rad9-myc can be observed in *dpb11-1*, *dot1Δ* and the double mutant, although in this last case a little reduced in intensity. Conversely, after UV irradiation, Rad9 binding to chromatin becomes strictly dependent upon the presence of H3-K79 methylation and the residual binding observed in the *dot1Δ* strain is abolished by the *dpb11-1* mutation (FIGURE 2/B). Altogether these data suggest that, after UV irradiation, both Dpb11 and H3-K79 promote Rad9 binding to chromatin, and that this binding is functionally required for Rad53 activation.

In order to better understand the mechanisms of Dpb11 function in promoting both Rad9 recruitment to chromatin and Rad9 and Rad53 phosphorylation we looked for mutants in Rad9 that, although being proficient in Rad53 phosphorylation, displayed a synergic defect if combined with *DOT1* deletion.

Previously published evidence demonstrated that an N-terminal truncation of Rad9 is defective in Chk1 phosphorylation, despite being able to phosphorylate and activate Rad53 (BLANKLEY and LYDALL, 2004). In *S.pombe*, phosphorylation of T215 of Crb2 — the orthologue of Rad9 — is required for the histone-independent recruitment of Crb2 itself to IR-induced foci (DU *et al.*, 2006); moreover the region containing T215 is homologous to the N-terminus of *S. cerevisiae* Rad9.

For these reason, we took advantage of a yeast strain expressing a version of Rad9 deleted in its N terminus (1–231), called *rad9ΔNT*, under its own promoter. We combined this mutation with the deletion of *DOT1* and we monitored Rad53 phosphorylation after UV irradiation. As it is shown in FIGURE 2/C, deletion of Rad9 N-terminus causes a defect in the rapid activation of Rad53, which is not relevant for survival to DNA damage, as the *rad9ΔNT* strain is only mildly sensitive to UV light (BLANKLEY and LYDALL, 2004). Indeed this defect completely disappears 30 minutes after irradiation.

Differently, deletion of *DOT1* in this background causes an almost completely inability to activate Rad53, even 30 minutes after UV irradiation, in a manner that is similar to the one of the *dot1Δdpb11-1*, suggesting that this region could be important for the Dot1-independent function of Rad9.

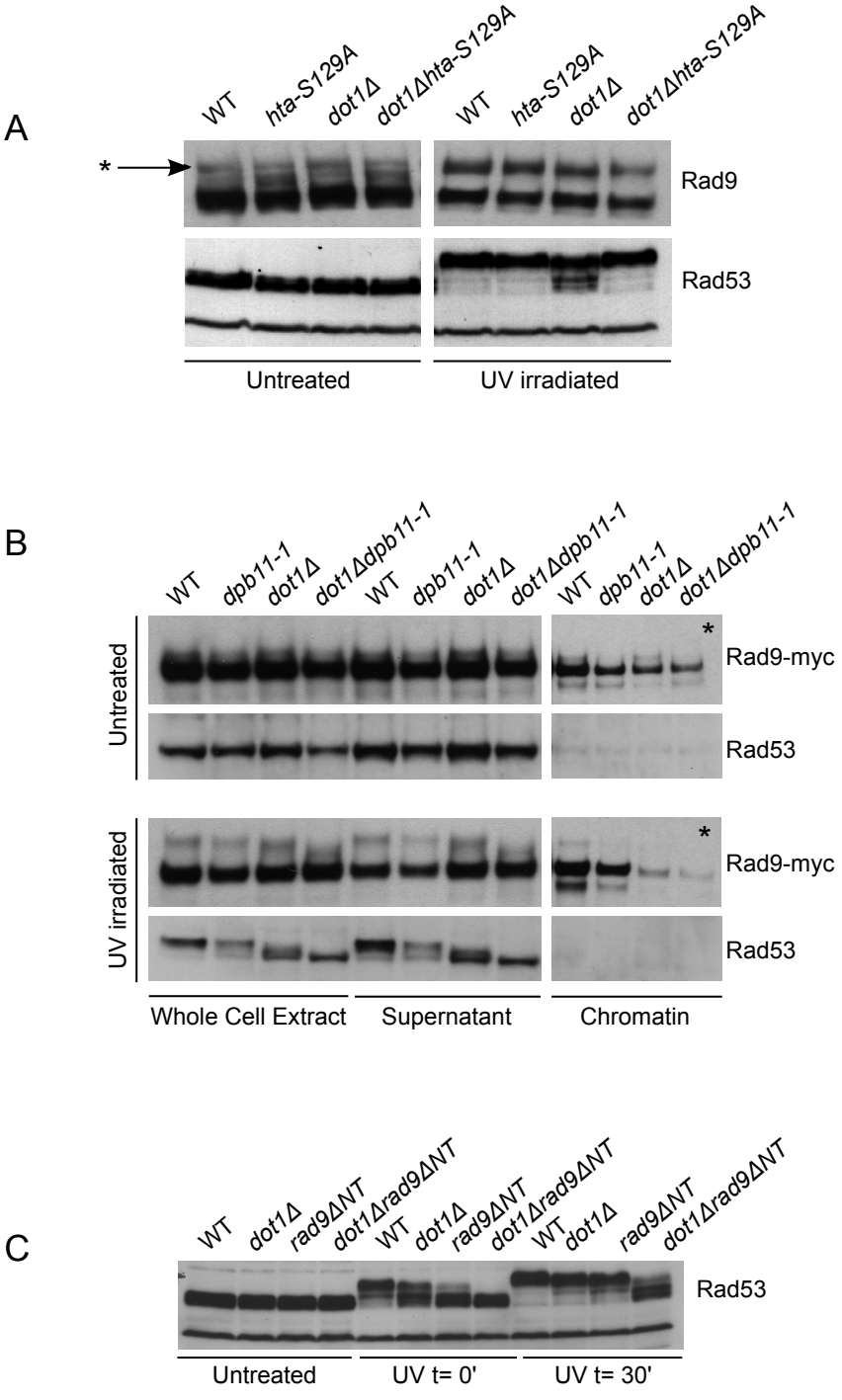

Figure 2. Dpb11 and H3-K79 promote Rad9 binding to chromatin after UV irradiation.

(A) YMAG149/7b (WT), YMAG168 (*hta-S129A*), YMAG150/4A (*dot1Δ*) and YMAG170 (*dot1Δhta-S129A*) were arrested with nocodazole and either mock or UV irradiated (75 J/m2); 30 minutes after irradiation, Rad53 and Rad9 proteins were analyzed by SDS PAGE and western blotting; the background band of the α-Rad9 antibodies is marked with an asterisk. **(B)** Chromatin binding assay: WT, *dpb11-1*, *dot1Δ* and *dot1Δdpb11-1*, all carrying a myc tagged version of Rad9, were arrested in M phase with nocodazole and either mock treated or UV irradiated. After 30 minutes, whole cell extracts were prepared and separation of chromatin enriched fraction and supernatant was carried out by centrifugation. The obtained samples were analyzed by SDS page and wester blotting, to assess the relative abundance of Rad9. Rad53 was used as a negative control. The images marked with an asterisk are overexposed to better appreciate the results. **(C)** SY2080 (WT), YFP90 (*dot1Δ*), DLY2236 (*rad9ΔNT*) and YFP91 (*dot1Δrad9ΔNT*) were arrested in M phase with nocodazole and either left untreated or subjected to UV irradiation. Immediatly (t=0') or 30 minutes (t=30') after treatment phosphorylation of Rad53 was analyzed by SDS-PAGE and western blot.

Ddc1 phosphorylation and *DOT1* are required for an effective UV response

Previously published evidence indicates that Dpb11 interacts physically and genetically with the Ddc1 subunit of the 9-1-1 checkpoint clamp: this interaction seems to involve the last BRCT domain of Dpb11, which is a phospho-protein binding motif (Wang and Elledge, 2002). Since Ddc1 is subject to cell cycle-dependent and DNA damage-dependent phosphorylation (Longhese *et al.*, 1997; Paciotti *et al.*, 1998), we decided to test whether Ddc1 phosphorylation plays any role in controlling this Dpb11-dependent pathway.

The deduced protein sequence of Ddc1 reveals the presence of three consensus phosphorylation sites for cyclin-dependent kinases ([S/T]P) and eight putative target sites for Mec1 ([S/T]Q). By site-specific mutagenesis, we converted the phosphorylatable residues to alanine and constructed the *ddc1-M3* allele, lacking the three putative CdK target sites; the *ddc1-M8* allele, lacking the eight Mec1 target sites; and the *ddc1-M11* allele, lacking all sites (Figure 3/A).

In order to determine the contribution of these phosphorylation sites to DNA damage-induced Ddc1 phosphorylation, the phosphorylation state of these mutant proteins was analysed by western blotting, after treatment with UV light. While mutations in the CdK consensus sites do not affect the UV-induced phosphorylation of Ddc1, the damage-dependent mobility shift of Ddc1 is lost in *ddc1-M8* and *ddc1-M11* mutant strains (Figure 3/B), indicating that at least one of the eight sites is phosphorylated in a DNA damage dependent manner.

The role of these phosphorylation sites in the downstream events in the DNA damage checkpoint cascade was further investigated by analysing the effects of the *ddc1-M3*, *ddc1-M8*, and *ddc1-M11* mutations on Rad9 and Rad53 phosphorylation after UV irradiation in nocodazole-arrested cells. Our results show that none of the *DDC1* phosphorylation mutant alleles affects the checkpoint response when H3-K79 can be methylated. On the other hand, both *ddc1-M8* and *ddc1-M11* produce a synthetic phenotype when combined with a *dot1Δ* mutation: in fact, both *dot1Δddc1-M8* and *dot1Δddc1-M11* mutant strains lose the ability to hyper-phosphorylate Rad9 and Rad53 (Figure 3/C and data not shown) and acquire a UV hypersensitivity similarly to what we observed in *dot1Δdpb11-1* mutant cells (Figure 3/D and data not shown).

Such observations suggest that a pathway requiring Dpb11 and Mec1-dependent phosphorylation

of Ddc1 collaborates with methylated H3-K79 in checkpoint activation and is required to phosphorylate Rad9 in the absence of the histone-mediated pathway. These results are in agreement with data obtained in other eukaryotic systems showing that the interaction of TopBP1 and Cut5 with the 9-1-1 complex requires the phosphorylation of the Ddc1 orthologues (Furuya *et al.*, 2004; Delacroix *et al.*, 2007; Lee *et al.*, 2007).

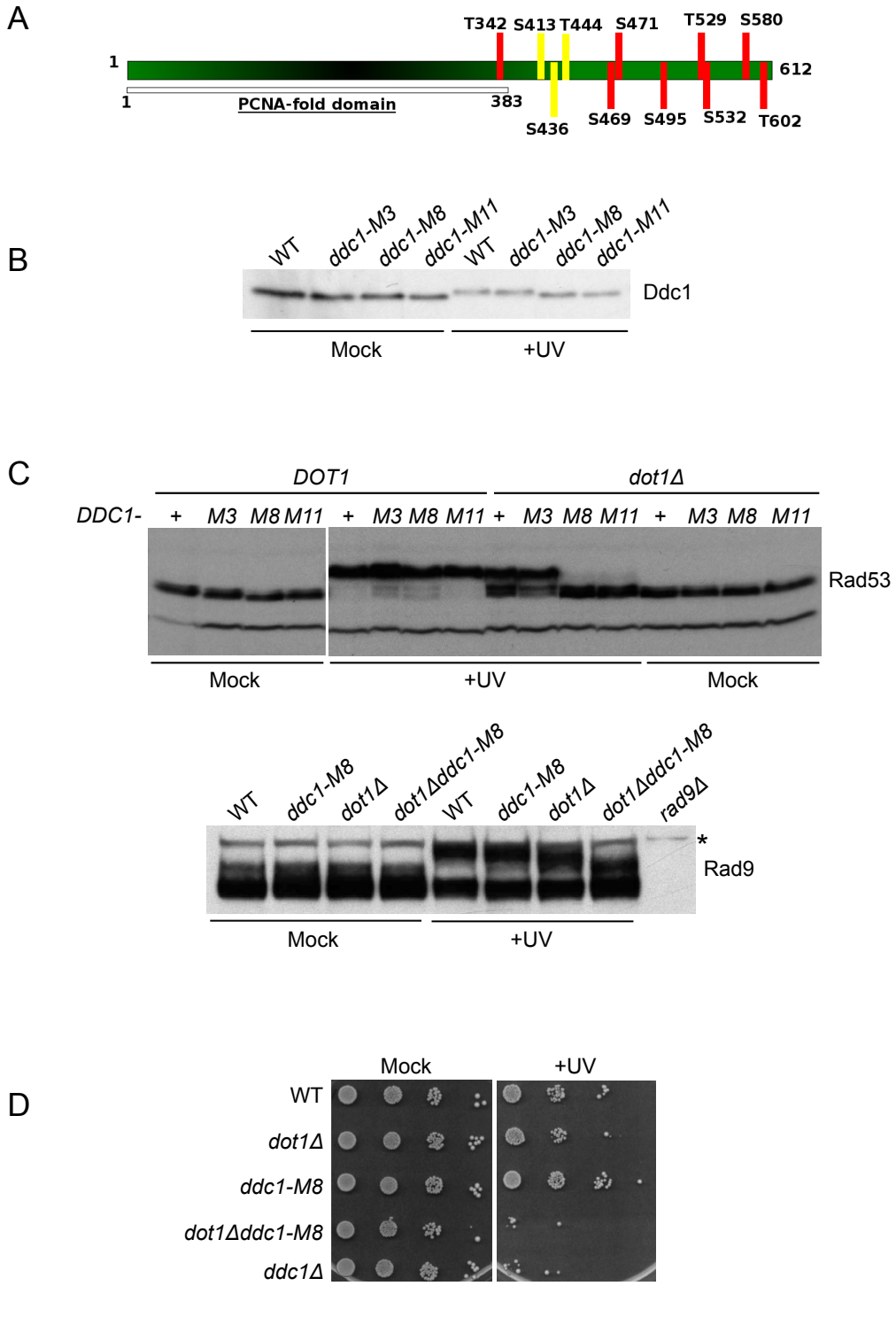

Figure 3. Ddc1 phosphorylation and DOT1 are required for an effective UV response.

(A) Outline of the Cdc28 (yellow) and Mec1 (red) putative phosphorylation target sites in Ddc1. Cdc28 and Mec1 target sites were mutated to alanine in ddc1-M3 and ddc1-M8 mutant strains, respectively. The ddc1-M11 mutant strain contains a combination of all of these mutations. **(B)** Strains YLDN25 (WT), YLDN17 (*ddc1-M3*), YLDN23 (*ddc1-M8*), and YLDN24 (*ddc1-M11*) were arrested with nocodazole and either UV irradiated (75 J/m2) or mock treated. Protein extracts prepared immediately after UV treatment were separated by SDS-PAGE and analyzed with anti-Ddc1 antibodies. **(C)** Strains YLDN25 (WT), YLDN17 (*ddc1-M3*), YLDN23 (*ddc1-M8*), YLDN24 (*ddc1-M11*), YFP27 (*dot1Δ*), YFP28 (*dot1Δddc1-M3*), YFP29 (*dot1Δddc1-M8*), and YFP30 (*dot1Δddc1-M11*) were arrested with nocodazole and either UV irradiated (75 J/m2) or mock treated. Rad9 and Rad53 phosphorylations were analyzed 30 min after irradiation. A protein extract from YMIC4E8 (rad9Δ) was loaded onto the same gel in order to identify the anti-Rad9 cross-reacting band, indicated by an asterisk. **(D)** In order to measure sensitivity to UV irradiation, 10-fold serial dilutions of overnight cultures of the strains from panel C and strain YFP152 (*ddc1Δ*) were spotted onto plates, which were then either mock or UV irradiated. Images of the plates were taken after 3 days to assess cell survival.

Ddc1 T602 phosphorylation allows checkpoint activation in the absence of H3-^{me}K79

In order to gain more insight into the mechanism of this pathway, we investigated the individual roles of the putative Mec1-dependent phosphorylation sites by testing the effect of the mutation of each site on the activation of Rad9. For this purpose, we combined *dot1Δ* with *ddc1* mutant alleles carrying different serine/threonine-to-alanine point mutations in each of the eight Mec1 target sites and monitored the activation of Rad53 and the phosphorylation of Rad9 after UV irradiation.

With this analysis, we were able to determine that threonine 602 is the critical residue for the function of Ddc1 in this pathway. In fact, FIGURE 4/A shows that, when arrested in M phase and UV irradiated, *ddc1-T602A* has the same synthetic effect, in combination with *dot1Δ*, as the one displayed by *ddc1-M8*; this is the only mutation, of the eight that were tested, which was able to abolish the residual Rad53 phosphorylation and to prevent Rad9 phosphorylation in a *dot1Δ* mutant cell (FIGURE 4/A AND DATA NOT SHOWN).

To prove that this synthetic effect is indeed due to a loss of Ddc1 T602 phosphorylation, we analysed both a putative phospho-mimicking mutant (*ddc1-T602E*) and a mutation that restores a different phosphorylatable residue (*ddc1-T602S*). In FIGURE 4/B it is shown that the T602E mutation is not able to sustain the Dot1-independent Rad53 phosphorylation pathway, indicating that, as it often happens, glutamic acid does not efficiently mimic the presence of a phosphorylated residue. On the contrary, the *dot1Δddc1-T602S* double mutant and the *dot1Δ* single mutant display a similar level of Rad53 phosphorylation, which means that *ddc1-T602S* mutation can almost completely rescue the defect observed in the *ddc1-T602A* mutant. These observations indicates that suggest that Dpb11-mediated recruitment of Rad9 requires Mec1 to phosphorylate Ddc1 on threonine 602.

The notion that phosphorylation of Ddc1 on threonine 602 and Dpb11 act in the same pathway is also supported by the fact that *ddc1-T602A* and *dpb11-1* are in the same epistasis group for what concern DNA damage-induced Rad53 activation. In fact, combining the *ddc1-T602A* and *dpb11-1* mutations does not cause any aggravation in the ability to phosphorylate Rad53 after UV damage (FIGURE 4/C).

Moreover these two mutations are epistatic also for sensitivity to UV irradiation: as it is shown in FIGURE 4/D, the *ddc1-T602Adpb11-1* double mutant is as sensitive to UV as either single mutants,

while a combination of *dot1Δ* with either *ddc1-T602A* or *dpb11-1* is more sensitive than any single mutant and as sensitive as the *dot1Δddc1-T602Adpb11-1* triple mutant.

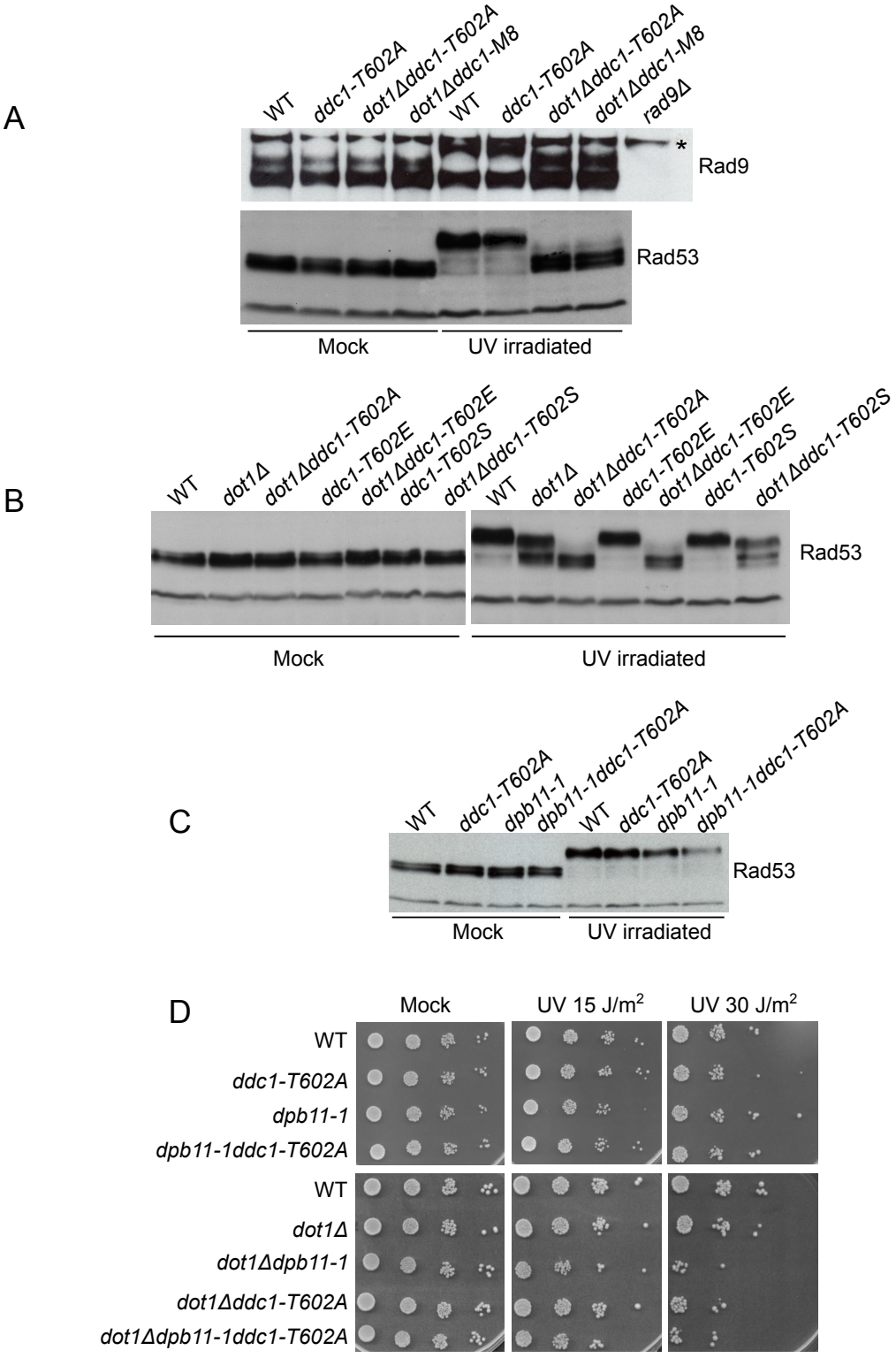

Figure 4. Ddc1 T602 phosphorylation allows checkpoint activation without H3-K79

(A) Strains YLDN25 (WT), YLDN9 (*ddc1-T602A*), YFP37 (*dot1Δddc1-T602A*) and YFP29 (*dot1Δddc1-M8*) were arrested with nocodazole and subjected to UV irradiation (75 J/m2) or mock treated. Rad53 phosphorylation was analyzed 30 min after UV treatment. A protein extract from strain YMIC4E8 (*rad9Δ*) was loaded onto the same gel in order to identify the anti-Rad9 cross-reacting band, indicated by an asterisk. **(B)** Strains YLDN25 (WT), YFP27 (*dot1Δ*), YFP37 (*dot1Δddc1-T602A*), YFP146 (*ddc1-T602E*), YFP147 (*dot1Δddc1-T602E*), YFP148 (*ddc1-T602S*) and YFP149 (*dot1Δddc1-T602S*) were arrested in M phase with nocodazole and either UV irradiated (75 J/m2) or mock treated. Rad53 phosphorylation was analyzed 30 min after treatment. **(C)** Strain YFP63 (WT), YFP64 (*ddc1-T602A*), YFP65 (*dpb11-1*), and YFP66 (*dpb11-1ddc1-T602A*) cells were arrested with nocodazole and UV irradiated. Rad53 phosphorylation was assayed 30 min after treatment. **(D)** Strains in panel C plus YFP27 (*dot1Δ*), YFP142 (*dot1Δdpb11-1*), YFP37 (*dot1Δddc1-T602A*), and YFP144 (*dot1Δdpb11-1ddc1-T602A*) were grown overnight to stationary phase, and then 10-fold dilutions were spotted onto appropriate plates and either mock treated or UV irradiated with the indicated dosages. Images were taken after 3 days to measure cell survival.

Mec1-dependent phosphorylation of Dpb11 is mediated by Ddc1-T602

Phospho-Ddc1 may be involved in recruiting Dpb11 to the lesion, bringing it close to the checkpoint kinases. We thus investigated the possibility that Dpb11 itself may be phosphorylated after DNA damage and whether this may be dependent upon phospho-Ddc1. We used a myc-tagged version of Dpb11 which does not affect cell viability, growth, or genotoxin sensitivity (DATA NOT SHOWN). After UV irradiation of nocodazole-arrested cells, we detected a modification of Dpb11 which is induced by DNA damage and is dependent upon Mec1 kinase and Ddc1; interestingly, under these experimental conditions, Rad53 also seems to play a partial role in this modification (FIGURE 5/A).

The data presented in FIGURE 5/A show that in cells with a *ddc1-T602A* phosphorylation site mutation, the DNA damage-induced modification of Dpb11 described above is greatly reduced. The effect of *ddc1-T602A* is even more evident when using a gel that takes advantage of Phos-tag technology, which is designed to retard the mobility of phosphorylated proteins (FIGURE 7/B). The defective Dpb11 phosphorylation detected in this mutant background can be explained if phosphorylation of Ddc1-T602 is required to recruit Dpb11 in the vicinity of the lesion. Consistent with this hypothesis, the interaction between Dpb11 and Ddc1 requires Mec1 activity.

The physical interaction between these two factors has been previously shown by using a two-hybrid assay and glutathione S-transferase pull-down experiments, while it seems to be undetectable by co-immunoprecipitation (WANG and ELLEDGE, 2002). We confirmed these findings and tested whether the interaction between Dpb11 and Ddc1 was dependent upon Mec1 kinase by performing two-hybrid experiments with yeast cells carrying a WT or a *mec1-1* mutant allele and expressing either full-length Ddc1 or a Ddc1 C-terminal fragment (amino acids 309–612).

FIGURE 7/C shows that a strong positive interaction signal can be detected in WT cells expressing both the full-length and truncated Ddc1 versions; on the other hand, this interaction is lost in a *mec1-1* mutant. When we tried a two-hybrid experiment with a Ddc1-T602A construct, we could not detect any effect on the interaction (NOT SHOWN). We then tested the interaction between Dpb11 and a Ddc1 mutant (*ddc1-M8*) lacking eight consensus sites for Mec1-dependent phosphorylation. FIGURE 7/D shows that under these conditions, the interaction is somewhat reduced, albeit not completely abolished, suggesting that, at least under the experimental conditions

of a two-hybrid experiment, there may be some other protein, perhaps Dpb11 itself, that is targeted by Mec1 kinase and plays a role in the interaction between Ddc1 and Dpb11. Moreover, even in the absence of Ddc1 phosphorylation, the highly expressed bait and prey can produce enough hybrid molecules to activate the reporter genes.

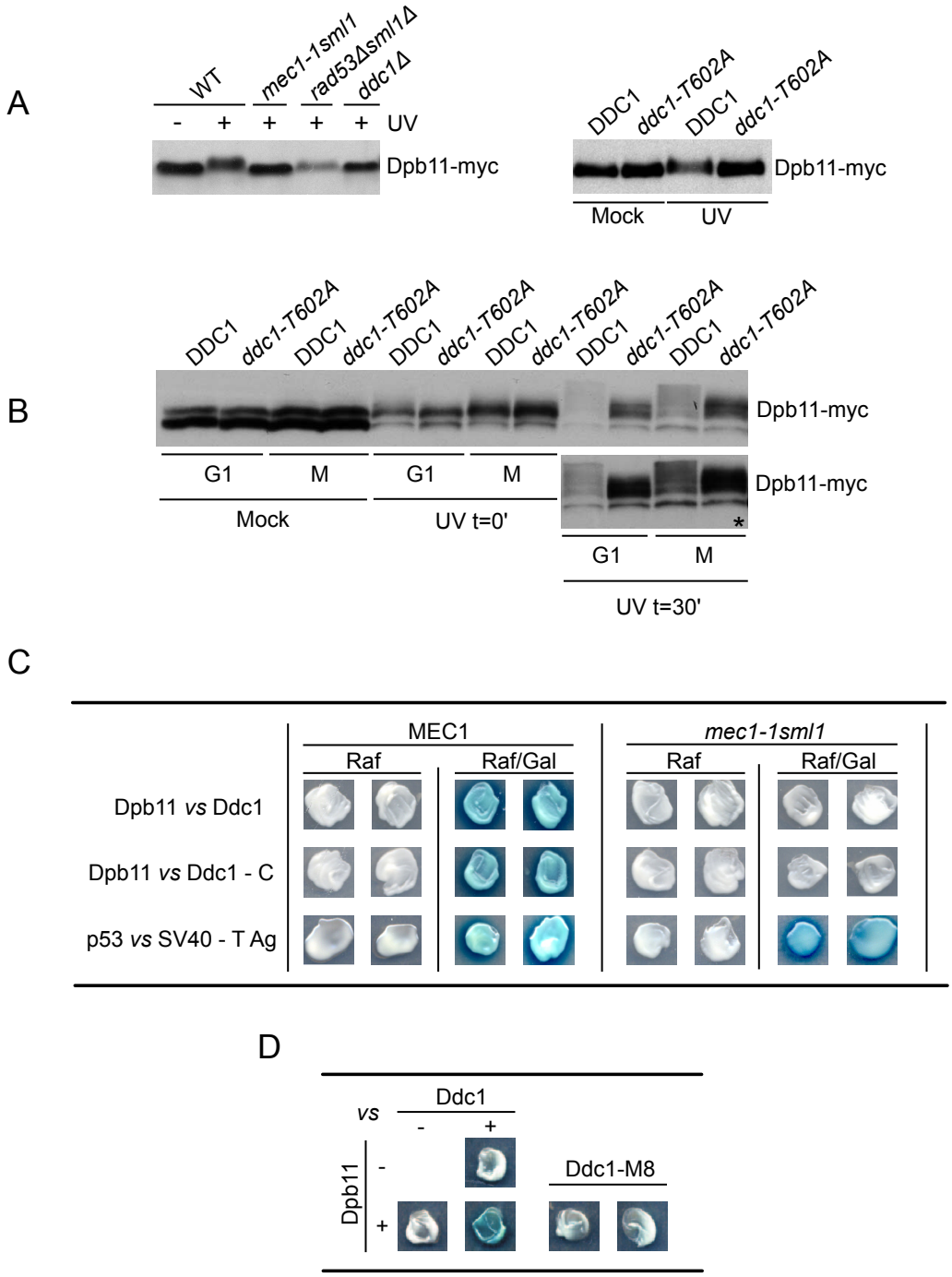

Figure 5. Mec1-dependent phosphorylation of Dpb11 is mediated by Ddc1-T602.

(A) Strains YFP38 (WT), YFP48/3a (*mec1-1sml1*), YFP49/1d (*rad53Δsml1Δ*), YFP55/6c (*ddc1Δ*), YFP63 (*DDC1*) and YFP64 (*ddc1-T602A*), all expressing a myc-tagged Dpb11 protein, were blocked in nocodazole and UV irradiated (75 J/m2). Dpb11 phosphorylation was assessed 30 min after UV irradiation by SDS-PAGE and Western blotting. **(B)** The indicated strains were arrested in either α-factor (G1) or nocodazole (M) and UV treated. Protein extracts prepared immediately (t=0') or 30 min (t=30') after UV irradiation were separated on Phos tag-conjugated acrylamide gels as described in Materials and Methods. **(C-D)** Plasmids pFP1 (pJG4-5-*DPB11*) and pFP2 (pEG202-*DDC1*) were cotransformed with pSH18-34, a β-galactosidase reporter plasmid, in either MEC1 or *mec1-1* mutant yeast cells. A similar strategy was adopted for pFP4 (pEG202-*ddc1-C*), which carries only the C-terminal fragment (nucleotides 309 to 612) of Ddc1, containing the 8 putative Mec1 phosphorylation target sites and for pFP10 (pEG202-*ddc1M8*). To assess two-hybrid interaction, these strains were patched onto 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates containing either raffinose (Raf: Dpb11 prey repressed) or galactose (Gal: Dpb11 prey expressed) as a carbon source. After 3 days, the plates were analyzed. The strains in panel D are YFP50 (*MEC1*, top), YFP52 (*MEC1*, middle), YFP113 (*mec1-1*, top), and YFP114 (*mec1-1*, middle). A positive control (bottom) p53 versus SV40 large T antigen was also used. The strains in panel D are, from left to right, YFP50, YFP86 (top), YFP54 (bottom), and YFP153 (two independent clones).

5

Dpb11 promotes Mec1 activation after replication stress

Dpb11 & Ddc1 are both required for Mec1 activation after replication stress

Many reports demonstrated that one role of Dpb11/TopBP1 in checkpoint activation is to stimulate Mec1/ATR kinase activity; this function is mediated by the ATR activation domain, which is lacking in the protein encoded by the *dpb11-1* allele (Mordes *et al.*, 2008b; Navadgi-Patil and Burgers, 2008). Since in our hands this mutant did not apparently showed any defect in Mec1 activation, we decided to better characterise the role of Dpb11 in the activation of the apical kinase. We performed this task by studying the cell response to hydroxyurea (HU). After HU-induced replication fork stalling, Mec1 becomes active and signals replication stress to Rad53, independently of the presence of Rad9 (Pellicioli *et al.*, 1999). This fact allows us, while studying the function of Dpb11 in Mec1 activation, without the influence of its role in recruiting Rad9 to chromatin.

In vitro studies demonstrated that two factors are able to stimulate in vitro Mec1 kinase activity: the PCNA-like complex and Dpb11 (Majka *et al.*, 2006b; Mordes *et al.*, 2008b). In order to assess the *in vivo* relationships within this two actors we analysed checkpoint activation in WT, *ddc1Δ*, *dpb11-1* and in the double mutant *ddc1Δdpb11-1*. These strains were synchronised in G1, released in HU-containing medium and Rad53 phosphorylation was assayed at different time points after release. In a WT strain Rad53 becomes fully phosphorylated 30 minutes after G1 release, in concomitance with the entrance into S phase, as monitored by bud emergence (Figure 6/a and

DATA NOT SHOWN). We observed that the two single mutations showed only a minor defect in Rad53 activation, but when combined together, a synthetic effect could be detected. Indeed the level of Rad53 phosphorylation in *ddc1Δdpb11-1* is very low, even 90 minutes after release, and it is similar to the one observed in *mec1-1* control strain (FIGURE 6/A).

To exclude the possibility that the synthetic effect on Rad53 phosphorylation was due to a defective entry into S phase, the same G1 synchronised cultures were released in fresh medium without HU, and progression into cell cycle was monitored by FACS analysis. FIGURE 6/B shows that a strain carrying the *dpb11-1* allele delays entrance into S phase about 20 minutes with respect to *DPB11* cells, and exhibits a slower S phase, possibly because of a defect in the firing of replication origins. Since *dpb11-1* and *ddc1Δdpb11-1* display a different effect on Rad53 activation but have a very similar cell cycle kinetics, we conclude that, in this case, the former is not a consequence of the latter.

To confirm that Ddc1 and Dpb11 are working on Mec1 activation, we monitored the phosphorylation of histone H2A, which is dependent upon Mec1 and Tel1. In a wild type strain, histone H2A is not phosphorylated in an unperturbed S phase, but undergoes a phosphorylation on serine 129 in late S or G2/M phases of the cell cycle (FIGURE 6/C, TOP). Conversely, when cells are released from G1 in a medium additioned with HU, although until 90 minutes they remain arrested in early S phase (FIGURE 6/B, LAST PANEL), histone H2A becomes rapidly phosphorylated, indicating the activation of apical checkpoint kinases (FIGURE 6/C, BOTTOM). In the single mutants *ddc1Δ* and *dpb11-1* histone H2A is phosphorylated with the same kinetics and to the same extent of the WT strain. Differently, the double mutant shows both a delay in the appearance of this modification and a global reduction in its level (FIGURE 6/C, BOTTOM).

It is already known that if cells are unable to activate a proper replication checkpoint response — e.g. in the absence of the S phase adaptor Mrc1 — they are still able to phosphorylate Rad53, with the help of the Rad9 DNA damage-specific adaptor. It has thus been suggested that inability to activate replication checkpoint causes DNA damage that is sensed by the DNA damage checkpoint (ALCASABAS *et al.*, 2001). To exclude the possibility that we are observing a DNA damage response also in the *dpb11-1* strain, we decided to monitor Rad9 phosphorylation as a marker of damage checkpoint activation in S phase. WT, *ddc1Δ*, *dpb11-1*, *ddc1Δdpb11-1* and *mrc1Δ*, as a positive

control, were synchronised in G1, released in HU-containing medium and at different time points Rad9 phosphorylation was assessed. As it is shown in FIGURE 6/D neither in the single mutants, nor in the double mutant, Rad9 is hyperphosphorylated in a fashion similar to the positive control, indicating that in these strains the DNA damage checkpoint is not activated.

Altogether these data suggest that, following replication stress, the PCNA-like complex and Dpb11 cooperate for Mec1 activation and that they work independently one of another.

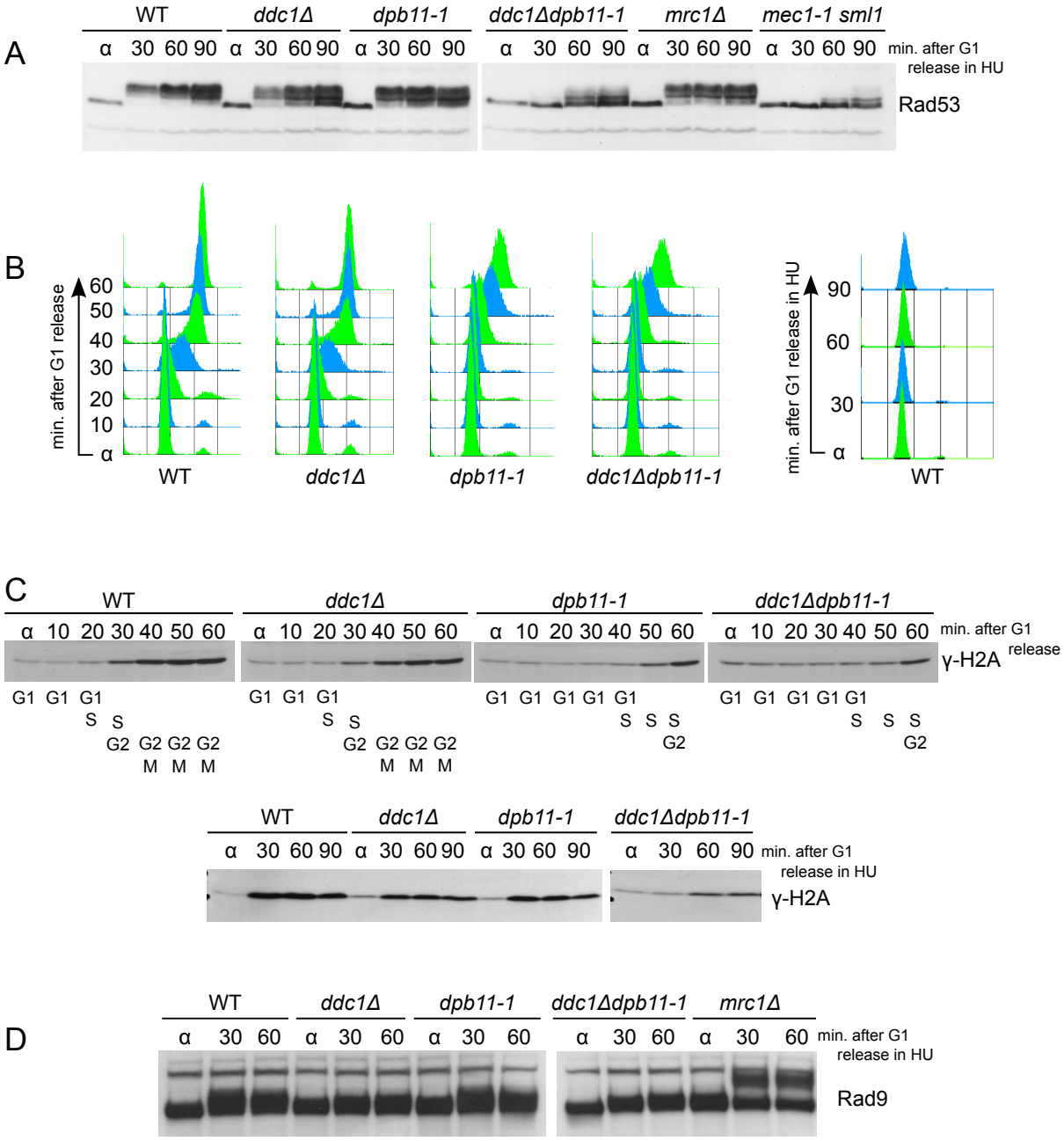

Figure 6. Dpb11 & Ddc1 are both required for Mec1 activation after replication stress.

(A) K699 (WT), YAN21/8d (*ddc1Δ*), YFP20 (*dpb11-1*), YFP62/1d (*ddc1Δdpb11-1*), YFP178/1a (*mrc1Δ*) and Y5A3 (*mec1-1sml1*) were synchronized in G1 with α-factor and then released in fresh YPD containing 200 mM hydroxyurea. 30, 60 and 90 minutes after the release protein extracts were prepared and Rad53 phosphorylation was asseyed by SDS-PAGE and western blotting. **(B)** K699 (WT), YAN21/8d (*ddc1Δ*), YFP20 (*dpb11-1*), YFP62/1d (*ddc1Δdpb11-1*) were syncronized in G1 and released in fresh YPD. Every 10 minutes progression into the cell cycle was monitored by FACS analysis. **(C)** At the same time protein extract were prepared and H2A phosphorylation was assayed using antibodies that specifically recognize phospho-serine 129 (top). The same cells were also released from G1 in YPD+200 mM HU and H2A phosphorylation was assayed 30, 60 and 90 minutes after release (bottom). **(D)** K699 (WT), YAN21/8d (*ddc1Δ*), YFP20 (*dpb11-1*), YFP62/1d (*ddc1Δdpb11-1*), YFP178/1a (*mrc1Δ*) were synchronized in G1 and release into S phase in YPD+200 mM HU. At the indicated timepoints Rad9 phosphorylation was assayed running protein extracts onto gradient Tris-Acetate gels.

Full Rad53 phosphorylation is not essential for an effective replication checkpoint

It has been widely demonstrated that Rad53 mutants lacking their kinase activity are sensitive to hydroxyurea, and that this sensitivity is due to the inability to stabilise stalled replication forks and to arrest cell cycle progression (Lopes *et al.*, 2001). Since the *ddc1Δdpb11-1* mutant appears to be greatly defective in Rad53 phosphorylation, we decided to analyse the sensitivity of the double mutant to HU. It has been previously reported that *ddc1Δ* and *dpb11-1* mutants are mildly sensitive to HU and that the double mutant displays a synergistic sensitivity (Wang and Elledge, 2002). We confirmed this results (Figure 7/A) and we determined that this sensitivity is intermediate between the one of a strain completely incapable of replication checkpoint activation (*mec1-1sml1*) and the one of a mutant lacking the replication-associated adaptor Mrc1 (*mrc1Δ*). To better characterise the inability to grow in the presence of hydroxyurea, we decided to analyse in more details the reason of this sensitivity.

The inability to stabilise stalled replication forks can be detected with the help of FACS analysis as the inability to resume DNA replication after the removal of hydroxyurea. To assess this phenotype, we synchronised in G1 wild type, *ddc1Δ*, *dpb11-1*, *ddc1Δdpb11-1* and a *mec1-1* strain used as a positive control. Cultures were released in HU for 90 minutes, allowing the cells to enter S phase and then HU was removed from the growth media and the ability to complete S phase was assayed. In these conditions a WT strain is able to restart DNA replication and completes S phase in about 80 minutes after the removal of HU (Figure 7/B); differently, a checkpoint mutant like *mec1-1* is unable to resume DNA replication and remains arrested in early S phase throughout all the experiment. The single mutants did not shown any difference from the wild type strain in their behaviour, but unexpectedly, also the double mutant *ddc1Δdpb11-1*, although being extremely defective in Rad53 phosphorylation and very sensitive to HU, was able to complete S phase with a kinetic similar to the wild type (Figure 7/B). This result indicates that the sensitivity of this strain is not due to an irreversible collapse of replication forks.

Another phenotype of checkpoint mutants is the inability to arrest cell cycle progression. Defects in Rad53 activation after replication stress correlates with premature entrance in mitosis, a phenotype that can be scored measuring the length of the mitotic spindle. To assess if *ddc1Δdpb11-1*

sensitivity was due to the inability to delay cell cycle progression we analysed by immunofluorescence spindle length in cells that were treated for 90 minutes with hydroxyurea after a G1 release. As it is shown in FIGURE 7/C after HU treatment more than 95% of wild type cells maintain short spindles (< 1.5 μm) while, in the same conditions, less than 45% of *mec1-1* cells does, with the most part of checkpoint null cells exhibiting an elongated spindle with a length between 1.5 μm and 4.5 μm . The behaviour of *ddc1Δ, dpb11-1* and the double mutant is similar to that of the wild type strain indicating that even with low levels of Rad53 phosphorylation cells are able to delay entrance into mitosis (FIGURE 7/C AND DATA NOT SHOWN). Confirming this results, the high sensitivity to HU of the *ddc1Δdpb11-1* strain is rescued if cells are subjected only to a 90 minutes pulse treatment with hydroxyurea (FIGURE 7/D).

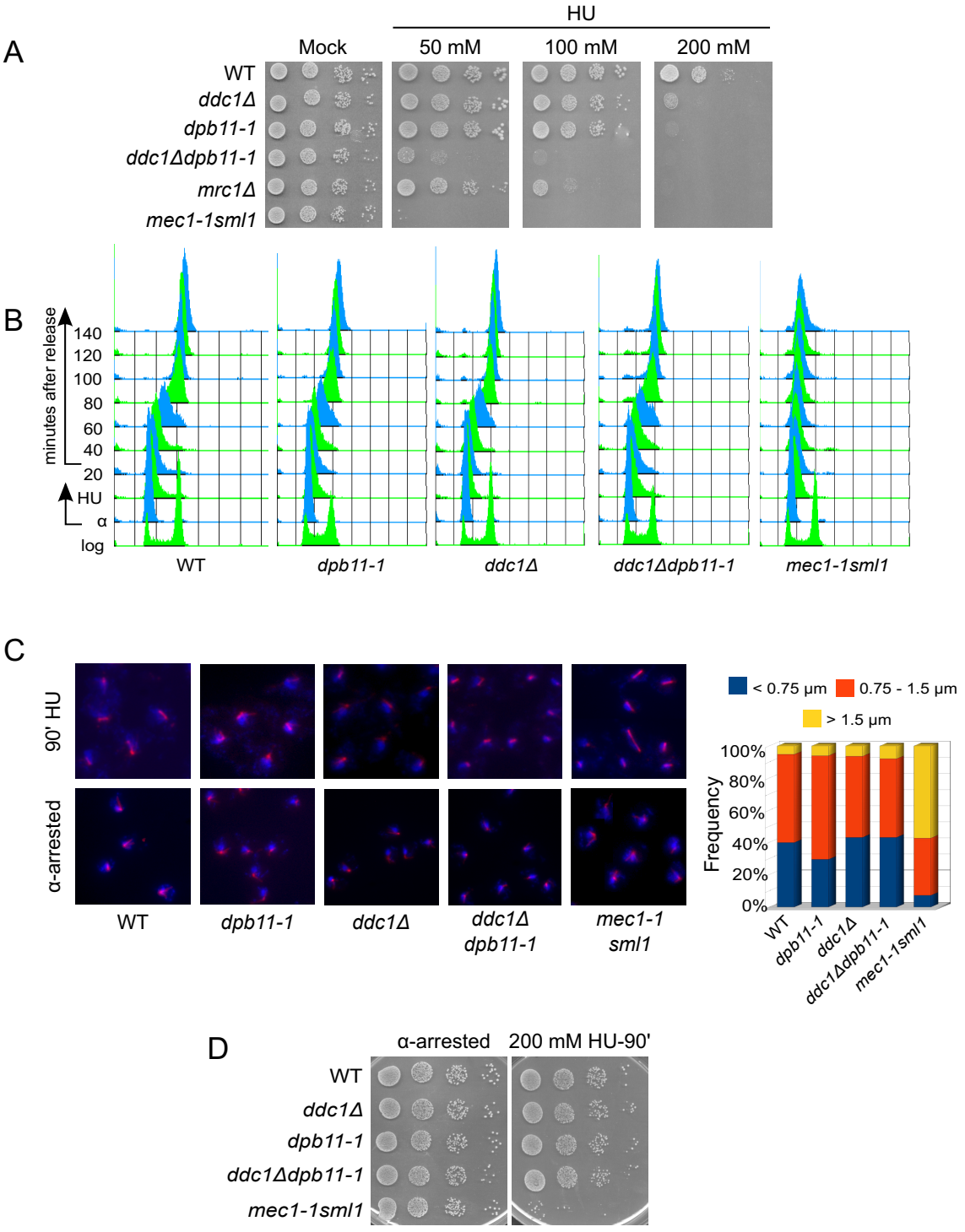

Figure 7. Full Rad53 phosphorylation is not essential for an effective replication checkpoint

(A) K699 (WT), YAN21/8d (*ddc1Δ*), YFP20 (*dpb11-1*), YFP62/1d (*ddc1Δdpb11-1*), YFP178/1a (*mrc1Δ*) and Y5A3 (*mec1-1sml1*) strains were grown overnight to stationary phase. 10 fold serial dilutions were prepared and spotted on YPD (Mock) or YPD+HU plates at the indicated concentrations. Survival was assessed after two to six days. **(B)** The indicated strains were synchronized in G1 with α-factor, released in 200 mM HU for 90 minutes and then released from the HU block in YPD + nocodazole. Every 20 minutes progression into S phase was followed by FACS scan analysis. **(C)** Cells from the same experiment were taken either from α-factor arrested samples or immediately before the release from the HU block, fixed and processed for immunofluorescence. DNA was stained with DAPI (blue) and the mitotic spindle was immunostained with α-tubulin primary antibodies and Alexa594 secondary antibodies (red). On the left are reported sample images from the immunofluorescence and on the right a quantification of cells belonging to each spindle length class 90 minutes after release in HU is shown. **(D)** Samples in the same conditions were also 10-fold serial dilluted and spotted onto YPD plates. Survival was assessed three days later.

Drc1 and Polε are required for Ddc1-independent Rad53 phosphorylation

If, as we hypothesise, PCNA-like and Dpb11 constitutes two different activation pathways for the apical kinase, they should be recruited in the proximity of ssDNA-bound Mec1. It has been demonstrated, both *in vitro* and *in vivo*, that the PCNA-like complex is loaded onto a 5' DNA primer-template junction in a reaction mediated by the RFC-like complex (Green *et al.*, 2000; Melo *et al.*, 2001; Majka and Burgers, 2003; Majka *et al.*, 2006a). On the other hand the requirements for Dpb11 function has not been thoroughly investigated.

Two other factors were demonstrated to be involved in sensing the replication stress: DNA polymerase ε (Navas *et al.*, 1995) and Drc1/Sld2 (Wang and Elledge, 1999). In order to obtain a better insight into the requirements for the function of Dpb11 in Mec1 activation and to establish its relationship with Pol ε, we deleted Dpb4, whose functions are currently unknown and is one of the two non-essential subunits of DNA polymerase ε.

dpb4Δ strains grow normally both at 25°C and at 37°C, but when combined with the deletion of *DDC1* we observed an unexpected synthetic thermosensitivity (Figure 8/A). For this reason, we carried out all the subsequent experiments at 25°C. As it is shown in Figure 8/B, *dpb4Δ* strain is mildly sensitive to hydroxyurea and only at high dosages: a behaviour similar to the one of a *ddc1Δ* strain. Conversely, the double mutant *dpb4Δddc1Δ* is extremely sensitive also at lower concentrations of HU and its sensitivity closely resembles that of a *ddc1Δdpb11-1* strain.

To understand if this synthetic sensitivity was correlated with a defect in Rad53 phosphorylation, as it happens in *ddc1Δdpb11-1,* we synchronised in G1 WT, *ddc1Δ*, *dpb4Δ*, *ddc1Δdpb4Δ* and *ddc1Δdpb11-1* cultures. Cells were then released in a medium containing HU and Rad53 phosphorylation was assayed at different time points after release. We observed that the single mutants showed only a partial defect in Rad53 phosphorylation, whereas the level of hyper-phosphorylated Rad53 in the double mutant *ddc1Δdpb4Δ* resembles the one of the *ddc1Δdpb11-1* positive control, indicating that, also in this case, the inability to grow in the presence of hydroxyurea correlates with a defect in checkpoint signalling (Figure 8/C, Top). Moreover the absence of Dpb4 does not causes an HU-induced activation of the DNA damage checkpoint, as it is demonstrated by the lack of Rad9 hyper-phosphorylation (Figure 8/C, bottom).

To understand if the Rad53 activation defect was due to the inability to activate Mec1 we also monitored the levels of S129-phosphorylated histone H2A (γ-H2A). As it is shown in FIGURE 8/C the single *ddc1Δ* and *dpb4Δ* mutants displayed a level of γ-H2A that is similar to the wild type. Phosphorylation of histone H2A in *ddc1Δdpb4Δ* is greatly defective, similarly to that of *ddc1Δdpb11-1* (FIGURE 8/C, MIDDLE).

During S phase, Cdk1-dependent phosphorylation of Drc1/Sld2 induces the formation of a complex between Drc1/Sld2 itself and Dpb11, which is required for the firing of replication origins (TAK *et al.*, 2006; ZEGERMAN and DIFFLEY, 2007). Moreover it has been demonstrated that, like the *dpb11-1* mutant, *drc1-1* cells are unable to delay mitosis if treated with hydroxyurea at non-permissive temperature (36°C): a condition in which both mutants cannot sustain DNA replication (WANG and ELLEDGE, 1999; ARAKI *et al.*, 1995). Since our experiments with *dpb11-1* were performed at 25°C, where replication is allowed, we decided to determine if Drc1 works with Dpb11 in supporting full checkpoint activation, in a condition that does not impair replication itself.

For this reason we analysed the sensitivity and the phosphorylation state of checkpoint proteins in the double mutant *ddc1Δdrc1-1*. As it is shown in FIGURE 8/D, mutations in *DRC1* and *DDC1* are synergic for what concern sensitivity to hydroxyurea, although the *drc1-1* mutant is by itself more sensitive than the wild type at elevated concentrations of HU. Moreover also in this case this sensitivity correlates with a synthetic defect on Rad53 phosphorylation (FIGURE 8/E), suggesting that, as expected, the defect in checkpoint signalling of the *drc1-1* mutant reflects a defect in the Dpb11-dependent activation of Mec1.

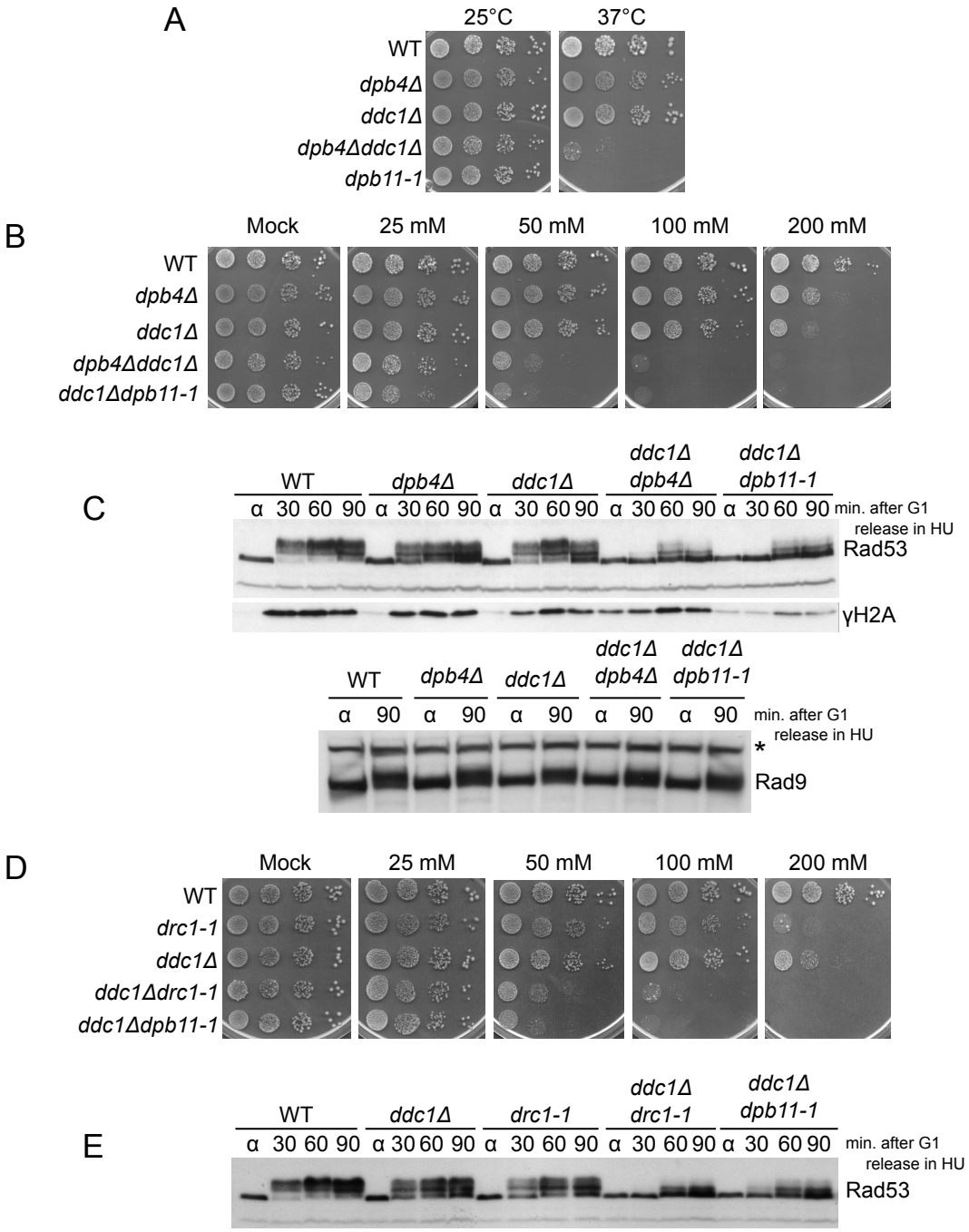

Figure 8. Drc1 and Polε are required for Ddc1-independent Rad53 phosphorylation

(A) K699 (WT), YFP167/1a (*dpb4Δ*), YAN21/8d (*ddc1Δ*), YFP206/1a (*ddc1Δdpb4Δ*) and YFP20 (*dpb11-1*) as positive control were grown overnight to stationary phase. 10-fold serial dilutions were then prepared and spotted onto YPD plates and allowed to grow at different temperatures. Images were taken two days later. **(B)** The same strains plus YFP62/1d (*ddc1Δdpb11-1*) were treated in the same conditions and spotted onto YPD or YPD + HU plates. Survival was assayed from three to six days later. **(C)** K699 (WT), YFP167/1a (*dpb4Δ*), YAN21/8d (*ddc1Δ*), YFP206/1a (*ddc1Δdpb4Δ*) and YFP62/1d (*ddc1Δdpb11-1*) were grown overnight to mid log phase, synchronized in G1 with α-factor and released in YPD+200mM HU. 30, 60 and 90 minutes after the release Rad53 and H2A phosphorylation was assayed. Rad9 phosphorylation was also analyzed in extracts from the α and 90' points; the backgroung cross-reacting band is indicated with an asterisk. **(D)** K699 (WT), Y799 (*drc1-1*), YAN21/8d (*ddc1Δ*), YFP218/1a (*ddc1Δdrc1-1*) and YFP62/1d (*ddc1Δdpb11-1*) were grown overnight to stationary phase. 10-fold serial dilutions were then prepared and spotted onto YPD or YPD+HU plates and allowed to grow. Images were taken three to six days later. **(E)** The same strains were grown overnight to mid log phase, synchronized in G1 with α-factor and released in YPD+200mM HU. 30, 60 and 90 minutes after the release Rad53 phosphorylation was assayed.

Part III

Discussion

Dpb11 acts as an adaptor during the DNA damage checkpoint response

Loss of genome integrity is a hallmark of cancer cells, and maintenance of genome stability is fundamental to the prevention of tumour development (Kastan and Bartek, 2004). Eukaryotic cells possess a set of complex pathways devoted to monitoring the presence of different kinds of genomic lesions and signalling their presence to downstream effectors. The output of these checkpoint pathways is cell cycle arrest, DNA repair, modifications of the transcriptional program, and apoptosis (McGowan and Russell, 2004).

The DNA damage checkpoint pathways are triggered by the activity of apical phosphoinositide-3-kinase-like kinases, namely, Mec1 and Tel1 in budding yeast and ATM and ATR in higher eukaryotes. ATM is recruited to DSBs through the Mre11-Rad50-Nbs1 (MRN) complex, while the ATR/ATRIP heterodimer (Mec1/ Ddc2 in budding yeast) is recruited by RPA-covered ssDNA filaments generated after nucleolytic processing of damaged DNA (Zou and Elledge, 2003). The order of function of the players in the checkpoint signal transduction cascade has been defined by monitoring the phosphorylation status of individual proteins. The availability of yeast mutants affected in different factors has greatly aided in this task (Carr, 2002; Longhese *et al.*, 1998).

In budding yeast, once Mec1 kinase has been brought onto damaged DNA, it phosphorylates a series of targets, among which are Ddc2, the Ddc1 subunit of the 9-1-1 complex, the Rad9 mediator, and the Rad53 and Chk1 downstream kinases (Longhese *et al.*, 1998; Melo and Toczyski, 2002).

Phosphorylation of Rad9, an event that is necessary to relay the signal to the downstream effectors, is strongly influenced by histone modifications. Indeed, monoubiquitination of H2B and methylation of H3 on lysine 79 are required for Rad9 phosphorylation and checkpoint activation in the G1 phase of the cell cycle, while they have only a partial role in the G2/M checkpoint response, which in budding yeast arrests the cell cycle at the metaphase to anaphase transition.

The mechanism through which histones contribute to Rad9 activation seems to involve the recognition of methylated H3-K79 by the Tudor domain of Rad9, which helps bringing Rad9 into proximity to the active Mec1 kinase (Giannattasio *et al.*, 2005; Grenon *et al.*, 2007; Hammet *et al.*, 2007; Wysocki *et al.*, 2005). A similar pathway has been described in fission yeast and in higher eukaryotes (Botuyan *et al.*, 2006; Du *et al.*, 2006; Huyen *et al.*, 2004; Sanders *et al.*,

2004).

Given the facts that the G2/M checkpoint response is still functional in cells lacking the histone H3-K79 methyltransferase Dot1 and that Rad9 is still highly hyperphosphorylated after UV irradiation of M-phase-arrested *dot1Δ* cells (Giannattasio *et al.*, 2005; Wysocki *et al.*, 2005), a parallel, partially redundant pathway leading to the recruitment of Rad9 to damaged chromatin must exist in later stages of the cell cycle.

We analysed in more detail the signalling after UV irradiation of M-phase-arrested *dot1Δ* cells and showed that the residual phosphorylation of Rad9 and Rad53 in this mutant is still dependent upon Mec1 kinase and independent of Tel1 or Chk1 checkpoint kinases. One possible mechanism for recruiting Rad9 to damaged chromatin in the absence of H3-K79 methylation could involve the modification of some other histone residues. We tested the contribution of histone H2A phosphorylation on serine 129, which has been shown to be relevant for Rad9 phosphorylation in G1 cells (Hammet *et al.*, 2007), and we confirmed that in G2 this histone modification plays a minor role (Javaheri *et al.*, 2006; Toh *et al.*, 2006; Hammet *et al.*, 2007).

Evidence coming from other eukaryotic systems has suggested a role in the DNA damage checkpoint for Dpb11 (Rad4/Cut5 in S. pombe and TopBP1 in higher eukaryotes). This factor plays different roles in DNA metabolic processes (reviewed in Garcia *et al.*, 2005), particularly in DNA replication. Moreover, TopBP1 can also interact with the 9-1-1 checkpoint clamp (Delacroix *et al.*, 2007; Lee *et al.*, 2007). In *S.pombe*, Rad4/Cut5 cooperates in the activation of Chk1 by interacting with the 9-1-1 complex and, in the absence of H2A C-terminal phosphorylation and H4-K20 methylation, it is involved in accumulating the Crb2 mediator at a single persistent DSB. These functions of Rad4/Cut5 are modulated by protein phosphorylation events (Du *et al.*, 2006; Furuya *et al.*, 2004).

We combined a *dpb11-1* allele with a deletion of *DOT1* and analysed the DNA damage checkpoint response after UV irradiation of M-phase-arrested cells. Our results show that, after treatment with UV or induction of DSBs, *dpb11-1* by itself has no major effects on cellular survival; on Ddc2, Rad9, and Rad53 phosphorylation; or on G2/M checkpoint arrest.

On the other hand, when *dpb11-1* is combined with a *dot1Δ* allele, the G2/M checkpoint is not functional and cells become quite sensitive to UV irradiation and the DNA damage-dependent

phosphorylation of Rad9 and Rad53 is abolished, while Mec1 activity does not seem to be significantly reduced. These data can be explained if, in the absence of H3-K79 methylation, Rad9 can be recruited to the kinase through a Dpb11-dependent pathway.

We next demonstrated that, after UV damage, Rad9 persistence onto chromatin is almost completely dependent upon H3-^{me}K79, and that *dpb11-1* mutation is able to abrogate the residual binding observed in *dot1Δ* cells. The fact that *dot1Δ* and *dpb11-1* mutants display a similar level of Rad53 phosphorylation, but in one case Rad9 is present in the chromatin-enriched fraction and in the other case it is absent, can be explained if Dpb11 holds Rad9 near the Mec1 kinase less tightly than the H3-K79 does.

How does Dpb11 mediate Rad9 recruitment and hyper-phosphorylation? Using a version of Rad9 which lacks the first 231 amino acids acids — the Chk1 Activation Domain — we were able to demonstrate that this region of Rad9 is involved in the Dot1-independent pathway for Rad53 phosphorylation. In fission yeast, the interaction between the two orthologous factors depends upon the activity of Cdk1 (Du *et al.*, 2006) and the N-terminus of Rad9 contain at least three consensus sites for Cdc28, *S.cerevisiae* most important CdK. Moreover, Dpb11 contains four BRCT domains, which are phospho-residues binding motifs and if the interaction of Dpb11 with Rad9 is regulated by the binding of BRCTs to a residue phosphorylated by CdK on Rad9, this would give an explanation of why this pathway is predominant in G2/M cells and it is absent in G1 cells.

Dpb11 has been reported to interact with the Ddc1 subunit of the 9-1-1 complex (Wang and Elledge, 2002; Ogiwara *et al.*, 2006). In order to investigate the molecular details of this pathway, we analysed a collection of *DDC1* mutants. Ddc1 sequence analysis revealed the presence of eight consensus sites for Mec1-dependent phosphorylation and three consensus sites for Cdc28-dependent phosphorylation; accordingly, Ddc1 has been reported to be phosphorylated in a cell cycle- and DNA damage-dependent manner (Longhese *et al.*, 1997; Paciotti *et al.*, 1998).

We generated a *ddc1-M3* allele lacking the three Cdk1 sites, a *ddc1-M8* version lacking the consensus sites for Mec1 kinase-dependent phosphorylation, and *ddc1-M11*, where all putative phosphorylation sites have been mutated. Both *ddc1-M8* and *ddc1-M11* have lost the DNA damage-dependent phosphorylation of Ddc1. While these mutations, by themselves, do not visibly affect the checkpoint response to DNA damage, when combined with *dot1Δ*, these mutants also eliminate

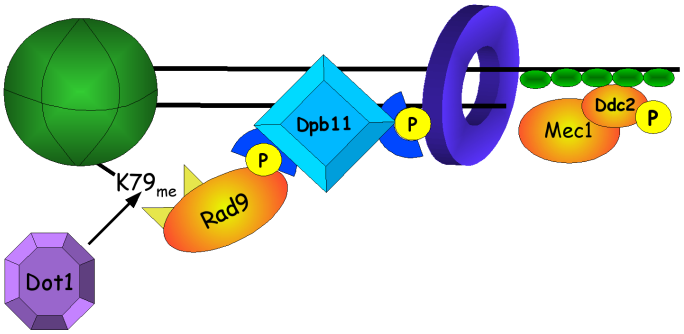

Figure 5.1: Model of the function of Dpb11 after UV irradiation in M phase: the blue ring represents the PCNA-like complex and the small green ovals are RPA.

the UV-induced phosphorylation of Rad9 and Rad53 and displayed a synthetic lethality after UV irradiation.

Moreover this phenotype can be recapitulated by the single *ddc1-T602A* mutation and strongly resembles the phenotype encoded in the *dpb11-1* mutation, which has been described above. Consistently, *ddc1-T602A* and *dpb11-1* appear to be in the same epistasis group for both Rad53 phosphorylation and DNA damage sensitivity, which suggest the hypothesis that phosphorylation of Ddc1-T602 by Mec1 provides a means to recruit Dpb11 and that the physical interaction between Dpb11 and Ddc1 requires functional Mec1.

To provide further evidences of this model we analysed Dpb11 protein by western blotting and we determined that Dpb11 undergoes a DNA damage-dependent and Mec1-dependent mobility shift on SDS-PAGE that increases by using a technology specifically designed to retard the mobility of phosphorylated proteins. Despite at the moment we do not know the functional significance of this modification, which is likely a phosphorylation event, the fact that it appears to be greatly reduced in a *ddc1-T602A* mutant strain, suggest that phosphorylation of T602 is an event that promote either the recruitment of Dpb11 or its phosphorylation by Mec1.

Altogether, our data support a model (represented in figure 5.2) in which UV-induced lesions activate the checkpoint cascade, likely by bringing Mec1 to RPA-covered ssDNA via a Ddc2-RPA

interaction. Loading of the PCNA-like complex and the subsequent phosphorylation of Ddc1 by the Mec1 kinase, allows recruitment of Dpb11 to chromatin, where it can be phosphorylated by Mec1. Dpb11 cooperates with methylated H3-K79 to allow a proper recruitment and phosphorylation of Rad9 to damaged chromatin, allowing signal amplification and a complete response to DNA damage.

Dpb11 promotes Mec1 activation after replication stress

Apical kinases ATM and ATR convert a structural signal coming from damaged DNA to a phosphorylation based signalling cascade. A lot of work has been done to understand how they perform this task, but the attention has mostly been concentrated on the physical recruitment of these kinases to the damaged DNA (Zou and Elledge, 2003), suggesting that their binding to the site of damage would turn them active.

More recently, the identification of proteins — among which Dpb11/TopBP1 — that are able to stimulate Mec1 activity in the presence or even in the absence of DNA, indicated that a more complex game is taking place around the damaged DNA (Majka *et al.*, 2006b; Mordes *et al.*, 2008b; Navadgi-Patil and Burgers, 2008).

The experiments performed with the *dpb11-1* allele did not indicate defective activation of Mec1 kinase following UV damage, in contrast to the *in vitro* data obtained with Xenopus egg extracts and in mammalian cells (Kumagai *et al.*, 2006; Mordes *et al.*, 2008a). This could be due to a TopBP1 function which is specific for higher eukaryotes, but evidence accumulated in the last years suggested that an interaction between Rad4/Cut5 and the checkpoint sensor kinase Rad3-Rad26 also exists in *S. pombe* (Furuya *et al.*, 2004; Taricani and Wang, 2006). Indeed, it has been recently demonstrated that also Dpb11 contains an ATR activation domain, that this domain is sufficient for Mec1 activation *in vitro* and that a C-terminus truncated version of Dpb11, similar to the *dpb11-1* allele, does not displays this *in vitro* activity (Mordes *et al.*, 2008b; Navadgi-Patil and Burgers, 2008).

Given these facts, it is even more difficult to understand why, in a *dpb11-1* mutant, Mec1 can still phosphorylate Ddc2 after UV damage in M phase. At least two, non-mutually exclusive, explanations are possible: the first is that Ddc2 is too sensitive to be used as marker of Mec1 kinase activity and in *dpb11-1* mutants Mec1 activity is sufficiently high to phosphorylate Ddc2, while is

defective towards other checkpoint targets; the second is that after UV damage, in M phase cells, Dpb11 may play only a marginal role in Mec1 activation.

We favour this second hypothesis because *dpb11-1* strains are not sensitive to UV irradiation, are proficient in a G2/M checkpoint assay and because, in yeast, the PCNA-like complex has also been identified as a putative activator of Mec1 *in vitro* (Majka *et al.*, 2006b). If this hypothesis is correct, Dpb11 could play its role in Mec1 activation in response to a different kind of damage or its function could be regulated during the cell cycle, as it is suggested by the fact that a defect in Ddc2 phosphorylation, after DSBs, can indeed be observed in G1-arrested *dpb11-1* cells (Data not shown). Moreover it has been demonstrated that *dpb11-1* thermosensitive mutant is incapable of checkpoint activation after a treatment that induces replication stress at 36°C, but is only mildly sensitive to the same treatment at the permissive temperature (Araki *et al.*, 1995; Wang and Elledge, 1999). To obtain a clearer understanding of the process of Mec1 activation as a result of replication stress we decided to assess the relative functions of Dpb11 and of the other putative Mec1 activator, the PCNA-like complex, in response to hydroxyurea treatment.

Hydroxyurea induces replication stress by reducing the cellular concentration of deoxyribonucleotides available for DNA synthesis. In order to grow in these conditions, yeast cells activate a checkpoint cascade known as the replication checkpoint, in which Mec1 kinase phosphorylates the adaptor Mrc1 — a constitutive member of the replisome progression complex — which recruits Rad53 and facilitates its phosphorylation; such task is executed by Rad9 in the DNA damage response (Alcasabas *et al.*, 2001; Chen and Zhou, 2009). The fact that Rad9 is not required for Rad53 phosphorylation after HU treatment allows us also to study the effect of the *dpb11-1* mutation on Mec1 activation by looking at Rad53, without being influenced by Rad9 recruitment.

In order to analyse the relative roles of Dpb11 and of the PCNA-like complex, we arrested cells in G1 and allowed them to enter synchronously into S phase in the presence of hydroxyurea.

In vivo analysis of the phosphorylation state of two Mec1 substrates, H2A and Rad53, allowed us to demonstrate that, in the contemporary absence of the AAD domain of Dpb11 and the Ddc1 subunit of the PCNA-like complex, Mec1 activity is extremely low in conditions of replication stress. This defect is not just the consequence of a different timing of S phase entrance, since *dpb11-1* and *ddc1Δdpb11-1* show a similar cell cycle kinetics in the absence of hydroxyurea but have different

levels of Mec1 activity when HU is present in the medium.

A plausible explanation for these results is that *dpb11-1* is defective *per se* in replication checkpoint activation and, in a condition of replication stress, DNA damage is generated and sensed by the PCNA like-dependent damage checkpoint, as it happens in an *mrc1Δ* strains. We excluded the possibility of an unscheduled DNA damage checkpoint activation monitoring the phosphorylation state of the damage-specific adaptor Rad9, which remains dephosphorylated in both *dpb11-1* and *ddc1Δ* single mutants.

Inability to activate the replication checkpoint results in replication fork breakdown (Lopes *et al.*, 2001) and incapacity to prevent spindle elongation (Weinert *et al.*, 1994). Indeed, as it has been previously reported, the inability to activate Mec1 characteristic of a *ddc1Δdpb11-1*, results in a high sensitivity of the double mutant to growth on plates supplemented with hydroxyurea (Wang and Elledge, 2002). We analysed in more details the reasons of this sensitivity, and we determined that it is not due to replication fork collapse or precocious elongation of the mitotic spindle, two phenotypes characteristic of mutants lacking Mec1 kinase.

Moreover *ddc1Δdpb11-1* sensitivity, differently from that of a *mec1-1sml1* strain, could be completely rescued if cells are treated with hydroxyurea for only 90 minutes after a G1 release. This suggests possibly that another function, induced by the replication checkpoint, is essential for sustaining growth in the presence of hydroxyurea but not to respond to temporary fork arrest.

In order to obtain more insights in the pathways leading to Ddc1-dependent and Dpb11-dependent activation of replication checkpoint, we analysed mutants in the genes coding for proteins that are currently known to be involved in the checkpoint response to hydroxyurea.

During the process of replication initiation, Dpb11 interacts with both Sld2/Drc1 and Sld3 in a phosphorylation-dependent manner, which is required for the process of origin firing (Zegerman and Diffley, 2007). Moreover *drc1-1* mutant, as well as *dpb11-1* mutants, when treated with hydroxyurea at their non-permissive temperature, display the same checkpoint-deficient phenotype (Wang and Elledge, 1999). We checked if Drc1 functions in the same Dpb11-dependent pathway for Mec1 activation by combining the *drc1-1* allele with *DDC1* deletion and we showed that *ddc1Δdrc1-1* double mutants display the same Rad53 phosphorylation defect and the same HU sensitivity of a *ddc1Δdpb11-1* control strain.

Mutants in the C-terminus of Pol2 has been shown to be defective in the establishment of replication checkpoint (Navas *et al.*, 1995) and this region of the protein has been suggested to be involved in the interaction of Pol2 with the other three subunits of DNA polymerase ε: the essential Dpb2 and the non-essential Dpb3 and Dpb4 (Dua *et al.*, 1998, 2000).

To demonstrate that Pol ε is performing its signalling function through its non-essential subunits and to try to identify if it is working in the Dpb11- or Ddc1-mediated pathway for Mec1 activation, we deleted the gene *DPB4* and we planned to combine this mutation with either *DDC1* deletion or *dpb11-1* mutation. *dpb4Δ* and *ddc1Δ*, as well as the double mutant strain, grow normally on YPD plates but when they are cultured on a medium containing hydroxyurea they show a synergic sensitivity, that resembles the one of a *ddc1Δdpb11-1* double mutant. Moreover, in both the double mutants this high HU sensitivity correlates with the inability to properly hyper-phosphorylate Rad53 after treatment with this drug, suggesting that Dpb4, and thus Pol ε, signals the presence of replication stress in the Dpb11-dependent pathway. If this model is correct a *dpb4Δdpb11-1* double mutant should display an almost normal level of Rad53 phosphorylation, similar to the one of the single mutants.

Unfortunately the ultimate demonstration that Drc1 and Dpb4 function in the Dpb11-1 dependent branch is lacking because *dpb4Δ* and *drc1* mutant alleles are synthetic lethal with the mutation *dpb11-1* (Ohya *et al.*, 2000; Kamimura *et al.*, 1998), a phenotype that could be expected if the three proteins are part of the same essential protein complex and each mutation weakens its stability. However, we are trying to overcome this problem either by using a degron-tag approach or by obtaining a mutation in *DPB11* that confers a defect in Mec1 activation, but not synthetic lethality with *dpb4Δ* and *drc1*.

In conclusion our data can be interpreted with the model shown in figure 5.2. Inhibition of ribonucleotide reductase induces a reduction of the dNTPs pool, causing a contemporary stalling of both the leading and the lagging strands at each active replication fork. On the lagging strand, discontinuous synthesis of the Okazaki fragments provides a 5' DNA end close to ssDNA generated by the inability of DNA polymerase to complete replication. The Rad24/Rfc2-5 complex can use this end to load the PCNA-like complex, which can in turn activate ssDNA-bound Mec1.

On the other hand, the high processivity of the synthesis of the leading strand, makes it likely

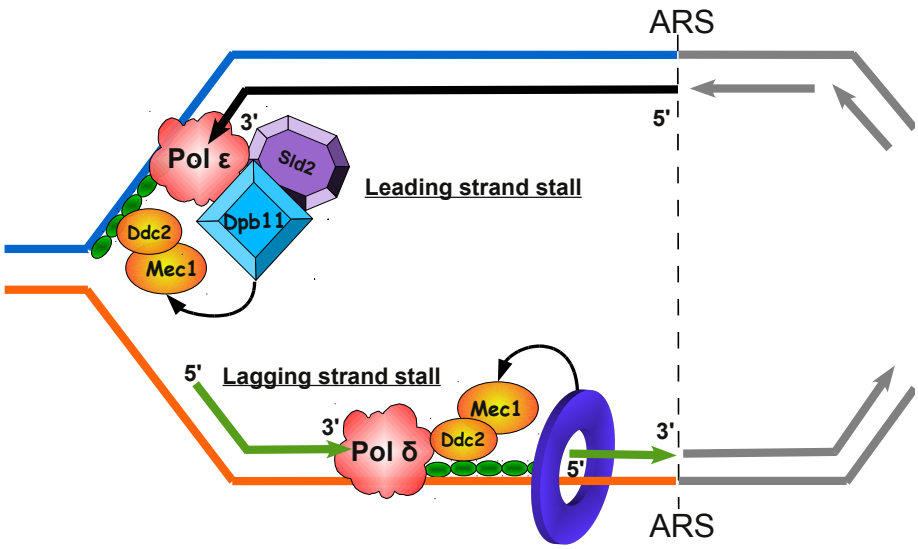

Figure 5.2: Model of the redundant Mec1-activating function of Dpb11 and the PCNA-like complex in conditions of replication stress: the blue ring represents the PCNA-like complex and the small green ovals are RPA.

that the nearest 5' end is quite far from the site of polymerase stalling, where ssDNA is generated and Mec1-Ddc2 complex is recruited by RPA. The absence of a 5' DNA end close to ssDNA-RPA-Mec1 could make impossible the PCNA-like dependent Mec1 activation. In this case the Dpb11-dependent pathway for the activation of Mec1 is invoked. In the leading strand pathway Sld2/Drc1 and the non-essential subunit of DNA polymerase ε, Dpb4, are also involved. Further supporting this model is the fact that the two replicative polymerases were recently assigned to the synthesis of each of the two DNA strands (McElhinny *et al.*, 2008) with Pol δ working on the lagging strand and Pol ε synthesising the leading strand.

Part IV

Articles

Part V

Materials & Methods

Abbreviations

BFB	Bromophenol Blue
bp	Base Pairs
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
kb	Kilobase Pairs
kDa	KiloDalton
PEG	Polyethylene Glycol
rpm	Revolutions Per Minute
SDS	Sodium Dodecyl Sulphate
Tris	Tris(hydroxymethyl)aminomethane
YNB	Yeast Nitrogen Base
YPD	Yeast Extract - Peptone - Dextrose
TCA	Trichloroacetic acid
OD	Optical Density
ON	Overnight
RT	Room Temperature

Growth Media

Escherichia coli Growth Media

LD :	Bacto Tryptone (DIFCO)	10 g
	Yeast Extract (DIFCO)	5 g
	NaCl	5 g
	H_2O	up to 1000 ml
	pH	7.25
LD-Agar:	LD medium	
	Agar	1%

LD-Amp:	LD medium	
	Ampicillin	50 μg/ml

Saccharomyces cerevisiae Growth Media

Common media

YPD:	Yeast extract	10 g
	Peptone	20 g
	H_2O	up to 1000 ml
	pH	5.4-5.7
	25X Glucose (50% w/v solution)	final conc. 2%
YPD-Agar:	YPD medium	
	Agar	2%
SD:	10X YNB (DIFCO) Solution [1]	40 ml
	25X Nutrient Mixture w/o His, Trp, Ura, Leu [2]	16 ml
	25X Glucose (50% w/v solution)	16 ml
	200X Histidine, Tryptophan, Uracil, Leucine solution [3]	2 ml
	H_2O	up to 400 ml

The medium is then filtered immediately after preparation. Different sugars can substitute glucose if needed: in this case 27 ml of raffinose or galactose 15X solutions (30% w/v) are added.

SD-Agar is prepared dissolving 8 g of Agar in 320 ml of water and autoclaving the suspension. After sterilisation and before solidification, the mixture of ingredients for SD medium is added and the content is poured into Petri dishes.

[1] Prepared dissolving 6.7 g in 100 ml of sterile water and sterilising by filtration

[2] See preparation of 25X Nutrient Mixture w/o His, Trp, Ura, Leu on the next page

[3] Prepared by dissolving the powders at a final concentration of 5 mg/ml and sterilising by filtration or with ethanol (for uracil)

Medium for Two Hybrid Analysis

The following nutrients are added to a sterile bottle containing 8 g of Agar and 320 ml of water:

10X BU Salt	40 ml
10X YNB (DIFCO) Solution	40 ml
25X Nutrient Mixture w/o His, Trp, Ura, Leu	16 ml
200X Histidine, Tryptophan, Uracil, Leucine solution	4 ml
30% Raffinose Solution (filter-sterilized)	33 ml
30% Galactose Solution(filter-sterilized), if required	33 ml
20 mg/ml X-Gal	1.6 ml

FOA medium

This medium is used to counter-select for the *URA3* marker. Ura^+ yeast cells die on this medium, whereas Ura^- cells are can live.

The following solution is added, after filter-sterilisation, to a sterile bottle containing 8 g of Agar and 200 ml of water:

10X YNB (DIFCO) Solution	40 ml
25X Nutrient Mixture w/o His, Trp, Ura, Leu	16 ml
200X Histidine, Tryptophan, Uracil, Leucine solution	4 ml
50% Glucose	16 ml
FOA	400 mg
H_2O	up to 200 ml

Preparation of 25X Nutrient Mixture w/o His, Trp, Ura, Leu

Is usually prepared in a final volume of 800 ml. Amino acids and Nitrogen Bases indicated below are added to a sterile 800 ml bottle at the final concentration indicated.

L-Thr	1.25mg/ml	L-Tyr	0.625mg/ml
L-Ile	0.625mg/ml	L-Arg	0.625mg/ml
L-Phe	0.625mg/ml	L-Met	0.625mg/ml
L-Lys	0.625mg/ml	Ade	0.625mg/ml

40 ml of absolute ethanol are added to the bottle, being careful of washing all its inner surface and of covering all the powders at the bottom. The bottle is closed and leaved ON at RT for complete sterilisation of the powders. In the morning 760 ml of sterile water are added to the bottle which is then conserved at 4°C.

Microbial strains

Escherichia coli strains

DH5α^{TM}: F Φ 80 dlacZ ΔM15 (lacZYA-argF) U169 deoR recA1 endA1 hsdR17 (rK,mK+) supE44 λ-thi-1 qyrA96 relA1.

This strain has been used as bacterial host for plasmid construction and has been purchased from Invitrogen. The cells used are already chemically competent for transformation.

Saccharomyces cerevisiae strains

Name	Relevant Genotype	Reference
K699	*MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100*	Kim Nasmith
K700	*MATalpha ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100*	Kim Nasmith
SY2080	*MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100* RAD5	M. Foiani
YFP20	(K699) *dpb11-1*	Puddu *et al.* (2008)
YFL234	(K699) *dot1::kanMX6*	Giannattasio *et al.* (2004)
YFL438	(K699) *dot1Δ::kanMX6 mec1-1 sml1*	Puddu *et al.* (2008)
YFL499/3d	(K699) *dot1::kanMX6 chk1::kanMX6*	Puddu *et al.* (2008)

Name	Relevant Genotype	Reference
YMAG6	(K699) *dot1::kanMX6 dpb11-1*	Puddu *et al.* (2008)
YMIC4F6	(K699) *mec3::TRP1 rad9::URA3*	Puddu *et al.* (2008)
YLL683.8/3B	(K699) *ddc2::DDC2-3HA:URA3*	Paciotti *et al.* (2000)
YFP24/6b	(K699) *dpb11-1 ddc2::DDC2-3HA:URA3*	Puddu *et al.* (2008)
YFL403/10b	(K699) *dot1::kanMX6 ddc2::DDC2-3HA:URA3*	Lazzaro *et al.* (2008)
YFL687/2b	(K699) *dot1::kanMX6 dpb11-1 ddc2::DDC2-3HA:URA3*	Puddu *et al.* (2008)
YFL211/3a	(K699) *RAD9-13myc:TRP1 ddc1::DDC1-HA:LEU2*	Puddu *et al.* (2008)
YMAG48/5b	(K700) *dpb11-1 RAD9-13myc:TRP1 ddc1::DDC1-HA:LEU2*	Puddu *et al.* (2008)
YMAG34/4a	(K699) *dot1::kanMX6 RAD9-13myc:TRP1 ddc1::DDC1-HA:LEU2*	Puddu *et al.* (2008)
YMAG52/3d	(K699) *dot1::kanMX6 dpb11-1 RAD9-13myc:TRP1 ddc1::DDC1-HA:LEU2*	Puddu *et al.* (2008)
YMIC4E8	(K699) *rad9::URA3*	Lazzaro *et al.* (2008)
YMAG149/7B	(K699) *hta1-htb1::LEU2 hta2-htb2::TRP1 [pSAB6]*	Puddu *et al.* (2008)
YFP115/3a	(K699) *chk1::CHK1-3HA::TRP1*	This Work
YFP116/8c	(K699) *chk1::CHK1-3HA::TRP1 dpb11-1*	This Work
YFP123/3b	(K699) *chk1::CHK1-3HA::TRP1 dot1::KanMX6*	This Work
YFP118/1b	(K699) *chk1::CHK1-3HA::TRP1 dpb11-1 dot1::KanMX6*	This Work
YMAG168	(K699) *hta1-htb1::LEU2 hta2-htb2::TRP1 [pJD151]*	Puddu *et al.* (2008)
YMAG150/4A	(K699) *dot1::kanMX6 hta1-htb1::LEU2 hta2-htb2::TRP1 [pSAB6]*	Puddu *et al.* (2008)
YMAG170	(K699) *dot1::kanMX6 hta1-htb1::LEU2 hta2-htb2::TRP1 [pJD151]*	Puddu *et al.* (2008)
YFP90	(SY2080) *dot1Δ::KanMX6*	This Work
DLY2236	(SY2080) *rad9Δ::LEU2 ura3::rad9Δ1-231::URA3*	Blankley and Lydall (2004)
YFP91	(SY2080) *rad9Δ::LEU2 ura3::rad9Δ1-231::URA3 dot1Δ::KanMX6*	This Work

Name	Relevant Genotype	Reference
YLDN25	(K699) *ddc1:: kanMX4 [pML89]*	Puddu *et al.* (2008)
YLDN17	(K699) *ddc1:: kanMX4 [pLD12]*	Puddu *et al.* (2008)
YLDN23	(K699) *ddc1:: kanMX4 [pLD26]*	Puddu *et al.* (2008)
YLDN24	(K699) *ddc1:: kanMX4 [pLD31]*	Puddu *et al.* (2008)
YFP27	(K699) *ddc1:: kanMX4 dot1::HIS3 [pML89]*	Puddu *et al.* (2008)
YFP28	(K699) *ddc1:: kanMX4 dot1::HIS3 [pLD12]*	Puddu *et al.* (2008)
YFP29	(K699) *ddc1:: kanMX4 dot1::HIS3 [pLD26]*	Puddu *et al.* (2008)
YFP30	(K699) *ddc1:: kanMX4 dot1::HIS3 [pLD31]*	Puddu *et al.* (2008)
YLDN9	(K699) *ddc1:: kanMX4 [pLD9]*	Puddu *et al.* (2008)
YFP37	(K699) *ddc1:: kanMX4 dot1::HIS3 [pLD9]*	Puddu *et al.* (2008)
YFP38	(K699) *dpb11::DPB11-13myc:HIS3*	Puddu *et al.* (2008)
YFP48/3a	(K699) *dpb11::DPB11-13myc:HIS3 mec1-1 sml1-1*	Puddu *et al.* (2008)
YFP49/1d	(K699) *dpb11::DPB11-13myc:HIS3 rad53::kanMX6 sml1::HIS3*	Puddu *et al.* (2008)
YFP55/6c	(K699) *ddc1:: kanMX6 dpb11::DPB11-13myc:HIS3*	Puddu *et al.* (2008)
YFP56	(K699) *ddc1:: kanMX6 dpb11::DPB11-13myc:HIS3[pML89]*	Puddu *et al.* (2008)
YFP57	(K699) *ddc1:: kanMX6 dpb11::DPB11-13myc:HIS3 [pLD9]*	Puddu *et al.* (2008)
YFP63	(K699) *ddc1:: kanMX6 [pML89]*	Puddu *et al.* (2008)
YFP64	(K699) *ddc1:: kanMX6 [pLD9]*	Puddu *et al.* (2008)
YFP65	(K699) *ddc1:: kanMX6 dpb11-1 [pML89]*	Puddu *et al.* (2008)
YFP66	(K699) *ddc1:: kanMX6 dpb11-1 [pLD9]*	Puddu *et al.* (2008)
YFP146	(K699) *ddc1:: kanMX6 dpb11-1 [pFP8]*	This Work
YFP147	(K699) *ddc1:: kanMX6 dpb11-1 [pFP8]*	This Work

Name	Relevant Genotype	Reference
YFP148	(K699) *ddc1:: kanMX6 dpb11-1 [pFP9]*	Puddu *et al.* (2008)
YFP149	(K699) *ddc1:: kanMX6 dpb11-1 [pFP9]*	Puddu *et al.* (2008)
YFP152	(K699) *ddc1:: kanMX6 [Ycplac111]*	Puddu *et al.* (2008)
YFP 142	(K699) *dot1:: HIS3 dpb11-1 ddc1:: kanMX6 [pML89]*	Puddu *et al.* (2008)
YFP 144	(K699) *dot1:: HIS3 dpb11-1 ddc1:: kanMX6 [pLD9]*	Puddu *et al.* (2008)
YFP50	(EGY48) *[pSH18.34; pFP1; pFP2]*	Puddu *et al.* (2008)
YFP52	(EGY48) *[pSH18.34; pFP1; pFP4]*	Puddu *et al.* (2008)
YFP113	(K699) *mec1-1 sml1 [pSH18.34; pFP1; pFP2]*	Puddu *et al.* (2008)
YFP114	(K699) *mec1-1 sml1 [pSH18.34; pFP1; pFP4]*	Puddu *et al.* (2008)
YFP86	(EGY48) *[pSH18.34; pJG4-5; pFP2]*	Puddu *et al.* (2008)
YFP54	(EGY48) *[pSH18.34; pFP1; pEG202]*	Puddu *et al.* (2008)
YFP153	(EGY48) *[pSH18.34; pFP1; pFP10]*	Puddu *et al.* (2008)
YAN21/8d	(K699) *ddc1Δ::kanMX6*	A.Nespoli
YFP62/1d	(K699) *ddc1Δ::kanMX6 dpb11-1*	This work
Y5A3	(K699) *mec1-1 sml1*	M. Giannattasio
YFP178/1a	(K699) *mrc1Δ::HIS3*	This work
YFP167/1a	(K699) *dpb4Δ::HIS3*	This work
YFP206/1a	(K699) *ddc1Δ::kanMX6 dpb4Δ::HIS3*	This work
Y799	(K699) *drc1-1*	Wang and Elledge (1999)
YFP218/1a	(K699) *ddc1Δ::kanMX6 drc1-1*	This work

Oligonucleotides

Oligonucleotides for *DPB11* tagging and C-terminal deletion

- DPB11F2 GAGACGACAGACAAGAAATCAGACAAAGGAATTAGATTCTCGGATCCCCGGGTTAATTAA
- DPB11-1F2 ATCTGGAGAAAATAATGAAATCTTTTTAAACAATATCAAGCGGATCCCCGGGTTAATTAA
- DPB11R1 CGTATGTAAATGAATATCTTATAAAATTACGGACTACATTGAATTCGAGCTCGTTTAAAC
- DPB11F1 TCTAGTATGGCAGGTATTTTATCAGTAGCATTAATATTACTCGGATCCCCGGGTTAATTAA
- dpb11.3 TTGATTTTCAAAAATTGTGCG
- dpb11.4 AGATAGAAGCAAAGCAACCC

Oligonucleotides for *DPB11* sequencing

- Dpb11seqprom AAAGGAAGGATTCTTTTGCAGGC
- Dpb11seq0 TCAAATCTTTAGAAAATATACC
- Dpb11seq.1 CCATCGATTCATTGGTTAGG
- Dpb11seq.2 AAACTCTTGCGATTGCTGGG
- Dpb11seq.3 AATCTTCTAAAACCAATGGG
- Dpb11seq.4 CTGCCCAAGAGGACACAAGG

Oligonucleotides for Ddc1-T602 mutation to S and E

- DDC1-T602S-F TGGAAGATGGGCTGGGTCTATCACAAGTAGAAAAGCCAAGGGG
- DDC1-T602S-R CCCCTTGGCTTTTCTACTTGTGATAGACCCAGCCCATCTTCCA
- DDC1-T602E-F TGGAAGATGGGCTGGGTCTAGAACAAGTAGAAAAGCCAAGGGG
- DDC1-T602E-R CCCCTTGGCTTTTCTACTTGTTCTAGACCCAGCCCATCTTCCA

Oligonucleotides for *DDC1* deletion

- DDC1F1 TAGTGTAACAATAACACAGCATAACTTTGCTTAGACATATCGGATCCCCGGGTTAATTAA
- DDC1R1 TAATATTTACACGCCTTTATACTGATTTTGCATTATGGTTGAATTCGAGCTCGTTTAAAC
- DDC1.3 TGCAAGGTCTGTTGAATTCCC
- DDC1.4 GCCTAGAATGTCCATCACCC

Oligonucleotides for *DPB4* deletion

- DPB4F1 TCATTGCTTATTTATATCAGACCATATATTTTTACACACGCGGATCCCCGGGTTAATTAA
- DPB4R1 GAGTGGTGGCAAGCACTACTAGACAGTTTCCATAGCGGGGGAATTCGAGCTCGTTTAAAC
- DPB4.3 GATGGCGATATAGATATGGG
- DPB4.4 CAATATCAACTTCTTGTCCC

Oligonucleotides for *DPB11* and *DDC1* cloning in the Two Hybrid vectors

- DPB11_pJG_FOR TGCCAGATTATGCCTCTCCCGAATTCGGCCGACTCGAGATGAAGCCCTTTCAAGGAAT
- DPB11_pJG_REV CCAAACCTCTGGCGAAGAAGTCCAaagcttCTCGAGTCAAGAATCTAATTCCTTTG
- DDC1_pEG_FOR GGCGACTGGCTGGAATTCCCGGGGATCCGTCGACCATGGATGTCATTTAAGGCAACTAT
- Ddc1del1 CGGCGACTGGCTGGAATTCCCGGGGATCCGTCGACCATGGAAGCGATCACATAGTAAGCG
- Ddc1del5 AATTAGCTTGGCTGCAGGTCGACTCGAGCGGCCGCCATGGTTAGTCAAATATACCCCTTG

Plasmids

Plasmids for the Two Hybrid experiments

pJG4-5	This plasmid contains the gene for the B42-HA activation domain, under the expression of the inducible GAL1-10 promoter.
pFP1	Derived from pJG4-5 contains the coding sequence of *DPB11* fused in frame with the B42-HA.

pEG202	This plasmid contains the gene for the lexA DNA binding domain under the expression of the constitutive ADH promoter.
pFP2	Derived from pEG202, it contains the coding sequence of *DDC1* fused in frame with the lexA DNA binding domain.
pFP4	Derived from pEG202, it contains a truncated *DDC1* coding sequence (AA 309–612), fused in frame with the lexA DNA binding domain.
pFP10	Derived from pEG202, it contains the coding sequence of *DDC1*, mutated in the 8 consensus sites for Mec1 phosphorylation, fused in frame with the lexA DNA binding domain.
pSH18-34	In this reporter plasmid the lacZ regulatory sequence has been substituted with four lexA operator sequences, recognised by the lexA DNA binding domain.

DDC1 plasmids

pML89	This centromeric plasmid contains the *DDC1* gene under the expression of its own promoter (LONGHESE *et al.*, 1996).
pLD9	Derived from pML89, this plasmid carries a version of *DDC1* with T602 mutated to alanine (Lisa di Nola, Master Thesis).
pLD12	Derived from pML89, this plasmid carries a version of *DDC1* with S413, S436 and T444 (Cdc28 consensus sites) mutated to alanine (Lisa di Nola, Master Thesis).
pLD26	Derived from pML89, this plasmid carries a version of *DDC1* with T342, S469, S471, S495, T529, S532, S580 and T602 (Mec1 consensus sites) mutated to alanine (Lisa di Nola, Master Thesis).
pLD31	Derived from pML89, this plasmid carries a version of *DDC1* with T342, S413, S436, T444, S469, S471, S495, T529, S532, S580 and T602 (Mec1&Cdc28 consensus sites) mutated to alanine (Lisa di Nola, Master Thesis).

pFP9	Derived from pLD89, this plasmid carries a version of *DDC1* with T602 mutated to serine.
pFP8	Derived from pLD89, this plasmid carries a version of *DDC1* with T602 mutated to glutamic acid.

H2A plasmids

pSAB6	This centromeric plasmid contains the genes coding for histone H2A and histone H2B under the control of their own promoters (Hirschhorn *et al.*, 1995).
pJD151	This plasmid is derived from pSAB6 and carries a mutation of S129 of H2A to alanine (Downs *et al.*, 2000).

Solutions

10X Laemmli Buffer

Tris-Base	302.8 g
Glycine	1440.3 g
H_2O	up to 10 l

SDS-PAGE running buffer (SPAG) 1X

10X Laemmli Buffer	1 l
SDS	10 g
H_2O	up to 10 l

1X Transfer Buffer

10X Laemmli Buffer	0.5 l
Methanol	1 l
H_2O	up to 5 l

10X Phosphate Buffered Saline (PBS)

NaCl	80 g
KCl	2 g
KH_2PO_4	2 g
$Na_2HPO_4 . 2H_2O$	11.4 g
H_2O	up to 1 l

Immediately after preparation the solution is autoclaved or filter-sterilized.

PBST

This solution is prepared by diluting 10X PBS to 1X and adding Tween-20 to a final concentration of 0.2%.

PBST-milk

This solution is prepared by dissolving non-fat milk (Genespin) 5% w/v in PBST solution.

1X TE

Tris-HCl pH 7.4	10 mM
EDTA	1 mM

1X TAE

Tris-Acetate pH 8.0	40 mM
EDTA	10 mM

5X TBE

Tris-base	540 g
Boric Acid	275 g
EDTA 0.5 M pH 8	200 ml
H_2O	up to 10 l

Coomassie stain for 1 litre

Coomassie brilliant Blue R250	2.5 g
Glacial Acetic Acid	100 ml
H_2O:MetOH 1:1	900 ml

Coomassie destain I for 1 litre

MetOH	500 ml
Glacial Acetic Acid	100 ml
H_2O	up to 1 l

Coomassie destain II for 1 litre

MetOH	50 ml
Glacial Acetic Acid	70 ml
H_2O	up to 1 l

6X Blue Sample Buffer for Protein SDS-PAGE

0.5 M Tris-HCl pH 6.8	7 ml
Glycerol	3 ml
SDS	1 g
DTT	0.93 g
BFB	1.2 mg

6X DNA Loading Dye

BFB	0.125 g
87% Glycerol	17.24 ml
H_2O	up to 50 ml

1 Kb DNA Ladder

500 ng/µl DNA Ladder (NEB)	60 µl
6X DNA Loading Dye	100 µl
H_2O	440 µl

Ponceau S solution

0.2% Ponceau S 3% TCA

10X BU-salt

$Na_2HPO_4.2H_2O$	35 g
$NaH_2PO_4.H_2O$	15 g
H_2O	up to 500 ml

Immediately after preparation the solution is autoclaved or filter-sterilized.

500 X Ampicillin

It is prepared by dissolving ampicillin at a concentration of 25 mg/ml.

Protocols

PCR (Polymerase chain reaction)

PCR is carried out using plasmid or genomic DNA preparations ad template. Amplification of a target DNA fragment requires the use of two oligonucleotides flanking the target that functions as primers for the reaction catalysed by DNA polymerase. The DNA polymerases currently used are two: Taq polymerase (Genespin); Pfu Ultra II Fusion HS DNA Polymerase (Stratagene).

Reaction mix contains:

- Oligonucleotides: 20 pmol each
- Template DNA: 25-100 ng depending if plasmid or genomic DNA
- 10X DNA Polymerase Rxn buffer: 5 µl

- dNTPs (2mM each): 5 µl
- DNA polymerase: 2 units
- dH_2O: up to 50µl

Reactions are made using either the Mastercycler (Eppendorf) or Robocycler (Stratagene) apparatus; they consist of the following steps:

1. First denaturation: 2' @ 94°C
2. Denaturation: 1' @ 94°C
3. Annealing: 1' @ Tm - 5° C
4. Extension 1' per Kilobase of amplification target + 2' @ 72°C
5. Repeat passages from 2 to 4 for 25–30 cycles
6. Final extension: 10' @ 72°C.

Tm is the lower melting temperature for the couple of oligonucleotides used. Conditions are to be adjusted depending on the template, the purpose of the PCR and the DNA polymerase used. Differently from this protocol colony based PCRs, which are for diagnostic purposes, are carried out by substituting the template with a small amount of a yeast patch. To induce the break of the cell wall and liberation of genomic DNA initial denaturation time is prolonged to 7' minutes, denaturation and annealing last 30'' and the program is repeated for 45 cycles.

DNA restriction and agarose gel electrophoresis

DNA is digested with the proper restriction endonucleases, following the indications of the supplier (New England Biolabs). 1/6 volume of 6X DNA loading dye (0.25% BFB in 30% glycerol) is then added to digested samples, which are loaded on an agarose gel (0.6%-2%). Fragment are separated depending on their molecular weight by electrophoresis in a 1X TAE buffer. DNA is then visualised by adding ethidium bromide before pouring the gel at a final concentration of 5 µg/ml. This molecule intercalates in DNA and emit fluorescent light if stimulated with a 260 nm UV radiation. To estimate

the molecular weight of the fragments a molecular weight marker (MWM, New England Biolabs) is loaded in parallel.

DNA purification by agarose gel extraction

After electrophoresis a small slice of agarose gel, containing the DNA fragment to be purified, is excised, weighed and closed in an Eppendorf tube. DNA is then extracted from the gel slice using the Wizard Plus Gel Extraction Kit (Promega). An aliquot of the extracted DNA is then loaded on a new gel to evaluate purity and extraction efficiency.

DNA precipitation

1/10 volume of NaAc 3M pH 5.0 and 2 volumes of EtOH 100% (cold, @ -20°C) are added to the DNA solution that has to be purified. Samples are then incubated for at least 2h @ -20°C or $\frac{1}{2}$ hour @ -80°C. Then they are centrifuged for 30' in a refrigerated centrifuge (4°C) and supernatant is removed. The pellet is washed with 1 ml of 70% EtOH (-20°C) and let dry in a Savant centrifuge. Finally the pellet is resuspended in 10-15 µl of water or TE buffer.

E.coli transformation

Chemical competent DH5α cells (Invitrogen), kept at –80°C, are thawed on ice for 20 minutes. After mixing the cells, 50 µl are aliquoted in Eppendorf tubes. DNA is then added (100 ng-1 µg) and after 30' on ice cells are subjected to a 2' heath shock at 37°C. Cells are then cooled on ice for 2 minutes, diluted in 950 µl of LD and incubated 1 hour at 37°C. Depending on the starting DNA the whole suspension, concentrated in 100 µl, or 1/10 of the whole suspension is plated on LD plates supplemented with the selective agent (usually ampicillin). Plates are incubated overnight at 37°C.

Gap repair cloning

Starting from the observation that linear DNA fragments are able to stimulate homologous recombination in yeast, in 1987 a quicker *in vivo* strategy for the construction of plasmids has been set up (Ma *et al.*, 1987). Briefly it consist in linearising the vector with the desired restriction enzyme, and then in generating the insert by PCR using oligonucleotides that carry a 40 nt region homologous

to the vector DNA ends. The following step is the co-transformation of the two DNA molecules: the homology between the vector and the PCR promotes two recombination events and as a result a circular DNA molecule carrying the insert is obtained. Cells in which this event happened are selected by plating on a medium that allows the growth only to the cells that harbour a circular plasmid that can be kept along generations.

S.cerevisiae transformation

The strain to be transformed is growth overnight in 50 ml of the appropriate medium at 28°C until the culture has reached a concentration between $5{\cdot}10^6$ and $1{\cdot}10^7$ cells/ml. Then, the suspension is centrifuged 5 minutes at 4000 rpm and cells are washed with 25 ml of sterile H_2O; cells are subsequently resuspended in 500 µl of sterile water, and 100 µl are used for each transformation. Cells are then pelleted and the pellet is resuspended in 360 µl of Tmix (33% PEG-4000, 0.1 M LiAc, 0.27 mg/ml ssDNA(Eppendorf)) containing the appropriate amount of transforming DNA. The suspension is incubated at 42°C for different times, depending on the DNA transformed: 5' for a plasmid, 20' for more than one plasmid, 40' for transformations that requires recombination events. Cells are then pelleted and washed with sterile water, resuspended in 200 µl H_2O and finally plated on selective medium. If the selection marker requires some time for the expression of the resistance (for example G418 resistance), before plating, cells are resuspended in rich medium and left 2 hours at 28°C.

Plasmids extraction from yeast

Yeast cells are grown ON in 10 ml of an appropriate medium, collected by centrifugation and resuspended in 1ml of Zymobuffer (0.9M Sorbitol, 0.1M EDTA). Cell are transferred to Eppendorf tubes, pelleted and resuspended in 400 µl of Zymobuffer supplemented with 14 mM β-mercaptoethanol. 100 µl of 2 mg/ml Zymolyase is then added and samples are incubated at 37°C until complete spheroplastization. Spheroplasts are collected by centrifugation for 1 minute at 4000 rpm and resuspended in 250 µl of Cell Resuspension Solution of the Wizard Plus Miniprep kit (Promega). The protocol of the kit is then followed.

Synchronisation of yeast cells in different phases of the cell cycle

A log phase culture of yeast contains cells that are in all the different phases of cell cycle. Sometimes it is useful to obtain cultures where all the cells are in the same stage of cell cycle. This synchronisation is obtained with the use of α-factor (G1) or nocodazole (mitosis).

α-factor α-factor is a pheromone produced by Mat α cells whose presence is sensed only by Mat a cells. It activates a signalling cascade which ultimately interfere with the level of G1 cyclins, preventing the exit from G1. α-factor is dissolved in sterile water at the final concentration of 1 mg/ml (500X) and conserved at -20°C. To obtain G1 synchronisation, yeast cells are exposed for 1.5 - 2 hours to α-factor at a concentration ranging from 1X to 2.5 X. Synchronisation is checked by counting the number of single unbudded (G1) cells in the culture. After the synchronisation is reached, cells can be collected and resuspended in fresh medium (release) to have a synchronised entrance into S-phase. To obtain, instead, a stable arrest, α-factor is used at a final concentration of 10X, for the same time.

Nocodazole Nocodazole destabilises microtubules preventing tubulin polymerisation, and thus preventing the formation of the mitotic spindle. The nocodazole treatment synchronise yeast cells in mitosis at the transition from metaphase to anaphase. Nocodazole is dissolved in DMSO at a final concentration of 2 mg/ml (1000X) and conserved at -20°C. To obtain M synchronisation yeast cells are exposed for 1.5 - 2 hours to nocodazole at a 2.5 X concentration. Synchronisation is checked by counting the number of double dumbbell (M) cells in the culture. After the synchronisation is reached, cells can be collected and resuspended in fresh medium (release) to have a synchronised execution of mitosis. To obtain, instead, a stable arrest, nocodazole is used at a final concentration of 10X, for the same time.

Analysis of Rad53 phosphorylation after UV in arrested cells

After arresting the cells as described before, the culture is centrifuged and the collected cells are spread on YPD plates (Ø = 14 cm) being careful to plate the equivalent of 50 ml at $1{\cdot}10^7$ cell/ml each plate. Plates are then irradiated with the desired UV dosage (2-200 J/m^2, usually 75 J/m^2)

and recovered in a medium containing the relevant cell cycle blocking agent. 30 minutes later, cells are collected for TCA protein extracts preparation (see below on this page).

DAPI nuclear staining

1 ml of a yeast culture at a concentration of $5{\cdot}10^6$ cell/ml is fixed for at least 30 minutes in 2 ml of 100% EtOH. Cells are then washed two times with PBS, collected, resuspended in 50 µl of 0.2 µg/ml DAPI and left in the dark for at least 30 minutes. Cells are washed two times with deionised water and before the last centrifuge sonicated for 4-5 seconds. Cells are then centrifuged 5 minutes at 4000 rpm and resuspended in 50 µl of 50% glycerol. Samples can then be observed using a fluorescence microscope and scored for mono-nucleated vs. bi-nucleated cells.

In situ immunofluorescence

1 ml of cells is fixed ON at 4°C with fixation buffer (3,7% formaldehyde, 0,1 M K-phosphate pH 6,4, 0,5 mM $MgCl_2$); after fixation cells were washed three times with 1 ml of wash buffer (0,1 M K-phosphate pH 6,4, 0,5 mM $MgCl_2$), one time with spheroplasting solution (1,4 M sorbitol, 0,1 M K-phosphate pH 6,4, 0,5 mM $MgCl_2$) and resuspended in 200 µl of the same buffer. Spheroplasts were prepared using 5 µl of Zymolyase 10 mg/ml, at 37°C monitoring spheroplastisation by microscopic observation and then washed one time with spheroplasting solution. Spheroplasts were used to prepare a multi-well glass for immunofluorescence. Primary antibody was used ON at 4°C.

Primary Antibody	Dilution	Secondary Antibody	Dilution
α-γ-tubulin YOL1/34	1:100	Goat-α-Rat/Rhodamine	1:100

Preparation of protein extracts with Trichloroacetic acid (TCA)

Protein extraction from yeast cells is carried out using TCA as described in Falconi *et al.* (1993). This method is particularly useful to avoid protein degradation during extraction. A sample consisting of 10 - 20 ml of a yeast culture at the concentration of about $1{\cdot}10^7$ cells/ml is centrifuged at 4000 rpm to collect the cells. The pellet is washed with 1 ml of 20% TCA, transferred to a 2 ml Eppendorf tube and finally resuspended in 50 µl of 20% TCA. An equal volume of acid washed glass

	Running Gel		
	10%	***15%***	**Stacking Gel**
40% acrylamide	5 ml	7.5 ml	1.25 ml
2% N'-N-methylenbisacrylamide	1.29 ml	1.94 ml	0.7 ml
0.5 M Tris- HCl pH 6.8			2.5 ml
1.5 M Tris-HCl pH 8.8	5 ml	5 ml	
10% SDS	200 µl	200 µl	100 µl
10% APS	200 µl	200 µl	100 µl
Temed	20 µl	20 µl	10 µl
H_2O	8.29 ml	5.14 ml	5.4 ml

Table 5.2: Preparation of PAA gels

beads (Ø=425 - 600 µm, Sigma) is then added to the tube and samples are vortexed for 5 minutes, breaking the cells. The obtained lysate is supplemented with 100 µl of 5% TCA and transferred to a new Eppendorf tube which is centrifuged 10 minutes at 3000 rpm. Protein pellets are resuspended in 100 µl of 2X Sample buffer, prepared by diluting the 6X Stock described on page 117. The TCA residues are neutralised by adding 60 µl of 2M Tris-base. The extract is then boiled 3 minutes to allow protein dissolution and the insoluble material is discarded after a 2 minutes centrifugation at maximum speed.

Denatured protein electrophoresis in polyacrylamide gel (SDS-PAGE)

Polyacrylamide (PAA) gel electrophoresis is a technique used to separate proteins depending on their molecular weight. SDS is an anionic detergent that binds to proteins denaturing them and conferring to proteins a global negative charge, which is constant per mass unit. This makes the proteins move towards the anode with a speed proportional to their molecular weight.

The technique used is discontinuous electrophoresis, in which PAA gel is made by two different regions: the stacking gel, whose function is to concentrate the sample loaded in the well into a thin band and the running gel, which is the one that actually separates the proteins.

Stacking and running mix were prepared as it is indicated in table 5.2:

Protein samples, resuspended in 2X sample buffer, are loaded in wells obtained in the stacking

Primary Antibody	Dilution	Secondary Antibody	Dilution
α Rad53 (courtesy of C. Santocanale)	1:5,000	GAR	1:50,000
α Ddc1 (raised in the lab)	1:30	GAM	1:25,000
α Rad9 (courtesy of D. Stern)	1:7,500	GAR	1:25,000
α MYC (9E10)	1:30	GAM	1:50,000
α HA (12CA5)	1:30	GAM	1:25,000
α LexA (Santa Cruz 7544)	1:500	GAM	1:25,000
α γH2A (Abcam 15083)	1:1,500	GAR	1:25,000

Table 5.3: Antibodies used in this thesis (GAR: Goat-α-Rabbit; GAM: Goat-α-Mouse; RAG: Rabbit-α-Goat)

gel. Electrophoresis has been carried out in Euroclone setup, using as running buffer 1X SPAG (see page 115). A molecular weight marker (MWM, NEB) is loaded next to the samples to follow the run. Gels were run as follows: 120V until proteins are in stacking gel, 170V until the desired MWM exit from the gel (47 KDa for Rad53).

Nitrocellulose membrane transfer and western blotting

Once the electrophoretic run has ended, proteins are electro-blotted on a nitrocellulose membrane. The transfer process is carried out at 400 mA for 2 hours or at 200 mA ON, in the transfer buffer described on page 115.

At the end of the transfer, the nitrocellulose membrane is washed with deionised water and proteins are stained with Ponceau S solution (see page 118), allowing to assess the quality of the run and the transfer. Filters are then destained in PBST and incubated for one hour in PBST-milk at room temperature to allow saturation. Primary antibodies are then added at the desired dilution (see table 5.3) and filters are incubated for 2/3 hours in agitation at room temperature.

Afterwards membranes are washed three times, 10 minutes each, in PBST and subsequently hybridised for one hour with peroxidase-conjugated secondary antibodies (Pierce). Filters are then washed three times, 10 minutes each, in PBST and put in the developing solution, which contains the chemiluminescent substrates. Autoradiography film (Amersham) is then exposed to visualise the result.

Bibliography

Agarwal, R., Z. Tang, H. Yu, and O. Cohen-Fix, 2003 Two distinct pathways for inhibiting pds1 ubiquitination in response to DNA damage. J Biol Chem **278**: 45027–45033.

Alani, E., R. Thresher, J. D. Griffith, and R. D. Kolodner, 1992 Characterization of DNA-binding and strand-exchange stimulation properties of y-RPA, a yeast single-strand-DNA-binding protein. J Mol Biol **227**: 54–71.

Alcasabas, A. A., A. J. Osborn, J. Bachant, F. Hu, P. J. Werler, *et al.*, 2001 Mrc1 transduces signals of DNA replication stress to activate Rad53. Nat Cell Biol **3**: 958–965.

Allen, J. B., Z. Zhou, W. Siede, E. C. Friedberg, and S. J. Elledge, 1994 The SAD1/RAD53 protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast. Genes Dev **8**: 2401–2415.

Andrews, B., and V. Measday, 1998 The cyclin family of budding yeast: abundant use of a good idea. Trends Genet **14**: 66–72.

Araki, H., S. H. Leem, A. Phongdara, and A. Sugino, 1995 Dpb11, which interacts with DNA polymerase II(epsilon) in Saccharomyces cerevisiae, has a dual role in S-phase progression and at a cell cycle checkpoint. Proc Natl Acad Sci U S A **92**: 11791–11795.

Bellaoui, M., M. Chang, J. Ou, H. Xu, C. Boone, *et al.*, 2003 Elg1 forms an alternative RFC complex important for DNA replication and genome integrity. EMBO J **22**: 4304–4313.

Blankley, R. T., and D. Lydall, 2004 A domain of Rad9 specifically required for activation of Chk1 in budding yeast. J Cell Sci **117**: 601–608.

Bloom, J., and F. R. Cross, 2007 Multiple levels of cyclin specificity in cell-cycle control. Nat Rev Mol Cell Biol **8**: 149–160.

Boiteux, S., and M. Guillet, 2004 Abasic sites in DNA: repair and biological consequences in Saccharomyces cerevisiae. DNA Repair (Amst) **3**: 1–12.

Bonilla, C. Y., J. A. Melo, and D. P. Toczyski, 2008 Colocalization of sensors is sufficient to activate the DNA damage checkpoint in the absence of damage. Mol Cell **30**: 267–276.

Borde, V., W. Lin, E. Novikov, J. H. Petrini, M. Lichten, *et al.*, 2004 Association of Mre11p with double-strand break sites during yeast meiosis. Mol Cell **13**: 389–401.

Botuyan, M. V., J. Lee, I. M. Ward, J.-E. Kim, J. R. Thompson, *et al.*, 2006 Structural basis for the methylation state-specific recognition of histone H4-K20 by 53BP1 and Crb2 in DNA repair. Cell **127**: 1361–1373.

Boulton, S. J., and S. P. Jackson, 1996 Identification of a Saccharomyces cerevisiae Ku80 homologue: roles in DNA double strand break rejoining and in telomeric maintenance. Nucleic Acids Res **24**: 4639–4648.

Breeden, L., 1996 Start-specific transcription in yeast. Curr Top Microbiol Immunol **208**: 95–127.

Brewer, B. J., and W. L. Fangman, 1988 A replication fork barrier at the 3' end of yeast ribosomal RNA genes. Cell **55**: 637–643.

Brush, G. S., and T. J. Kelly, 2000 Phosphorylation of the replication protein A large subunit in the Saccharomyces cerevisiae checkpoint response. Nucleic Acids Res **28**: 3725–3732.

Brush, G. S., D. M. Morrow, P. Hieter, and T. J. Kelly, 1996 The ATM homologue MEC1 is required for phosphorylation of replication protein A in yeast. Proc Natl Acad Sci U S A **93**: 15075–15080.

Bylund, G. O., and P. M. J. Burgers, 2005 Replication protein A-directed unloading of PCNA by the Ctf18 cohesion establishment complex. Mol Cell Biol **25**: 5445–5455.

Carr, A. M., 2002 DNA structure dependent checkpoints as regulators of DNA repair. DNA Repair (Amst) **1**: 983–994.

Cha, R. S., and N. Kleckner, 2002 ATR homolog Mec1 promotes fork progression, thus averting breaks in replication slow zones. Science **297**: 602–606.

Chen, L., K. Trujillo, W. Ramos, P. Sung, and A. E. Tomkinson, 2001 Promotion of Dnl4-catalyzed DNA end-joining by the Rad50/Mre11/Xrs2 and Hdf1/Hdf2 complexes. Mol Cell **8**: 1105–1115.

Chen, S.-H., and H. Zhou, 2009 Reconstitution of Rad53 activation by Mec1 through adaptor protein Mrc1. J Biol Chem **284**: 18593–18604.

Clerici, M., D. Mantiero, G. Lucchini, and M. P. Longhese, 2005 The Saccharomyces cerevisiae Sae2 protein promotes resection and bridging of double strand break ends. J Biol Chem **280**: 38631–38638.

Clerici, M., D. Mantiero, G. Lucchini, and M. P. Longhese, 2006 The Saccharomyces cerevisiae Sae2 protein negatively regulates DNA damage checkpoint signalling. EMBO Rep **7**: 212–218.

Cohen-Fix, O., and D. Koshland, 1997 The anaphase inhibitor of Saccharomyces cerevisiae Pds1p is a target of the DNA damage checkpoint pathway. Proc Natl Acad Sci U S A **94**: 14361–14366.

Cotta-Ramusino, C., D. Fachinetti, C. Lucca, Y. Doksani, M. Lopes, *et al.*, 2005 Exo1 processes stalled replication forks and counteracts fork reversal in checkpoint-defective cells. Mol Cell **17**: 153–159.

Critchlow, S. E., and S. P. Jackson, 1998 DNA end-joining: from yeast to man. Trends Biochem Sci **23**: 394–398.

D'Amours, D., and S. P. Jackson, 2002 The Mre11 complex: at the crossroads of dna repair and checkpoint signalling. Nat Rev Mol Cell Biol **3**: 317–327.

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

Desany, B. A., A. A. Alcasabas, J. B. Bachant, and S. J. Elledge, 1998 Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway. Genes Dev **12**: 2956–2970.

Diffley, J. F. X., 2004 Regulation of early events in chromosome replication. Curr Biol **14**: R778–R786.

Donovan, S., J. Harwood, L. S. Drury, and J. F. Diffley, 1997 Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. Proc Natl Acad Sci U S A **94**: 5611–5616.

Doré, A. S., M. L. Kilkenny, N. J. Rzechorzek, and L. H. Pearl, 2009 Crystal Structure of the Rad9-Rad1-Hus1 DNA Damage Checkpoint Complex- Implications for Clamp Loading and Regulation. Mol Cell **34**: 735–745.

Downs, J. A., N. F. Lowndes, and S. P. Jackson, 2000 A role for Saccharomyces cerevisiae histone H2A in DNA repair. Nature **408**: 1001–1004.

Drury, L. S., G. Perkins, and J. F. Diffley, 1997 The Cdc4/34/53 pathway targets Cdc6p for proteolysis in budding yeast. EMBO J **16**: 5966–5976.

Du, L.-L., T. M. Nakamura, and P. Russell, 2006 Histone modification-dependent and -independent pathways for recruitment of checkpoint protein Crb2 to double-strand breaks. Genes Dev **20**: 1583–1596.

Dua, R., S. Edwards, D. L. Levy, and J. L. Campbell, 2000 Subunit interactions within the Saccharomyces cerevisiae DNA polymerase epsilon (pol epsilon) complex. Demonstration of a dimeric pol epsilon. J Biol Chem **275**: 28816–28825.

Dua, R., D. L. Levy, and J. L. Campbell, 1998 Role of the putative zinc finger domain of Saccharomyces cerevisiae DNA polymerase epsilon in DNA replication and the S/M checkpoint pathway. J Biol Chem **273**: 30046–30055.

Eker, A. P. M., C. Quayle, I. Chaves, and G. T. J. van der Horst, 2009 DNA repair in mammalian cells: Direct DNA damage reversal: elegant solutions for nasty problems. Cell Mol Life Sci **66**: 968–980.

Evans, T., E. T. Rosenthal, J. Youngblom, D. Distel, and T. Hunt, 1983 Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. Cell **33**: 389–396.

Falconi, M. M., A. Piseri, M. Ferrari, G. Lucchini, P. Plevani, *et al.*, 1993 De novo synthesis of budding yeast DNA polymerase alpha and POL1 transcription at the G1/S boundary are not required for entrance into S phase. Proc Natl Acad Sci U S A **90**: 10519–10523.

Fousteri, M., and L. H. F. Mullenders, 2008 Transcription-coupled nucleotide excision repair in mammalian cells: molecular mechanisms and biological effects. Cell Res **18**: 73–84.

Friedberg, E. C., 2001 How nucleotide excision repair protects against cancer. Nat Rev Cancer **1**: 22–33.

Friedel, A. M., B. L. Pike, and S. M. Gasser, 2009 ATR/Mec1: coordinating fork stability and repair. Curr Opin Cell Biol **21**: 237–244.

Furuya, K., M. Poitelea, L. Guo, T. Caspari, and A. M. Carr, 2004 Chk1 activation requires Rad9 S/TQ-site phosphorylation to promote association with C-terminal BRCT domains of Rad4TOPBP1. Genes Dev **18**: 1154–1164.

Futcher, B., 1996 Cyclins and the wiring of the yeast cell cycle. Yeast **12**: 1635–1646.

Garcia, V., K. Furuya, and A. M. Carr, 2005 Identification and functional analysis of TopBP1 and its homologs. DNA Repair (Amst) **4**: 1227–1239.

Gardner, R., C. W. Putnam, and T. Weinert, 1999 RAD53, DUN1 and PDS1 define two parallel G2/M checkpoint pathways in budding yeast. EMBO J **18**: 3173–3185.

Garvik, B., M. Carson, and L. Hartwell, 1995 Single-stranded DNA arising at telomeres in cdc13 mutants may constitute a specific signal for the RAD9 checkpoint. Mol Cell Biol **15**: 6128–6138.

Giannattasio, M., F. Lazzaro, M. P. Longhese, P. Plevani, and M. Muzi-Falconi, 2004 Physical and functional interactions between nucleotide excision repair and DNA damage checkpoint. EMBO J **23**: 429–438.

Giannattasio, M., F. Lazzaro, P. Plevani, and M. Muzi-Falconi, 2005 The DNA damage checkpoint response requires histone H2B ubiquitination by Rad6-Bre1 and H3 methylation by Dot1. J Biol Chem **280**: 9879–9886.

Giannattasio, M., E. Sommariva, R. Vercillo, F. Lippi-Boncambi, G. Liberi, *et al.*, 2002 A dominant-negative MEC3 mutant uncovers new functions for the Rad17 complex and Tel1. Proc Natl Acad Sci U S A **99**: 12997–13002.

Gilbert, C. S., C. M. Green, and N. F. Lowndes, 2001 Budding yeast Rad9 is an ATP-dependent Rad53 activating machine. Mol Cell **8**: 129–136.

Girard, P. M., and S. Boiteux, 1997 Repair of oxidized DNA bases in the yeast Saccharomyces cerevisiae. Biochimie **79**: 559–566.

Green, C. M., H. Erdjument-Bromage, P. Tempst, and N. F. Lowndes, 2000 A novel Rad24 checkpoint protein complex closely related to replication factor C. Curr Biol **10**: 39–42.

Grenon, M., T. Costelloe, S. Jimeno, A. O'shaughnessy, J. Fitzgerald, *et al.*, 2007 Docking onto chromatin via the Saccharomyces cerevisiae Rad9 Tudor domain. Yeast **24**: 105–119.

Guzder, S. N., Y. Habraken, P. Sung, L. Prakash, and S. Prakash, 1995 Reconstitution of yeast nucleotide excision repair with purified Rad proteins, replication protein A, and transcription factor TFIIH. J Biol Chem **270**: 12973–12976.

Guzder, S. N., C. H. Sommers, L. Prakash, and S. Prakash, 2006 Complex formation with damage recognition protein Rad14 is essential for Saccharomyces cerevisiae Rad1-Rad10 nuclease to perform its function in nucleotide excision repair in vivo. Mol Cell Biol **26**: 1135–1141.

Guzder, S. N., P. Sung, L. Prakash, and S. Prakash, 1998 Affinity of yeast nucleotide excision repair factor 2, consisting of the Rad4 and Rad23 proteins, for ultraviolet damaged DNA. J Biol Chem **273**: 31541–31546.

Haber, J. E., 1998 The many interfaces of Mre11. Cell **95**: 583–586.

Hammet, A., C. Magill, J. Heierhorst, and S. P. Jackson, 2007 Rad9 BRCT domain interaction with phosphorylated H2AX regulates the G1 checkpoint in budding yeast. EMBO Rep **8**: 851–857.

Hanna, J. S., E. S. Kroll, V. Lundblad, and F. A. Spencer, 2001 Saccharomyces cerevisiae CTF18 and CTF4 are required for sister chromatid cohesion. Mol Cell Biol **21**: 3144–3158.

Hardy, C. F., O. Dryga, S. Seematter, P. M. Pahl, and R. A. Sclafani, 1997 mcm5/cdc46-bob1 bypasses the requirement for the S phase activator Cdc7p. Proc Natl Acad Sci U S A **94**: 3151–3155.

Harrison, J. C., and J. E. Haber, 2006 Surviving the breakup: the DNA damage checkpoint. Annu Rev Genet **40**: 209–235.

Hartwell, L. H., J. Culotti, J. R. Pringle, and B. J. Reid, 1974 Genetic control of the cell division cycle in yeast. Science **183**: 46–51.

Hartwell, L. H., and M. B. Kastan, 1994 Cell cycle control and cancer. Science **266**: 1821–1828.

Hartwell, L. H., and T. A. Weinert, 1989 Checkpoints: controls that ensure the order of cell cycle events. Science **246**: 629–634.

Hirschhorn, J. N., A. L. Bortvin, S. L. Ricupero-Hovasse, and F. Winston, 1995 A new class of histone H2A mutations in Saccharomyces cerevisiae causes specific transcriptional defects in vivo. Mol Cell Biol **15**: 1999–2009.

Hoeijmakers, J. H., 1993a Nucleotide excision repair I: from E. coli to yeast. Trends Genet **9**: 173–177.

Hoeijmakers, J. H., 1993b Nucleotide excision repair. II: From yeast to mammals. Trends Genet **9**: 211–217.

Hoeijmakers, J. H., 2001 Genome maintenance mechanisms for preventing cancer. Nature **411**: 366–374.

Hollingsworth, N. M., L. Ponte, and C. Halsey, 1995 MSH5, a novel MutS homolog, facilitates meiotic reciprocal recombination between homologs in Saccharomyces cerevisiae but not mismatch repair. Genes Dev **9**: 1728–1739.

Hollingsworth, R. E., and R. A. Sclafani, 1990 DNA metabolism gene CDC7 from yeast encodes a serine (threonine) protein kinase. Proc Natl Acad Sci U S A **87**: 6272–6276.

Homesley, L., M. Lei, Y. Kawasaki, S. Sawyer, T. Christensen, *et al.*, 2000 Mcm10 and the MCM2-7 complex interact to initiate DNA synthesis and to release replication factors from origins. Genes Dev **14**: 913–926.

Hoyt, M. A., L. Totis, and B. T. Roberts, 1991 S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function. Cell **66**: 507–517.

Hsiang, Y. H., R. Hertzberg, S. Hecht, and L. F. Liu, 1985 Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. J Biol Chem **260**: 14873–14878.

Huyen, Y., O. Zgheib, R. A. Ditullio, V. G. Gorgoulis, P. Zacharatos, *et al.*, 2004 Methylated lysine 79 of histone H3 targets 53BP1 to DNA double-strand breaks. Nature **432**: 406–411.

Ivanov, E. L., N. Sugawara, C. I. White, F. Fabre, and J. E. Haber, 1994 Mutations in XRS2 and RAD50 delay but do not prevent mating-type switching in Saccharomyces cerevisiae. Mol Cell Biol **14**: 3414–3425.

Jackson, A. L., P. M. Pahl, K. Harrison, J. Rosamond, and R. A. Sclafani, 1993 Cell cycle regulation of the yeast Cdc7 protein kinase by association with the Dbf4 protein. Mol Cell Biol **13**: 2899–2908.

Jansen, L. E., R. A. Verhage, and J. Brouwer, 1998 Preferential binding of yeast Rad4.Rad23 complex to damaged DNA. J Biol Chem **273**: 33111–33114.

Javaheri, A., R. Wysocki, O. Jobin-Robitaille, M. Altaf, J. Cï¿œtï¿œ, *et al.*, 2006 Yeast G1 DNA damage checkpoint regulation by H2A phosphorylation is independent of chromatin remodeling. Proc Natl Acad Sci U S A **103**: 13771–13776.

Kamimura, Y., H. Masumoto, A. Sugino, and H. Araki, 1998 Sld2, which interacts with Dpb11 in Saccharomyces cerevisiae, is required for chromosomal DNA replication. Mol Cell Biol **18**: 6102–6109.

Kastan, M. B., and J. Bartek, 2004 Cell-cycle checkpoints and cancer. Nature **432**: 316–323.

Kelley, M. R., Y. W. Kow, and D. M. Wilson, 2003 Disparity between DNA base excision repair in yeast and mammals: translational implications. Cancer Res **63**: 549–554.

Kim, S. T., D. S. Lim, C. E. Canman, and M. B. Kastan, 1999 Substrate specificities and identification of putative substrates of ATM kinase family members. J Biol Chem **274**: 37538–37543.

Kitada, K., L. H. Johnston, T. Sugino, and A. Sugino, 1992 Temperature-sensitive cdc7 mutations of Saccharomyces cerevisiae are suppressed by the DBF4 gene, which is required for the G1/S cell cycle transition. Genetics **131**: 21–29.

Kleczkowska, H. E., G. Marra, T. Lettieri, and J. Jiricny, 2001 hMSH3 and hMSH6 interact with PCNA and colocalize with it to replication foci. Genes Dev **15**: 724–736.

Kumagai, A., and W. G. Dunphy, 2000 Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in Xenopus egg extracts. Mol Cell **6**: 839–849.

Kumagai, A., J. Lee, H. Y. Yoo, and W. G. Dunphy, 2006 TopBP1 activates the ATR-ATRIP complex. Cell **124**: 943–955.

Labib, K., S. E. Kearsey, and J. F. Diffley, 2001 MCM2-7 proteins are essential components of prereplicative complexes that accumulate cooperatively in the nucleus during G1-phase and are required to establish, but not maintain, the S-phase checkpoint. Mol Biol Cell **12**: 3658–3667.

Lazzaro, F., V. Sapountzi, M. Granata, A. Pellicioli, M. Vaze, *et al.*, 2008 Histone methyltransferase Dot1 and Rad9 inhibit single-stranded DNA accumulation at DSBs and uncapped telomeres. EMBO J **27**: 1502–1512.

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

Lee, S. D., and E. Alani, 2006 Analysis of interactions between mismatch repair initiation factors and the replication processivity factor PCNA. J Mol Biol **355**: 175–184.

Lei, M., Y. Kawasaki, M. R. Young, M. Kihara, A. Sugino, *et al.*, 1997 Mcm2 is a target of regulation by Cdc7-Dbf4 during the initiation of DNA synthesis. Genes Dev **11**: 3365–3374.

Lew, D. J., and S. I. Reed, 1995 A cell cycle checkpoint monitors cell morphogenesis in budding yeast. J Cell Biol **129**: 739–749.

Li, G.-M., 2008 Mechanisms and functions of DNA mismatch repair. Cell Res **18**: 85–98.

Llorente, B., and L. S. Symington, 2004 The Mre11 nuclease is not required for 5' to 3' resection at multiple HO-induced double-strand breaks. Mol Cell Biol **24**: 9682–9694.

Lobachev, K., E. Vitriol, J. Stemple, M. A. Resnick, and K. Bloom, 2004 Chromosome fragmentation after induction of a double-strand break is an active process prevented by the RMX repair complex. Curr Biol **14**: 2107–2112.

Lodish, H., A. Berk, L. S. Zipursky, P. Matsudaira, D. Baltimore, *et al.*, 1999 *Molecular cell biology*. 4th edition.

Longhese, M. P., M. Foiani, M. Muzi-Falconi, G. Lucchini, and P. Plevani, 1998 DNA damage checkpoint in budding yeast. EMBO J **17**: 5525–5528.

Longhese, M. P., D. Mantiero, and M. Clerici, 2006 The cellular response to chromosome breakage. Mol Microbiol **60**: 1099–1108.

Longhese, M. P., H. Neecke, V. Paciotti, G. Lucchini, and P. Plevani, 1996 The 70 kDa subunit of replication protein A is required for the G1/S and intra-S DNA damage checkpoints in budding yeast. Nucleic Acids Res **24**: 3533–3537.

Longhese, M. P., V. Paciotti, R. Fraschini, R. Zaccarini, P. Plevani, *et al.*, 1997 The novel DNA damage checkpoint protein ddc1p is phosphorylated periodically during the cell cycle and in response to DNA damage in budding yeast. EMBO J **16**: 5216–5226.

Lopes, M., C. Cotta-Ramusino, A. Pellicioli, G. Liberi, P. Plevani, *et al.*, 2001 The DNA replication checkpoint response stabilizes stalled replication forks. Nature **412**: 557–561.

Lucca, C., F. Vanoli, C. Cotta-Ramusino, A. Pellicioli, G. Liberi, *et al.*, 2004 Checkpoint-mediated control of replisome-fork association and signalling in response to replication pausing. Oncogene **23**: 1206–1213.

Lörincz, A. T., and S. I. Reed, 1984 Primary structure homology between the product of yeast cell division control gene CDC28 and vertebrate oncogenes. Nature **307**: 183–185.

Ma, H., S. Kunes, P. J. Schatz, and D. Botstein, 1987 Plasmid construction by homologous recombination in yeast. Gene **58**: 201–216.

Macdougall, C. A., T. S. Byun, C. Van, M.-C. Yee, and K. A. Cimprich, 2007 The structural determinants of checkpoint activation. Genes Dev **21**: 898–903.

Majka, J., S. K. Binz, M. S. Wold, and P. M. Burgers, 2006a RPA directs loading of the DNA damage checkpoint clamp to 5'-DNA junctions. J Biol Chem **281**: 27855–27861.

Majka, J., and P. M. J. Burgers, 2003 Yeast Rad17/Mec3/Ddc1: a sliding clamp for the DNA damage checkpoint. Proc Natl Acad Sci U S A **100**: 2249–2254.

Majka, J., A. Niedziela-Majka, and P. M. J. Burgers, 2006b The Checkpoint Clamp Activates Mec1 Kinase during Initiation of the DNA Damage Checkpoint. Mol Cell **24**: 891–901.

Marini, F., A. Pellicioli, V. Paciotti, G. Lucchini, P. Plevani, *et al.*, 1997 A role for DNA primase in coupling DNA replication to DNA damage response. EMBO J **16**: 639–650.

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

McElhinny, S. A. N., D. A. Gordenin, C. M. Stith, P. M. J. Burgers, and T. A. Kunkel, 2008 Division of labor at the eukaryotic replication fork. Mol Cell **30**: 137–144.

McGowan, C. H., and P. Russell, 2004 The DNA damage response: sensing and signaling. Curr Opin Cell Biol **16**: 629–633.

Melo, J., and D. Toczyski, 2002 A unified view of the DNA-damage checkpoint.

Melo, J. A., J. Cohen, and D. P. Toczyski, 2001 Two checkpoint complexes are independently recruited to sites of DNA damage in vivo. Genes Dev **15**: 2809–2821.

Mendenhall, M. D., 1993 An inhibitor of p34CDC28 protein kinase activity from Saccharomyces cerevisiae. Science **259**: 216–219.

Mendenhall, M. D., and A. E. Hodge, 1998 Regulation of Cdc28 cyclin-dependent protein kinase activity during the cell cycle of the yeast Saccharomyces cerevisiae. Microbiol Mol Biol Rev **62**: 1191–1243.

Michael, W. M., R. Ott, E. Fanning, and J. Newport, 2000 Activation of the DNA replication checkpoint through RNA synthesis by primase. Science **289**: 2133–2137.

Mordes, D. A., G. G. Glick, R. Zhao, and D. Cortez, 2008a TopBP1 activates ATR through ATRIP and a PIKK regulatory domain. Genes Dev **22**: 1478–1489.

Mordes, D. A., E. A. Nam, and D. Cortez, 2008b Dpb11 activates the Mec1-Ddc2 complex. Proc Natl Acad Sci U S A **105**: 18730–18734.

Moreau, S., J. R. Ferguson, and L. S. Symington, 1999 The nuclease activity of Mre11 is required for meiosis but not for mating type switching, end joining, or telomere maintenance. Mol Cell Biol **19**: 556–566.

Moreau, S., E. A. Morgan, and L. S. Symington, 2001 Overlapping functions of the Saccharomyces cerevisiae Mre11, Exo1 and Rad27 nucleases in DNA metabolism. Genetics **159**: 1423–1433.

Morin, I., H.-P. Ngo, A. Greenall, M. K. Zubko, N. Morrice, *et al.*, 2008 Checkpoint-dependent phosphorylation of Exo1 modulates the DNA damage response. EMBO J **27**: 2400–2410.

Morrow, D. M., D. A. Tagle, Y. Shiloh, F. S. Collins, and P. Hieter, 1995 TEL1, an S. cerevisiae homolog of the human gene mutated in ataxia telangiectasia, is functionally related to the yeast checkpoint gene MEC1. Cell **82**: 831–840.

Naiki, T., T. Kondo, D. Nakada, K. Matsumoto, and K. Sugimoto, 2001 Chl12 (Ctf18) forms a novel replication factor C-related complex and functions redundantly with Rad24 in the DNA replication checkpoint pathway. Mol Cell Biol **21**: 5838–5845.

Nakada, D., Y. Hirano, and K. Sugimoto, 2004 Requirement of the Mre11 complex and exonuclease 1 for activation of the Mec1 signaling pathway. Mol Cell Biol **24**: 10016–10025.

Nakamura, T. M., L.-L. Du, C. Redon, and P. Russell, 2004 Histone H2A phosphorylation controls Crb2 recruitment at DNA breaks, maintains checkpoint arrest, and influences DNA repair in fission yeast. Mol Cell Biol **24**: 6215–6230.

Nasmyth, K., 1996 At the heart of the budding yeast cell cycle. Trends Genet **12**: 405–412.

Navadgi-Patil, V. M., and P. M. Burgers, 2008 Yeast DNA replication protein Dpb11 activates the Mec1/ATR checkpoint kinase. J Biol Chem **283**: 35853–35859.

Navadgi-Patil, V. M., and P. M. Burgers, 2009 A tale of two tails: Activation of DNA damage checkpoint kinase Mec1/ATR by the 9-1-1 clamp and by Dpb11/TopBP1. DNA Repair (Amst) **8**: 996–1003.

Navas, T. A., Z. Zhou, and S. J. Elledge, 1995 DNA polymerase epsilon links the DNA replication machinery to the S phase checkpoint. Cell **80**: 29–39.

Noton, E., and J. F. Diffley, 2000 CDK inactivation is the only essential function of the APC/C and the mitotic exit network proteins for origin resetting during mitosis. Mol Cell **5**: 85–95.

Ogiwara, H., A. Ui, F. Onoda, S. Tada, T. Enomoto, *et al.*, 2006 Dpb11, the budding yeast homolog of TopBP1, functions with the checkpoint clamp in recombination repair. Nucleic Acids Res **34**: 3389–3398.

Ohya, T., S. Maki, Y. Kawasaki, and A. Sugino, 2000 Structure and function of the fourth subunit (Dpb4p) of DNA polymerase epsilon in Saccharomyces cerevisiae. Nucleic Acids Res **28**: 3846–3852.

Osborn, A. J., and S. J. Elledge, 2003 Mrc1 is a replication fork component whose phosphorylation in response to DNA replication stress activates Rad53. Genes Dev **17**: 1755–1767.

Paciotti, V., M. Clerici, G. Lucchini, and M. P. Longhese, 2000 The checkpoint protein Ddc2, functionally related to S. pombe Rad26, interacts with Mec1 and is regulated by Mec1-dependent phosphorylation in budding yeast. Genes Dev **14**: 2046–2059.

Paciotti, V., G. Lucchini, P. Plevani, and M. P. Longhese, 1998 Mec1p is essential for phosphorylation of the yeast DNA damage checkpoint protein Ddc1p, which physically interacts with Mec3p. EMBO J **17**: 4199–4209.

Pang, D., S. Yoo, W. S. Dynan, M. Jung, and A. Dritschilo, 1997 Ku proteins join DNA fragments as shown by atomic force microscopy. Cancer Res **57**: 1412–1415.

Pasero, P., B. P. Duncker, E. Schwob, and S. M. Gasser, 1999 A role for the Cdc7 kinase regulatory subunit Dbf4p in the formation of initiation-competent origins of replication. Genes Dev **13**: 2159–2176.

Paulovich, A. G., and L. H. Hartwell, 1995 A checkpoint regulates the rate of progression through S phase in S. cerevisiae in response to DNA damage. Cell **82**: 841–847.

Pellicioli, A., C. Lucca, G. Liberi, F. Marini, M. Lopes, *et al.*, 1999 Activation of Rad53 kinase in response to DNA damage and its effect in modulating phosphorylation of the lagging strand DNA polymerase. EMBO J **18**: 6561–6572.

Pereira, G., T. Höfken, J. Grindlay, C. Manson, and E. Schiebel, 2000 The Bub2p spindle checkpoint links nuclear migration with mitotic exit. Mol Cell **6**: 1–10.

Piatti, S., C. Lengauer, and K. Nasmyth, 1995 Cdc6 is an unstable protein whose de novo synthesis in G1 is important for the onset of S phase and for preventing a 'reductional' anaphase in the budding yeast Saccharomyces cerevisiae. EMBO J **14**: 3788–3799.

Piggott, J. R., R. Rai, and B. L. Carter, 1982 A bifunctional gene product involved in two phases of the yeast cell cycle. Nature **298**: 391–393.

Puddu, F., M. Granata, L. D. Nola, A. Balestrini, G. Piergiovanni, *et al.*, 2008 Phosphorylation of the budding yeast 9-1-1 complex is required for Dpb11 function in the full activation of the UV-induced DNA damage checkpoint. Mol Cell Biol **28**: 4782–4793.

Pâques, F., and J. E. Haber, 1999 Multiple pathways of recombination induced by double-strand breaks in Saccharomyces cerevisiae. Microbiol Mol Biol Rev **63**: 349–404.

Rademakers, S., M. Volker, D. Hoogstraten, A. L. Nigg, M. J. Moné, *et al.*, 2003 Xeroderma pigmentosum group A protein loads as a separate factor onto DNA lesions. Mol Cell Biol **23**: 5755–5767.

Rattray, A. J., C. B. McGill, B. K. Shafer, and J. N. Strathern, 2001 Fidelity of mitotic double-strand-break repair in Saccharomyces cerevisiae: a role for SAE2/COM1. Genetics **158**: 109–122.

Reed, S. I., and C. Wittenberg, 1990 Mitotic role for the Cdc28 protein kinase of Saccharomyces cerevisiae. Proc Natl Acad Sci U S A **87**: 5697–5701.

Richardson, H. E., C. Wittenberg, F. Cross, and S. I. Reed, 1989 An essential G1 function for cyclin-like proteins in yeast. Cell **59**: 1127–1133.

Ross-Macdonald, P., and G. S. Roeder, 1994 Mutation of a meiosis-specific MutS homolog decreases crossing over but not mismatch correction. Cell **79**: 1069–1080.

Sanchez, Y., J. Bachant, H. Wang, F. Hu, D. Liu, *et al.*, 1999 Control of the DNA damage checkpoint by chk1 and rad53 protein kinases through distinct mechanisms. Science **286**: 1166–1171.

Sanders, S. L., M. Portoso, J. Mata, J. Bähler, R. C. Allshire, *et al.*, 2004 Methylation of histone H4 lysine 20 controls recruitment of Crb2 to sites of DNA damage. Cell **119**: 603–614.

Santocanale, C., and J. F. Diffley, 1998 A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication. Nature **395**: 615–618.

Schwob, E., and K. Nasmyth, 1993 CLB5 and CLB6, a new pair of B cyclins involved in DNA replication in Saccharomyces cerevisiae. Genes Dev **7**: 1160–1175.

Schärer, O. D., 2008 Hot topics in DNA repair: the molecular basis for different disease states caused by mutations in TFIIH and XPG. DNA Repair (Amst) **7**: 339–344.

Segurado, M., and J. F. X. Diffley, 2008 Separate roles for the DNA damage checkpoint protein kinases in stabilizing DNA replication forks. Genes Dev **22**: 1816–1827.

Shuck, S. C., E. A. Short, and J. J. Turchi, 2008 Eukaryotic nucleotide excision repair: from understanding mechanisms to influencing biology. Cell Res **18**: 64–72.

Sia, E. A., and D. T. Kirkpatrick, 2005 The yeast MSH1 gene is not involved in DNA repair or recombination during meiosis. DNA Repair (Amst) **4**: 253–261.

Sidorova, J. M., and L. L. Breeden, 1997 Rad53-dependent phosphorylation of Swi6 and down-regulation of CLN1 and CLN2 transcription occur in response to DNA damage in Saccharomyces cerevisiae. Genes Dev **11**: 3032–3045.

Siede, W., A. S. Friedberg, I. Dianova, and E. C. Friedberg, 1994 Characterization of G1 checkpoint control in the yeast Saccharomyces cerevisiae following exposure to DNA-damaging agents. Genetics **138**: 271–281.

Siede, W., A. S. Friedberg, and E. C. Friedberg, 1993 RAD9-dependent G1 arrest defines a second checkpoint for damaged DNA in the cell cycle of Saccharomyces cerevisiae. Proc Natl Acad Sci U S A **90**: 7985–7989.

Sogo, J. M., M. Lopes, and M. Foiani, 2002 Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. Science **297**: 599–602.

Soulier, J., and N. F. Lowndes, 1999 The BRCT domain of the S. cerevisiae checkpoint protein Rad9 mediates a Rad9-Rad9 interaction after DNA damage. Curr Biol **9**: 551–554.

Sugasawa, K., J. M. Ng, C. Masutani, S. Iwai, P. J. van der Spek, *et al.*, 1998 Xeroderma pigmentosum group C protein complex is the initiator of global genome nucleotide excision repair. Mol Cell **2**: 223–232.

Sugiyama, T., and S. C. Kowalczykowski, 2002 Rad52 protein associates with replication protein A (RPA)-single-stranded DNA to accelerate Rad51-mediated displacement of RPA and presynaptic complex formation. J Biol Chem **277**: 31663–31672.

Sugiyama, T., E. M. Zaitseva, and S. C. Kowalczykowski, 1997 A single-stranded DNA-binding protein is needed for efficient presynaptic complex formation by the Saccharomyces cerevisiae Rad51 protein. J Biol Chem **272**: 7940–7945.

Sung, P., 1997 Yeast Rad55 and Rad57 proteins form a heterodimer that functions with replication protein A to promote DNA strand exchange by Rad51 recombinase. Genes Dev **11**: 1111–1121.

Sweeney, F. D., F. Yang, A. Chi, J. Shabanowitz, D. F. Hunt, *et al.*, 2005 Saccharomyces cerevisiae Rad9 acts as a Mec1 adaptor to allow Rad53 activation. Curr Biol **15**: 1364–1375.

Tak, Y.-S., Y. Tanaka, S. Endo, Y. Kamimura, and H. Araki, 2006 A CDK-catalysed regulatory phosphorylation for formation of the DNA replication complex Sld2-Dpb11. EMBO J **25**: 1987–1996.

Takisawa, H., S. Mimura, and Y. Kubota, 2000 Eukaryotic DNA replication: from pre-replication complex to initiation complex. Curr Opin Cell Biol **12**: 690–696.

Tanaka, S., and J. F. X. Diffley, 2002 Interdependent nuclear accumulation of budding yeast Cdt1 and Mcm2-7 during G1 phase. Nat Cell Biol **4**: 198–207.

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

Taricani, L., and T. S. F. Wang, 2006 Rad4TopBP1, a scaffold protein, plays separate roles in DNA damage and replication checkpoints and DNA replication. Mol Biol Cell **17**: 3456–3468.

Tishkoff, D. X., N. S. Amin, C. S. Viars, K. C. Arden, and R. D. Kolodner, 1998 Identification of a human gene encoding a homologue of Saccharomyces cerevisiae EXO1, an exonuclease implicated in mismatch repair and recombination. Cancer Res **58**: 5027–5031.

Toh, G. W.-L., A. M. O'shaughnessy, S. Jimeno, I. M. Dobbie, M. Grenon, *et al.*, 2006 Histone H2A phosphorylation and H3 methylation are required for a novel Rad9 DSB repair function following checkpoint activation. DNA Repair (Amst) **5**: 693–703.

Toone, W. M., B. L. Aerne, B. A. Morgan, and L. H. Johnston, 1997 Getting started: regulating the initiation of DNA replication in yeast. Annu Rev Microbiol **51**: 125–149.

Tran, P. T., J. A. Simon, and R. M. Liskay, 2001 Interactions of Exo1p with components of MutLalpha in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A **98**: 9760–9765.

Tsubouchi, H., and H. Ogawa, 2000 Exo1 roles for repair of DNA double-strand breaks and meiotic crossing over in Saccharomyces cerevisiae. Mol Biol Cell **11**: 2221–2233.

Unal, E., A. Arbel-Eden, U. Sattler, R. Shroff, M. Lichten, *et al.*, 2004 DNA damage response pathway uses histone modification to assemble a double-strand break-specific cohesin domain. Mol Cell **16**: 991–1002.

Usui, T., S. S. Foster, and J. H. J. Petrini, 2009 Maintenance of the DNA-damage checkpoint requires DNA-damage-induced mediator protein oligomerization. Mol Cell **33**: 147–159.

Usui, T., H. Ogawa, and J. H. Petrini, 2001 A DNA damage response pathway controlled by Tel1 and the Mre11 complex. Mol Cell **7**: 1255–1266.

Visintin, R., K. Craig, E. S. Hwang, S. Prinz, M. Tyers, *et al.*, 1998 The phosphatase Cdc14 triggers mitotic exit by reversal of Cdk-dependent phosphorylation. Mol Cell **2**: 709–718.

Volker, M., M. J. Moné, P. Karmakar, A. van Hoffen, W. Schul, *et al.*, 2001 Sequential assembly of the nucleotide excision repair factors in vivo. Mol Cell **8**: 213–224.

Wakasugi, M., and A. Sancar, 1999 Order of assembly of human DNA repair excision nuclease. J Biol Chem **274**: 18759–18768.

Wang, H., and S. J. Elledge, 1999 DRC1, DNA replication and checkpoint protein 1, functions with DPB11 to control DNA replication and the S-phase checkpoint in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A **96**: 3824–3829.

Wang, H., and S. J. Elledge, 2002 Genetic and physical interactions between DPB11 and DDC1 in the yeast DNA damage response pathway. Genetics **160**: 1295–1304.

Wang, Z., X. Wu, and E. C. Friedberg, 1993 DNA repair synthesis during base excision repair in vitro is catalyzed by DNA polymerase epsilon and is influenced by DNA polymerases alpha and delta in Saccharomyces cerevisiae. Mol Cell Biol **13**: 1051–1058.

Weinert, T., and D. Lydall, 1993 Cell cycle checkpoints, genetic instability and cancer. Semin Cancer Biol **4**: 129–140.

Weinert, T. A., and L. H. Hartwell, 1988 The RAD9 gene controls the cell cycle response to DNA damage in Saccharomyces cerevisiae. Science **241**: 317–322.

Weinert, T. A., G. L. Kiser, and L. H. Hartwell, 1994 Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. Genes Dev **8**: 652–665.

Wu, X., E. Braithwaite, and Z. Wang, 1999a DNA ligation during excision repair in yeast cell-free extracts is specifically catalyzed by the CDC9 gene product. Biochemistry **38**: 2628–2635.

Wu, X., and Z. Wang, 1999 Relationships between yeast Rad27 and Apn1 in response to apurinic/apyrimidinic (AP) sites in DNA. Nucleic Acids Res **27**: 956–962.

Wu, X., T. E. Wilson, and M. R. Lieber, 1999b A role for FEN-1 in nonhomologous DNA end joining: the order of strand annealing and nucleolytic processing events. Proc Natl Acad Sci U S A **96**: 1303–1308.

Wysocki, R., A. Javaheri, S. Allard, F. Sha, J. Côté, *et al.*, 2005 Role of Dot1-dependent histone H3 methylation in G1 and S phase DNA damage checkpoint functions of Rad9. Mol Cell Biol **25**: 8430–8443.

You, Z., L. Kong, and J. Newport, 2002 The role of single-stranded DNA and polymerase alpha in establishing the ATR, Hus1 DNA replication checkpoint. J Biol Chem **277**: 27088–27093.

Zachariae, W., and K. Nasmyth, 1999 Whose end is destruction: cell division and the anaphase-promoting complex. Genes Dev **13**: 2039–2058.

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

Zhou, B. B., and S. J. Elledge, 2000 The DNA damage response: putting checkpoints in perspective. Nature **408**: 433–439.

Zou, L., and S. J. Elledge, 2003 Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science **300**: 1542–1548.

Zou, L., D. Liu, and S. J. Elledge, 2003 Replication protein A-mediated recruitment and activation of Rad17 complexes. Proc Natl Acad Sci U S A **100**: 13827–13832.

Zou, L., and B. Stillman, 2000 Assembly of a complex containing Cdc45p, replication protein A, and Mcm2p at replication origins controlled by S-phase cyclin-dependent kinases and Cdc7p-Dbf4p kinase. Mol Cell Biol **20**: 3086–3096.

Acknowledgements

This work would not have been possible without the help of a number of people that I would like to thank. First of all, my student Gabriele: together we did most of the experiments collected in this thesis. He was the best student I've ever had (don't get a big head, you were just the first...) and he is also a good friend.

Then I have to thank Magda/PubMeg, who worked on a parallel story, for her help and her suggestions, and because joining our data we managed to publish our work. Thanks to Lisa and Bale, even if they were not present anymore in the lab, because if they hadn't prepared all the Ddc1 mutants, it would have been very difficult to arrive at the end of this story. I cannot forget also the elderly of the lab, Federico "the motivator" and Michele "the demotivator" who, carefully balanced, gave us the right spur to go through this work.

Thanks also to all the the other members of Lab 15: Paolo "Jean Plugs" and Ale "Big" for getting Laura angry whenever possible, and Laura for standing them. Daniele, for finding me every other day a new computer issue to deal with, and Panigades for... let me think... what do I have to thank him for? Thanks to Flavio, the only PhD student with a bench *and* a desk, for the time we shared discussing how many ubiquitin bands could be seen in his films.

I must not forget the people in Lab6. The yeast side: Roberto, Ben and Matteo; and the human side: Manu, Sarah and Giuseppe.

And finally, I have to thank Marco and Paolo because they gave me the possibility to work in their lab, and to follow the topics I was interested in.

Thank you all! It's all your merit if these three years passed in a glimpse!

I'd like to dedicate the last lines to thank the ones who kept me company this last month: LaTeX and LyX. Thanks for allowing me to write these pages without being too worried about typographical matters.