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Histone-dependent and histone-independent pathways for Rad9 chromatin recruitment and checkpoint activation

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Se vuoi arrivare primo, corri da solo.

Se vuoi camminare lontano, cammina insieme.

PART I -

INTRODUCTION

I

THE CELL CYCLE AND ITS CONTROL

GENERAL ASPECTS OF THE EUKARYOTIC CELL CYCLE

The proliferation of eukaryotic cells relies on the accurate execution of the cell cycle, a series of interconnected and genetically controlled events which lead a mother cell to give birth to two daughter cells. It is therefore crucial that, during the cell cycle, the exact replication of the genetic material and its correct segregation into the two daughter cells are guaranteed. These two processes characterize the foremost phases of each cell cycle: the S - "synthesis"- phase, in which genomic DNA is faithfully replicated and the M - "Mitosis"- phase, during which replicated chromosomes segregate in the two daughter cells. The two moments are divided by two time lapses called "gap": the G1 phase, which elapses from the end of mitosis and the beginning of the S phase, and the G2 phase, encompassed between the end of replication and the beginning of mitosis (fig. I1). The two gaps have variable lengths among different organisms and also among different tissues; sometimes they could even be skipped during the course of the cell cycle, contrary to the S and M phases, which are essential for cell viability.

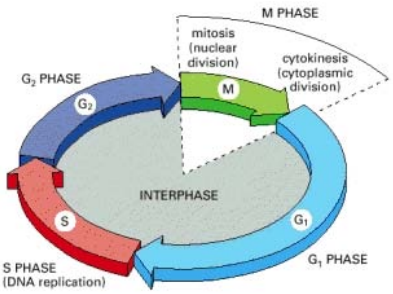

Fig.I 1 The eukaryotic cell cycle (Alberts et al. Molecular Biology of the cell, 4th ed., 2002).

During the G phases, the cell checks the environmental cues and its own metabolic conditions and prepares for the following phases, growing in mass and synthesizing the required proteins (Hartwell and Weinert, 1989). It is also essential that any damage in the genome is recognized and corrected before DNA replication or mitosis start. If these controls fail, the cell might meets catastrophic events which compromise its viability.

Lots of factors participate to the fine setting of cell cycle progression. In particular, the correct execution of the cell cycle is finely tuned by the eterodimeric CDK (Cyclin-Dependent Kinase) complexes, which are composed of a catalytic subunit, the kinase, and a regulatory subunit, the cyclin. Cyclins are unstable proteins which are periodically synthesized 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. Therefore 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.

The Cdk complexes are also subjected to the action of different CKI or CdK-Inhibitors (Mendenhall, 1993) that bind the catalytic subunit, inactivating it. A further level of regulation of CdK activity is represented by covalent modifications of the cyclins, in particular phosphorylations and dephosphorylations. (Mendenhall and Hodge, 1998)

CELL CYCLE PROGRESSION IN *Saccharomyces cerevisiae*

The first complete characterization of the eukaryotic cell cycle was performed at the beginning of the '70s by a pioneeristic study conducted by Lee Hartwell using the budding yeast *Saccharomyces cerevisiae* as a model organism (Hartwell et al., 1974)

Budding yeast is a non pathogenic unicellular fungus belonging to the ascomycetes family. It has a duplication time of 90 minutes and a small genome of 12 Mb, organized in 16 chromosomes which have been completely sequenced in 1996 (Goffeau et al., 1996).

Although small, this organism maintains much of the functional and structural complexity of higher eukaryotes. This aspect, combined with its genetic versatility, makes it an invaluable model organism to understand the molecular details of complex biochemical mechanisms, such as the control of the cell division, which occurs by budding. Yeast cells display morphological characters typical of the cell cycle phase in which they are. In particular, bud emergence is used as a standard marker for entry into S-phase and thus defines the G1/S transition. In large-budded cells, nuclear migration and spindle formation are markers for the G2/M transition, whereas completion of anaphase can be determined by the presence of divided nuclei (fig. I2).

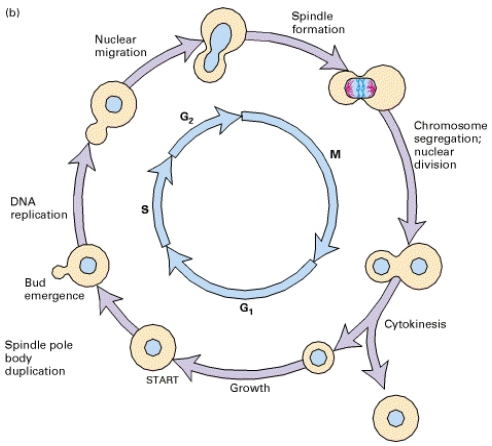

Fig.I 2 The budding yeast S. cerevisiae cell cycle (Lodish et al. Molecular Cell Biology, 4th ed., 2000)

In their study, Hartwell and colleagues isolated a set of conditional mutants that exhibited alterations in different stages of the cell cycle. The correspondenting genes were called for this reason *CDC* genes, for Cell Division Cycle (Hartwell et al., 1974).

Among the genes identified, *CDC28* turned out to be of capital importance for cell cycle progression (Lorincz and Reed, 1984). This gene encodes for a 34 kDa protein with serine/threonine kinase activity required for both G1/S and G2/M transitions, which is the only essential CdK present in this organism (Piggot et al., 1982; Reed and Wittenberg, 1990).

Cdc28 binds are at least nine cyclins whic belong to three distinct subclasses: G1 cyclins (Cln1, Cln2 and Cln3) (Hadwiger et al., 1989), S cyclins (Clb5 and Clb6) (Epstein and Cross, 1992; Scwob and Nasmyth, 1993) and G2 cyclins (Clb1, Clb2, Clb3 and Clb4) (Ghiara et al., 1991; Surana et al, 1991). In each subclass some cyclins seem to be at least partially redundant with others since none of the genes encoding for cyclins is essential for cell viability (Nasmyth, 1996),

whereas the contemporary absence of two or three cyclins belonging to the same class is lethal (Richardson et al., 1989).

The progression through the mitotic cycle requires the passage through a restriction point in G1, named START, during which the cell monitors parameters, such as completionof the previous cell cycle phase, nutritional compounds avaibility and the reaching of a critical mass. The satisfaction of the last requirements is essential to avoid the generation of daughter cells that are born smaller at each cell cycle.

In G1, just before START, the CKI Sic1 and the cyclins B proteolisis inactivate Cdc28. In G1 the only cyclin expressed is Cln3, which, unlike the other cyclins, does not fluctuate during the cell cycle. The activity of the Cdc28/Cln3 complex remains low until the cell does not reach the critical mass necessary to begin a new replicative cycle. When this condition is satisfied, Cln3 levels increase and the activity of Cdc28/Cln3 is now sufficient to allow the activation of a wide S-phase transcritptional programme, called also "*CLN2* cluster", responsible for the synthesis of Cln1, Cln2, Clb5 and Clb6. (Spellman et al., 1998).

The expression of these genes leads to the formation of the Cdc28/Cln1-2 complexes in G1, which allow cells to overcome the START and to initiate bud emergence and spindle pole body duplication. During the G1 phase, also the Cdc28/Clb5-6 complexes are formed, but their activity is initially inhibited until the beginning of the S phase by Sic1.

In late G1, the Cdc28/Cln1-2 complexes, present in large amount, phosphorylate Sic1 on at least six sites at the N-terminus, targeting it to the ubiquitin- and Cdc34-dependent degradation pathway (Nash et al., 2001). In these conditions, the Cdc28/Clb5-6 complexes become active and they allow the beginning of the DNA replication at ARSes, promoting the conversion of pre-replicative (pre-RC) complexes into post-replicative (post-RC) complexes (Andrews and Measday, 1998). The post-RC complexes are maintained until the end of M-phase by Cdc28 activity, in order to prevent the re-use of the same origin and thus rereplication within the same cell cycle (Noton and Diffley, 2000).

At the end of S phase, the transcription and subsequent synthesis of Clb3 and Clb4 begins. Just before the G2, the levels of Clb3 and Clb4 associated with Cdc28 peak, allowing the assembly of the mitotic spindle. In G2, a second set of genes, including *CLB1* and *CLB2* (the "*CLB2* cluster"), is transcribed. The correspondent products, in complex with Cdc28, are required for entry into mitosis, spindle elongation and the transcriptional repression of the *CLN2* cluster. In this phase Cdc28/Clb3-4 activates also the transcription of *ACE2*, *SWI5* and *APC1* (Lydall et al., 1991; Althoefer et al., 1995; Maher et al., 1995; Spellman et al., 1998). The products of the first two genes are transcription factors momentarily confined to the cytoplasm, while the latter encodes the largest subunit of the Anaphase Promoting Complex/Cyclosome (APC/C), a large multimeric complex with ubiquitin ligase activity.

During the last phases of mitosis, Cdc28/Clb1-2 complexes leads to increased activity of APC/C, which is required for the complete ubiquitin-dependent degradation of all B type cyclins and thus for the exit from mitosis.

In the meantime, at the end of mitosis, Swi5 and Ace2 enter the nucleus and activate the transcription of different genes, including *CTS1*, whose product is required for cytokinesis, and *SIC1*. The inhibition of Cdc28 activity and the presence of APC/C allow exit from mitosis and the re-establishment of the pre-replicative status on ARSes (Zachariae and Nasmyth, 1998).

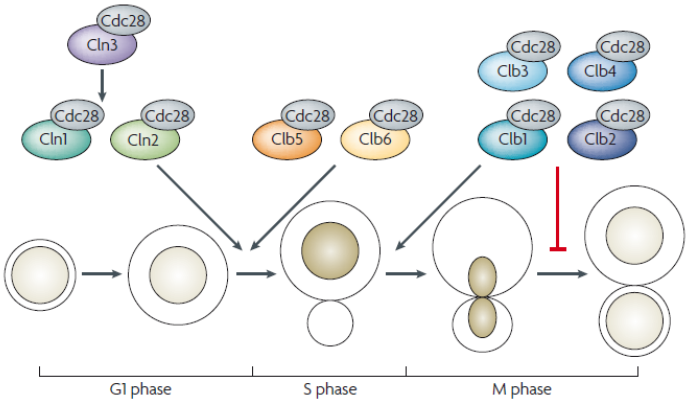

Fig.I 3: Cyclins in budding yeast cell cycle (from Bloom and Cross, 2007)

2

THE DNA DAMAGE RESPONSE - DNA REPAIR SYSTEMS

A low mutational rate is essential to guarantee the genetic variability necessary for the evolution; however, the survival of an organism depends, first of all, upon the stability of its genetic material. Life and biodiversity require therefore a proper balance between the onset of new mutations and the ability of repairing them.

Cells are continually exposed to genomic insults resulting from exposure to exogenous chemicals and physical agents (i.e. benzopyrene, polychlorinated biphenyls, dioxin, cigarette smoke, asbestos, ultraviolet light, radon). Even cell metabolism produces extremely toxic intermediates, such as reactive oxygen and nitrogen species (ROS and NOS), able to attack the DNA phosphodiesteric backbone or to produce base alterations. Moreover, the relative fidelity of DNA polymerases makes DNA replication itself a virtually mutagenic event: replicative errors produce a permanent DNA modification that could eventually result in the alteration of a coding sequence or within the regulatory regions of a gene.

When exposed with DNA damage, the eukaryotic cell activates a complex network of biochemical pathways, known as DNA Damage Response (DDR), which detects and propagates the initial DNA damage signal to elicit cellular responses that include cell cycle arrest, DNA repair and eventually

apoptosis. Disregulation of components involved in these processes contributes to genomic instability, which in turn leads to tumorigenesis.

The major objective of the cellular response to DNA damage is to repair the lesion and to restore the original DNA sequence. To maintain genomic integrity, prokaryotic and eukaryotic cells evolved numerous highly sophisticated systems, able to recognize and repair all the different damages DNA can suffer.

DNA REPAIR SYSTEMS

DNA repair systems (fig. I4) can be classified in three general categories:

- Direct Damage Reversal;
- Excision Repair, that includes three repair systems that achieve of direct excision of the lesion:
    - BER (Base Excision Repair);
    - NER (Nucleotide Excision Repair);
    - MMR (MisMatch Repair);
- Double Strand Break Repair (DSBR) and its two sub-pathways:
    - Non Homologous End Joining (NHEJ);
    - Homologous Recombination (HR);

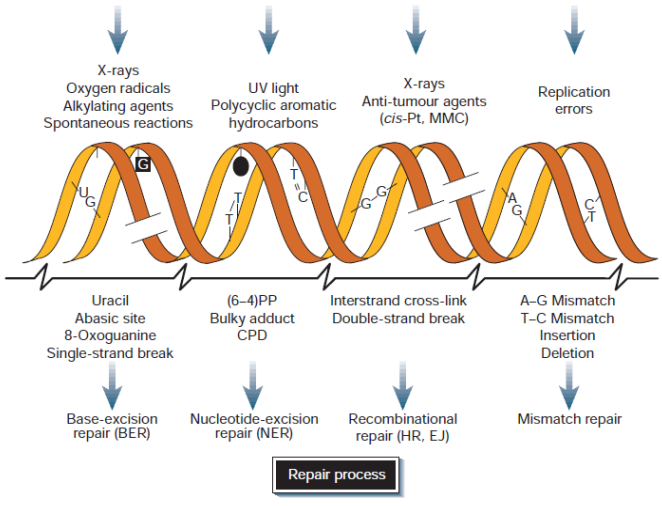

Fig.I 4 Common DNA damaging agents (top); examples of DNA lesions induced by these agents (middle) andmost relevant DNA repair mechanisms responsible for the removal of these lesions (bottom). (Hoeijmakers, 2001)

Direct Damage Reversal (DDR)

DNA Damage Reversal is a repair system which provides for the elimination of the damaged nucleotide in a one-step reaction, with the involvement of one, specific enzyme.

Thanks to its speed and low demand of energy, damage reversal is particularly important for coping with DNA lesions that occur fairly frequently, such as UV-induced pyrimidine dimers or alkylated bases, arisen after the transfer of a methylic or a ethylic group on a base.

The formation of pyrimidine dimers upon irradiation with UV light results in an abnormal covalent bond between adjacent pyrimidine bases that distort the DNA base pair structure. Cyclobutyl dimers are repaired by a light-dependent direct system called photoreactivation. This process involves an enzyme called photolyase. When stimulated by light with a wavelength between 300 and 500 nm the enzyme binds to cyclobutyl dimers and converts them back to the original monomeric nucleotides. Photoreactivation is a widespread but not universal type of repair: it is known in many but not all bacteria and also in quite a few eukaryotes, including some vertebrates, but is absent in humans and other placental mammals.

The base alkylation gives rise to O6 methylguanidines, guanine residues methylated on the O6 position, able to pair with thymine residues, rather than with cytosine ones. The elimination of these damaged nucleotides requires the action of the O6 methylguanine methyltransferase, which transfers the methylic group of the O6 methylguanidine on a cysteine within its active site. The S-methylcysteine which arises in the methylatrasferase catalytic domain is nevertheless particularly stable; the methylated enzyme can be hardly regenerated, causing the fast saturation of this repair system after the exposition to alkylating agents. (Friedberg E.C. et al, 2006).

Excision Repair

Excision repair systems represent the most important repair mechanisms for eukaryotic and prokaryotic cells; thanks to base excision repair, nucleotide excision repair and mismatch repair, cells are able to face with a large number and a wide range of DNA lesions.

BER: Base Excision Repair

The main targets of Base Excision Repair (BER) are oxidized, alkylated or ROS (Reactive Oxygen Species) damaged bases, moreover it repairs lesions caused by depurination of nucleotides and deamination of nitrogen bases.

The repair process begins with the intervention of a lesion- specific N-glycosylase, which recognizes a particular class of lesions and removes the damaged base through the hydrolysis of the N-glycosidic bond that anchors it to the sugar phosphodiesteric backbone.

The resulting apurinic or apyrimidinic site (which can also arise also after spontaneous hydrolysis) is than processedby an AP endonuclease which leaves a 3'OH end adjacent to a 5' deoxyribosephosphate 5'dRP. Thanks to its lyase domain, DNA Polb, removes the 5'dRP left behind by the AP endonuclease cleavage. Then,the replicative DNA polymerase polδ and polε along with the processivity factor PCNA attach a new nucleotide starting from the 3'OH end, using the complementary DNA strand as template. The completion of the repair is then achieved thanks to a DNA ligase which sticks the ends of the nick. (Friedberg E.C. et al., DNA repair and mutagenesis, 2006)

NER: Nucleotide Excision Repair

Nucleotide Excision Repair (NER) is the main repair system for UV-induced lesion (photoproducts, cyclobutane pyrimidine dimers and 6-4 pyrimidine-pyrimidone photoproducts) 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 recognize, rather than specific damaged nucleotides, the distortion of the double helix itself.

In *S. cerevisiae* the recognition of the lesion is made by Rad14, a protein that shows high affinity for UV damaged DNA, in collaboration with RPA and Rad4-Rad23 complexes (He et al. 1995; Burns et al., 1996; Guzder et al., 1998).

Once lesion has been recognized, the helicases Rad3 and Rad25 unwind the DNA respectively in 5' → 3' and in 3' → 5' direction, confining the lesion in a single stranded bubble. This structure represents the substrate for the endonucleolytic activity of the Rad1-Rad10 complex and for Rad2 which cut the ssDNA, respectively, at the 3' and at the 5' of the damage, releasing a 27-30 nt long fragment, which contains the lesion (Habraken et al., 1993; Davies et al., 1995).

The free ssDNA is quickly bound by RPA, which is then used as a template by DNA polymerases δ and ε for the synthesis of the complementary helix.

The ends of the fragment newly synthesized are then joined with the ends of the adjacent DNA thanks to the action of DNA ligase I, encoded by the *CDC9* gene (Shivji et al., 1995; Wu et al., 2001).

Biochemical and genetic studies, performed both in yeast and in higher eukaryotes, demonstrated that NER acts through two sub-pathways: the Global Genome Repair (GGR), which provides to the repair of the lesions which occur on the non-transcribed strand and non-codifying regions, and the

Transcription-Coupled Repair (TCR), which repairs the lesions which attend on the transcribed strand.

The main differences among the two sub-pathways relies in the factors required during the first recognition steps.(Hoeijmakers, 2001). In particular, it has been shown that the Rad7-Rad16 complex, that has both helicase and ATPase activity, plays a role in GGR, since it binds specifically non-transcribed DNA in an ATP-dependent way (Guzder et al., 1997). On the other hand, Rad26 turned out to be essential for the activation of TCR through physical and functional interactions with transcriptional complexes (van Gool et al., 1994).

The different enzymatic activities involved in NER can be associated in sub-complexes, called NEF, Nucleotide Excision Repair Factors, composed by several proteins which participate to a common function. In budding yeast four are the NEF described, whose order of arrival has still under investigation. It has been hypothesized that NEF4, composed by Rad7 and Rad16, is the first which binds the lesion; then NEF2 is recruited thanks to the physical interaction between Rad7 and the Rad4-Rad23 complexes which is established of. The simultaneous occurrence of the two NEFs reinforces synergically the binding of the complexes to the damaged site. Finally, the ability of Rad23 to interact with Rad14 and TFIIH (composed by Rad3, Rad25, SSL1, TBF1, TBF2, TBF3), might suggest that Rad1-Rad10 (NEF1) and Rad2-TFIIH (NEF3) are recruited in a following step, whereas RPA might join the repairosome last (Prakash and Prakash, 2000).

MMR: MisMatch Repair

Replication is an extraordinary faithful process; mutations occur at a frequency of roughly 1 in 10^9 to 10^{10} base pairs per cell division. Nucleotide selection at the base incorporation step and the proofreading function of DNA polymerases collectively result in an error rate of approximately 10^7 per bp per genome (Hsieh and Yamane, 2008). These rare polymerization errors that escape proofreading are mostly single base-base mismatches or one to a few unpaired nucleotides in the template strand (deletion mismatches) or in the primer strand (insertion mismatches). It is the responsibility of the general MMR pathway to remove these errors from the nascent strand in a manner that restores the parental genotype.

The mechanism of mismatch repair has been firstly described in E. coli, where it has been demonstrated that the protein MutS recognizes and binds the mismatched region of DNA as homodimer. Subsequently, the homodimer of MutL and MutH associate with MutS. The formation of this complex, which requires the expense of ATP, activated a latent endonucleolytic activity of MutH. This enzyme is bound to hemimethylated GATC sites. These sites are normally methylated on adenines, but because the modifying enzyme, deoxyadenine methylase (Dam), lags behind the replication fork by approximately 2 minutes, the newly synthesized strand is transiently unmethylated. Therefore, MutS/MutL-activated MutH uses this time window to incise the unmethylated - newly syntesized strand. The UvrD helicase, probably recruited by MutL, thanks to

its 5' → 3' activity, unwinds the ends of nicked error-containing strand from the template. This enables several exonucleases to digest the unwound DNA, either in the 5'→3' direction, when the nearest hemi-methylated GATC site lies 5' from the mismatch, or in the 3' → 5' direction if it lies 3' from the mismatch. The exonucleolytic degradation stops once the mismatch has been removed. The resulting gap is then filled by DNA polymerase III and the repair is completed when DNA ligase seals the remaining nick (Kunkel and Erie, 2005).

Eukaryotic MMR, although similar, is more complicated due to the presence of distinct partially redundant MutS homologues (MSH) which recognize different type of mismatches, and different MutL homologues (MLH). Moreover, there is no know MutH protein in eukaryotic cells; this leaves the problem to find an entry point for the strand excision activities.

In *S. cerevisiae* six homologues of MutS (Msh1-6) and four of MutL (Mlh1-3 and Pms1) has been identified. Msh2/3/6 form the heterodimeric complexes MutSα (Msh2-Msh6) and MutSβ (Msh2-Msh3) that specifically recognize mispairs and IDLs, respectively. The remaining MutS homologues seem not to be involved in MMR (Ross-Macdonald, 1994; Hollingsworth, 1995; Sia and Kirkpatrick 2005). The multiple MutL homologs form different heterodimers: MutLα (Mlh1-Pms1), MutLβ (Mlh1-Mlh2) and MutLγ (Mlh1-Mlh3); but mainly MutLα ,with whom MutSα and MutSβ interact, is involved in the repair of the majority of the mismatches (Kunkel and Erie, 2005).

The demonstration of a physical interaction between PCNA and the Mut factors Msh2 and Mlh1 (Umar et al., 1996) suggested a model in which DNA replication and MMR may be coupled. In particular, PCNA may help localize MutSα and MutSβ to mispairs in newly replicated DNA (Lau and Kolodner, 2003; Lee and Alani, 2006; Shell et al., 2007). The binding of MutSα to mismatched substrates was shown to lead to its dissociation from PCNA, indicating that the processivity factor might hand the mismatch over to the MMR machinery once the mismatch is detected (Lau and Kolodner, 2003).

The Msh proteins are ATPases that possess an highly conserved ATP-binding motif. It has been proposed that MutS initially binds to mismatched DNA in ADP bound state. Mismatched DNA binding then provokes an ADP-ATP exchange, resulting in conformational changes that form MutS-sliding clamp, which leaves the mismatch and diffuses on DNA bidirectionally, searching a signal that allows the discrimination between the template and the newly synthesized strand (Iaccarino et al., 2000). This process is suggested to occur iteratively to load multiple ATP-bound MutS clamps that can interact with MutL.

The nature of the signal allowing the discrimination between the parental and the newly synthesized strand is still under discussion, but the most favoured theory suggests that the repair machinery may recognize the discontinuity of the newly synthesized strand. In newly synthesized

strands, discontinuities can exist as 3'-ends or as 3'-ends or 5'-termini of Okazaki fragments (Jiricny, 2006).

Once the daughter molecule has been recognized, MutLα generates an incision on the discontinuous strand of the mismatch. It was demonstrated that MutLα incises the discontinuous strand at a distal site from the pre-existing strand break. The degradation of the fragment containing the misincorporated nucleotide is carried out by exonucleases, both in 3'→5' and 5'→3' directions. In budding yeast MMR, the only exonuclease certainly involved is Exo1 (Tishkoff et al., 1997), which, although has only 5' → 3' polarity, seems to participate in both the 3' → 5' and 5' → 3' degradation (Dzantiev et al., 2004). While Exo1 can readily carry out 5' directed mismatch excision in the presence of MutSα or MutSβ and RPA (Genschel and Modrich, 2003; Zhang and Paull, 2005) , its role in catalyzing 3' nick-directed excision requires the MutLα endonuclease, which is activated by PCNA and RFC (Dzantiev et al., 2004; Kadyrov et al., 2006). After the recognition of the 3' nick and the mismatch, MutLα endonuclease might make an incision 5' to the mismatch in a manner dependent on PCNA and RFC; Exo1 might then perform 5'→ 3' excision from the MutLα incision site through and beyond the site of the mismatch (Kunkel et al., 2005; Kadyrov et al., 2006). However, since *exo1* null mutants in yeast and in mice have a weak mutator phenotype (Amin et al., 2001; Wei et al., 2003), it is likely that additional unidentified exonucleases are involved in the excision step of eukaryotic MMR.

Once the mismatch is removed, Exo1 activity is actively inhibited by MutLα and RPA, bound to the ssDNA (Genschel and Modrich, 2003). Polδ and PCNA then fill the gap and DNA ligase I seals the remaining nick to complete the repair process.

Double Strand Break Repair

DNA double strand breaks (DSBs) represent a particular dangerous type of DNA damage which can arise from endogenous sources, including reactive oxygen species generated during cellular metabolism or when the DNA polymerase encounters a lesion in the template or a secondary DNA structure during DNA replication- DSBs are also generated by exogenous sources, including ionizing radiation (IR) and chemicals, that directly or indirectly, damage DNA and are often used in cancer therapy.

DSBs pose a particularly dangerous threat to cell viability and genome integrity, because, if left unrepaired or inappropriately repaired, they can result in cell death or can originate large-scale chromosome changes, including deletions, translocations, and chromosome fusions that enhance genome instability and are hallmarks of cancer cells.

Depending on the nature of the DSB and the cell cycle phase in which the damage is detected, eukaryotic cells have evolved two major pathways for repairing DSBs: non-homologous end joining (NHEJ) and homologous recombination (HR). The DSB repair pathways appear to compete for DSBs, but the balance between them differs widely among species, between different cell types or a single species, and during different cell cycle phases of a single cell type (see below).

NHEJ: Non Homologous End Joining

NHEJ allows the joining of two chromosomal ends with no, or minimal, base pairing at the junction (Moore and Haber, 1996) (Fig. I5, A). However, while its ability to ligate essentially any pair of DNA ends makes NHEJ a very effective mechanism for DSB repair, it also makes it intrinsecally mutagenic, because ligation of DNA ends with partially or fully non complementary overhangs might cause the loss of genetic information.

In both yeast and mammals, NHEJ begins when the DSB ends are bound by the Ku heterodimer, which consists of the Ku70 and Ku80 proteins. Ku is thought to form a ring-like structure that binds to DNA ends, holding them together so to facilitate the joining ligation. In mammalian cells, Ku interacts with the DNA-dependent protein kinase catalytic subunit (DNS-PKcs) and together they may act to synapse the two DNA ends to be repaired (DeFazio et al., 2004). DNA ends are then joined by DNA ligase IV (Dnl4 in yeast), helped by XRCC4 in human cells (Daley et al., 2005; Hefferin and Tomkinson, 2005). XRCC4 does not possess any enzymatic activity but acts as a scaffold that forms interactions with both Ku and DNA and therefore, stabilizes and stimulates the ligase activity (Grawunder et al., 1998).

NHEJ can join DNA ends with different structures, for this reason it may envisage a initial processing step. In human cells, it has been shown that the Artemis nuclease participates to this step. It is recruited to DSB sites by interactions with DNA-PKcs and it cleaves a variety of DNA overhangs (Ma et al., 2005). Also the scMRX/hMRN complex has been reported to be involved. This complex contains three subunits: Mre11, Rad50 and Xrs2 (human Nbs1), each one with a specific function. Mre11 contains a highly conserved phosphoesterase domain and possesses both exo- and endo-nucleolytic activities (Paull and Gellert, 1998). However, the nuclease activity does not appear to be necessary to process mismatched nucleotides of incompatible DNA ends prior to ligation, because NHEJ is not affected in the nuclease defective *mre11* mutants (Moreau et al., 1999; Zhang and Paull, 2005). Rad50 contains a split ATPase domain at its termini, separated by a long looped coiled-coil, which associates at its tip with another Rad50 molecule in a structure called the “Zn-hook” (Shin et al., 2004). Moreover, Rad50 belongs to the Structural Maintenance of Chromosome (SMC) protein family, whose members are implicated in sister chromatid cohesion. These physical features suggest that MRX might be involved in the tethering of DNA ends, together with Ku (Kaye et al., 2004; Lobachev et al., 2004; Wiltzius et al., 2005). Finally, Xrs2 (hNbs1) seems to be involved in the recruitment of the MRX complex to DSB (D’Amours and Jackson, 2002). The entire Mre11 complex acts as a single functional unit because loss of any of the three subunits

results in similar phenotypes, such as hypersensitivity to DNA.damaging agents, impaired HR and defective meiosis (Krogh and Symington, 2004).

Besides the tethering function, MRX might have a role in assisting yeast Ku and Dnl4, as suggested by the findings that MRX stimulates *in vitro* ligation by the Dnl4/Lif1 complex (Chen et al., 2001) and interacts with Ku and Lif1 (Palombos et al., 2005).

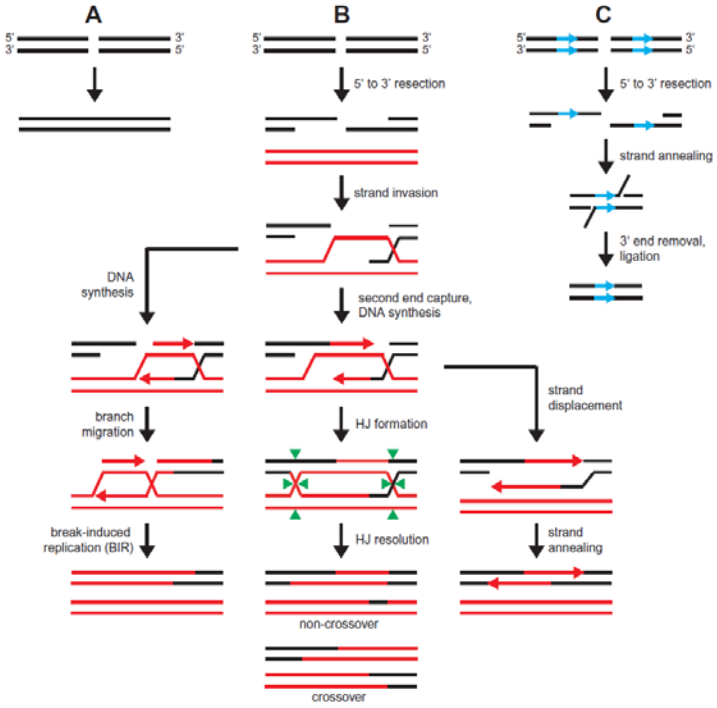

Fig.I 5 Different pathways of DSB repair: A = Non Homologous End Joining (NHEJ); B = Homologous Recombination pathways; B - left = Break Induced Recombination (BIR), B - centre = Double Strand Break Repair (DSBR), B - right = Single Strand Dependent Annealing (SDSA); C = Single Strand Annealing (SSA) (Longhese et al., 2006)

HR: Homologous Recombination

HR is considered a more accurate mechanism for DSB repair because broken ends use homologous sequences elsewhere in the genome (sister chromatids, homologous chromosomes or repeated region on the same or different chromosomes) to prime repair synthesis. If the repair template is perfectly homologous (as in the case of sister chromatids), repair can be 100% accurate; that's why this pathway is commonly described as "error-free". However, if the repair templates are not perfectly homologous, HR results in Gross Chromosomal Rearrangements (GCR), such as deletions, inversions or loss of heterozygosity.

HR initiates with extensive 5' to 3' end-processing at broken ends, carried out by specific nucleases (fig.I5, B). In yeast, resection is a two-step process catalyzed by numerous partially redundant nucleases, including Mre11, Sae2, Dna2 and Exo1 (Moreau et al., 2001; Clerici et al., 2005; Huertas et al., 2008; Mimitou et al., 2008, Zhu et al., 2008).

The model actually accepted proposes that the DSB-ends resection is initiated by the endonuclelytic activity of MRX complex in collaboration with Sae2, which are particularly important for the removal of hairpins, bulky adducts and other irregular end structures (Lengsfeld et a., 2007). This endonucleolytic activity might release small ssDNA oligonucleotides, which have been observed in yeast in the processing of meiotic DSBs (Neale et al., 2005) and in Xenopus egg extracts after DNA damage (Jazayeri et al., 2008).

Since DNA resection is slightly affected by the absence of Sae2 and in *mre11* nuclease-defective mutants, the existence of additional nucleases has been proposed. One of these is Exo1, a 5'→3' exonuclease conserved from yeast to human cells (Tran et al., 2004) and essential for end processing at uncapped telomeres and already described in the MMR section (Maringele and Lydall, 2004). *sae2 exo1* and *exo1 mre11* double mutants show a synergistic decrease in DNA-end resection and greater DNA-damage sensitivity than the single mutants (Mantiero et al., 2007). Moreover, overexpression of *EXO1* partially rescues the DNA sensitivity phenotype of *mre11* mutants, suggesting that Mre11 and Exo1 may function in parallel pathways (Moreau et al., 2001).

Since *exo1mre11* deletion mutants display a residual resection activity, it has been proposed that a third pathway exists (Moreau et al., 2001). This depends upon Dna2, a conserved endonuclease/helicase implicated in Okazaki fragment processing, working together with Sgs1 helicase. *sgs1* and *dna2* deletion mutants exhibit no defect in resection of sequences close to the DSB, but resection monitored far from the break site is reduced (Mimitou and Symington, 2008; Zhu et al., 2008)

The current model suggest that the DNA partially resected by MRX/Sae2 is further processed by the action of either of Exo1 or Dna2 in collaboration with the Sgs1 helicase. The initial processing by Mre11 and Sae2 can be bypassed in mitotic interphase, probably by the action of Exo1 or Sgs1/Dna2. In the absence of Exo1 and Sgs1, the endonucleolytic activity of Mre11 and Sae2 will be sufficient for short processing close to the ends (see fig. I6) (Huertas, 2010)

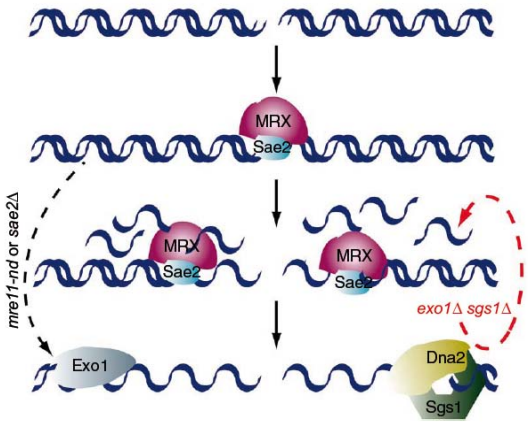

Fig.I 6 The two-step model for DSB end resection in budding yeast (Huertas, 2010)

Resection generates long stretches of 3' ssDNA tails, which are quickly covered by replication protein A (RPA) , whose role is to protect the DNA from further processing and to prevent formation of secondary structures in the DNA, which would inhibit the binding of HR factors (Alani et al., 1992; Sugiyama et al., 1997). RPA facilitates the recruitment of Rad52 to DSBs likely via a physical interaction. RPA is then removed and substituted by the recombinase Rad51 in a reaction mediated by Rad52 and two Rad51 paralogs, Rad55 and Rad57 (Lisby et al., 2004). Once recruited to the ssDNA filament, Rad51 catalyzes the strand exchange, during which ssDNA invades homologous duplex DNA forming a displacement loop (D-loop). Once formed, the D loop can have multiple fates, in fact it can be channeled into different recombination pathways (Fig. I5,B).

During BIR (Break Induced Replication) (fig.I5, B-left), which occurs generally at telomeres, the 3'-ended ssDNA tails invades the duplex homologous DNA region on a sister chromatid or on a homologous chromosome. Following strand invasion, the 3' end is extended by DNA synthesis, generating a unidirectional replication fork that migrates along the template chromosome (branch migration), copying the genetic information, until the DNA polymerase reaches the end of the chromosome.

During double-strand-break repair (DSBR) (fig.I5, B-centre), the second end is captured and extended by DNA synthesis. The newly synthesized DNA is ligated to the end of resected strands to form two cruciform structures known as Holliday junctions, which can be resolved to give either crossover or non-crossover products, depending on how the junction is cut.

Finally during the Synthesis Dependent Strand Annealing (SDSA) (fig.I5, B-right), the newly synthesized invading strand can be displaced from its template as soon as the region containing the break has been resynthesized. The displaced filament therefore captures the the 3' ssDNA end at the other side of the DSB and another synthesis event occurs, fillingl the gap and sealing the break .

If the DSB falls into a region which contains direct repeats, cell undergoes to Single Strand Annealing (SSA) (fig.I5,C). During SSA, DSBs ends are processed in 5' to 3' direction until complementary sequences flanking the break are exposed and can be annealed. The 3' tails in excess are removed by the endonuclease Rad1/Rad10, whereas the remaining nicks are sealed by DNA ligase. In this case the DSB repair occurs with concomitant deletion of one repeat and of the interventing sequence; for this reason SSA is described as an error-prone repair.

DSB repair pathway choice is regulated by several factors, including the nature of the lesion and the cell cycle phase in which repair occurs.

The cell cycle phase is a primary determinant: whereas NHEJ operates throughout the cell cycle, HR is restricted to the S and G2 phase of the cell cycle, where the sister chromatid is readily available. Accordingly, it has been reported that that DNA end resection takes place only when the cyclin-dependent kinases (CDKs) are active (Ira et al., 2004; Aylon et al., 2004; Huertas et al., 2008). In *S.cerevisiae*, CDK-dependent regulation of end resection has been shown to involve Rad9 and Sae2.

Rad9 seems to pose a physical obstacle for processive DNA resection, since *rad9Δ* mutants resect faster than wild type cells in G2; they can also resect in G1, when CDKs are not active (Lazzaro et al., 2008). Rad9 and its orthologs Crb2/53BP1 undergo multiple CDK-dependent phosphorylation (Grenon et al., 2007; Linding et al., 2007), but it is unknown whtehere these modifications affect resection. Sae2 is phosphorylated by CDK at Ser267 (Huertas et al., 2008). The non-phosphorylatable Sae2 mutant displays a phenotype similar to that of a *sae2* null mutant, including delayed HR and increased NHEJ. Interestingly, the phospho-mimicking *sae2-S267E* mutant, resects even in the absence of CDK activity, although the resection is limited to a few kilobases, suggesting a failure to activate Exo1 and Sgs1 pathways (Huertas et al., 2008). Analogously, CtIP, its human counterpart, is phosphorylated at the equivalent Thr847, and abrogation of CtIP phosphorylation impairs end resection (Huertas and Jackson, 2009).

Finally, NHEJ and HR compete *in vivo* for the same substrates. DSB end resection reduces the ability of Ku to bind DNA; indeed, lack of Mre11, Rad50, Xrs2 or Sae2 leads to increased amounts of Ku bound to DSBs (Zhang et al., 2007). On the contrary, in the absence of Ku or DNA ligase IV, the amount of Mre11 bound to the break is higher (Zhang et al., 2007; Clerici et al., 2008; Zierhut and Diffley., 2008). Moreover, the lack of Ku speeds up the resection in G2 arrested cells, and makes cells able to resect also in the G1 phase of the cell cycle (Zierhut and Diffley, 2008; Clerici et al., 2008).

3

THE DNA DAMAGE RESPONSE - THE DNA DAMAGE CHECKPOINT

The DNA damage response is considerably broader than DNA repair itself and actually encompasses additional processes. In particular, a hallmark of this response is the activation of surveillance mechanisms, named DNA damage checkpoints.

The concept of DNA damage checkpoint was initially developed in a study by Ted Weinert and Lee Hartwell, in the budding yeast *Saccharomyces cerevisiae,* where they described a G2/M cell-cycle arrest after X-ray irradiation. Since the arrest required *RAD9*, this led to the view that *RAD9* and similar genes defined control mechanisms that negatively regulate cell cycle progression in response to DNA damage (Weinert and Hartwell, 1988).

The large number of studies performed since then allowed us to understand that DNA damage checkpoints are highly conseverd signal transduction pathways (see Fig.I7) that sense the physical state of the genome and coordinate the orderly progression of the cell cycle with the completion of critical events such as DNA replication and repair (Shiloh, 2003; Kastan and Bartek, 2004). In response to DNA damage, these mechanisms temporary halt the cell cycle progression, providing time for DNA repair, thereby avoiding incorrect genetic information from being passed onto the progeny. Checkpoint activation frequently brings about changes in the transcriptional programme of the cell (Allen et al., 1994, Gasch et al., 2001; Sharma et al., 2007) and modifications of DNA

repair factors, resulting in a more efficient removal of the lesion (Zhao and Rothstein, 2002; Yao et al., 2003; Bashkirov et al., 2005).

In *S. cerevisiae,* DNA damage checkpoints delay the G1/S transitions and block the G2/M transitions of the cell cycle (Weinert and Hartwell., 1988; Siede et al., 1993). In addition, two types of S-phase checkpoints have been defined: the DNA replication checkpoint, which arrests cell cycle progression and inhibits firing of late replication origins in response to replication stress (Santocanale and Diffley., 1998), and the intra-S checkpoint, which slows DNA replication and cell cycle progression in response to DNA damage (Paulovich et al., 1997). Although these checkpoints are distinct, they share many components. Briefly, phosphatidylinositol 3 kinase-like kinases (PIKKs) comprising the *S. cerevisiae* Tel1 and Mec1 and their mammalian homologs ATR (Ataxia-Telangectasia Mutated) and ATR (ATM and Rad3-related), respectively, are a part of a sensor mechanism that detects DNA lesions or stalled replication forks and activates a pair of effector kinases Chk1 and Chk2 (called Rad53 in *S. cerevisiae*). For this task, they are assisted by numerous adaptor or mediator proteins including Rad9/53BP1, Mrc1/Claspin and Dpb11/TopBP1. Chk1 and Rad53/Chk2 then phosphorylate critical targets, which are responsible for the activation of the different cellular responses.

It has been convenient to think of checkpoints as unidirectional pathways, but this is an oversimplification. For example, DNA repair proteins can act as both sensors and effectors, and this may suggest that the checkpoint response is a complex regulatory network incorporating both feedback loops and threshold responses (Putnam et al., 2009).

A failure in checkpoint processes can lead to increasing mutation rate and genomic instability, and may facilitate the development of numerous disorders such as cancer (Hoeijmakers, 2001; O'Driscoll and Jeggo, 2006; Rass et al., 2007).

S. cerevisiae	*S. pombe*	Human/xenopus	Step	Function
Rpa1	Rpa1	RPA1	Initiation	RPA large subunit
Rpa2	Rpa2	RPA2	Initiation	RPA middle subunit, redirects RPA to DNA repair foci.
Rad24	Rad17	Rad17	Sensor	Rfc1 homolog; large subunit of Rad24/Rad17-RFC
Rad17	Rad1	Rad1	Sensor	Checkpoint clamp subunit
Mec3	Hus1	Hus1	Sensor	Checkpoint clamp subunit
Ddc1	Rad9	Rad9	Sensor	Checkpoint clamp subunit; activates Mec1; phosphorylated form binds Dpb11/Cut5/TopBP1
Dpb11	Cut5/Rad4	TopBP1	Sensor	Replication initiation protein; activates ATR; binds phosphorylated clamp; associates with Pol ε.
Pol2	Cdc20	Pol2	Sensor	Catalytic subunit of Pol ε
Mec1	Rad3	ATR	Transducer	PIKK catalytic subunit; phosphorylates Rad53/Chk1/Chk2, and factors upstream of it
Ddc2	Rad26	ATRIP	Transducer	Mec1/Rad3/ATR regulatory subunit; binds RPA
Tel1	Tel1	ATM	Transducer	PIKK; primarily in response to dsDNA breaks
Rad9	Crb2	53BP1 MDC1	Mediator	Scaffold for Rad53 or Chk1; facilitates transphosphorylation
Mrc1	Mrc1	Claspin	Mediator	Replication fork-associated scaffold
Rad53	Cds1	Chk2	Effector	FHA kinase; phosphorylates factors in effector pathways
Chk1	Chk1	Chk1	Effector	kinase; phosphorylates factors in effector pathways

Fig.I 7 Checkpoint proteins in the Mec1/ATR pathway (Navadgi-Patil and Burgers., 2009)

THE SIGNAL RESPONSIBLE FOR CHECKPOINT ACTIVATION

The DNA damage checkpoint is organized as a phosphorylation cascade initiated by upstream PIKK kinases that function as sensors in response to genotoxic stress.

A lot of work has been devoted to understand how cells become aware of the presence of a damage in their genome and how such event triggers checkpoint activation. The studies performed in the last years allowed to understand that although Tel1/ATM and Mec1/ATR share some of their downstream effectors, the DNA damage signals that evoke these two kinases are distinct. While human ATM plays a primary role in the response to DSBs, Mec1/ATR controls the response to a much broader spectrum of DNA lesions. Tel1/ATM association to the break site is transient and its ability to respond to the DSB is disrupted when DSB ends undergo the 5'→3' exonucleolytic degradation (Mantiero et al., 2007). It has thus been proposed that Tel1/ATM is recruited to blunt or minimally processed DSB ends and initiate DSB signalling through their interaction with the MRX/MRN complex. Indeed, studies in both human and yeast cells showed that Tel1/ATM binds a common motif in the C terminus of Xrs2/Nbs1 and that this interaction is specifically required for Tel1/ATM recruitment to a DSB (Nakada et al., 2003; Falck et al., 2005)

The versatility of ATR/Mec1 in the DNA damage response suggests that this pathway is able to sense a common signal generated by different types of DNA damage. At sites of DNA repair and stressed replication fork single stranded DNA (ssDNA) coated by RPA (Replicative Protein A) is frequently formed, and this structure is responsible for triggering checkpoint activities (Garvik et al., 1995). This model has been supported by a large amount of experimental data; in particular, *in vivo* studies in human cells demonstrated that exposure to IR induces the formation of RPA foci, indicative of the presence of ssDNA. The ATR-ATRIP apical complex (Mec1-Ddc2 in budding yeast) co-localizes with these foci, and RPA is necessary for both the localization of the complex and for its functional activation (Zou and Elledge, 2003). It has been also demonstrated that RPA stimulates the *in vitro* binding of human Rad9 and Rad17 and the *in vivo* binding of yeast Ddc1 to DNA (Zou, 2003).

The mechanism of ssDNA generation is different depending upon the original lesion, but, in general, endonuclease and exonuclease activities are required for this first step. DNA repair factors (described in the previous chapter) have a role in checkpoint activation either in the recruitment of checkpoint factors or in the generation of the ssDNA recognized by checkpoint proteins, suggesting that a tight connection between DNA repair pathways and DNA damage checkpoint activation exists. For example, as previously mentioned, the MRX complex, involved in the first steps of DSB repair, is also required for checkpoint activation after induction of DSBs

(Nakada et al., 2004); NER processing of UV lesion is necessary for UV-induced checkpoint activation and the NER factor Rad14 functionally and physically interact with the checkpoint protein Ddc1 (Giannattasio et al., 2004). Finally, exonucleases are also fundamental for the signalling, since they generate large amounts of ssDNA.

EARLY EVENTS IN CHECKPOINT ACTIVATION

In *S. cerevisiae* Mec1 is the main player in the DNA damage checkpoint. Independently of the presence of DNA damage, Mec1 forms a complex with Ddc2/Lcd1, that is essential for all known functions of the kinase (Paciotti et al., 2000; Rouse and Jackson, 2000; Wakayama et al., 2001; Majka et al., 2006).

MEC1, *DDC2* and *RAD53* are essential for cell viability in the absence of DNA damage. The lethality of a *mec1Δ*, *ddc2Δ* or *rad53Δ* strain is suppressed by increasing the activity of the Ribonucleotide Reductase (RNR), which can be achieved by overexpressing the *RNR1-3* genes, or deleting *SML1*, which encodes for a trepressor of RNR. (Desany et al., 1998; Zhao et al., 1998). This suggests that the essential role of these protein during a normal cell cycle may be the stabilization of the stalled replication forks (Lopes et al., 2001; Tercero and Diffley, 2001).

The role of Ddc2 in checkpoint activation is thought to be the recruitment of Mec1 to damaged DNA, in fact Ddc2 is required for association of Mec1 with single-stranded DNA coated with RPA (Rouse and Jackson, 2002; Zou and Elledge, 2003). The single-strand binding protein RPA is specifically required for the recruitment of Mec1-Ddc2 to ssDNA generated at a DSB (Zou et al., 2002): a specific point mutation in the large subunit of RPA, Rfa1-L45E (*rfa1-t11*), shows a partial reduction both in Ddc2 ChIP levels and in recruitment of a Ddc2-GFP fusion protein (Zou and Eleldge, 2003; Lisby et al., 2004; Nakada et al., 2004). Similarly, in human cells, depletion of the RPA70 subunit reduces ATR-ATRIP focus formation after irradiation and, consequently, the phosphorylation of the ATR target Chk1.

Ddc2 is phosphorylated by Mec1 during S phase in an unperturbed cell cycle and in response to DNA damage (Paciotti et al., 2000). Since Ddc2 phosphorylation does not require any other checkpoint factors, it is generally used as an *in vivo* marker for Mec1 kinase activation.

Purified Mec1-Ddc2 and ATR-ATRIP show a very low protein kinase activity, therefore, it has been assumed that the protein kinase is specifically activated as a regulated step during checkpoint function. The activity of Mec1/ATR is not directly regulated by the DNA intermediates responsible for checkpoint activation, rather it appears to be mediated by factors that interact with the signaling DNA substrates. In the last few years, two activators of Mec1/ATR were identified. One of these factors is the DNA damage checkpoint clamp (9-1-1 complex), a trimer composed of Ddc1, Rad17 and Mec3 in yeast, and their orthologs Rad9, Hus1 and Rad1 in *S. pombe* and

vertebrates, hence the designation 9-1-1 (Majka and Burgers, 2003; Parilla-Castellar et al., 2004; Majka and Burgers, 2004). The crystal structure of human 9-1-1 demonstrated a strong structural relationship between this factor and the replication clamp PCNA (Proliferating Cell Nuclear Antigen) (Dorè et al., 2009; Sohn and Cho, 2009). Rad17/Mec3/Ddc1 is loaded onto gapped DNA by its loader, Rad24-RFC (*S. pombe* and human Rad17-RFC), in an ATP-dependent manner. The Rad24-RFC clamp loader differs from the PCNA loader RFC, in that the Rad24 protein replaces the Rfc1 subunit in a heteropentameric complex with the Rfc2-5 subunits (Green et al., 2000). Whereas RFC loads PCNA specifically onto 3'-primer/template junctions, Rad24-RFC loads the 9-1-1 clamp specifically onto 5'-primer/template junctions (Majka et al., 2006).

The functional role of the 9-1-1 complex in Mec1-Ddc2 activation has been investigated and partially explained only recently. Burgers's group was the first to study the dynamics of Mec1 activation; they observed that colocalization of Mec1-Ddc2-RPA and of Ddc1-Mec3-Rad17 in the context of partial duplex DNA results in Mec1 activation (Majka et al., 2006). A recent paper supported this function for the 9-1-1 complex observing that forced localization of Mec1-Ddc2 and of 9-1-1 to chromosomal arrays of Lac operator sequences can trigger the DNA damage response in the absence of DNA lesion, proving that indeed the 9-1-1 complex is required for Mec1 kinase activation and, furthermore, that ssDNA might play only a passive role as a scaffold for the recruitment of checkpoint factors (Bonilla et al., 2008). Moreover, colocalization of only the Ddc1 subunit with Mec1-Ddc2 has been demonstrated to be sufficient to activate Mec1 (Bonilla et al., 2008), confirming the previous *in vitro* observation that under low salt conditions purified Ddc1 stimulates the kinase activity of Mec1(Majka et al., 2006). Once the 9-1-1 complex has been loaded in the proximity of the lesion, is phsophorylated by Mec1 (Longhese et al., 1997). The function of this phosphorylation event is currently unknown since it is not required for complex formation and depends upon the presence of a loaded complex (Paciotti et al., 1998). Moreover, Ddc1 is phosphorylated during an unperturbed cell cycle in S phase in at least one of the three Cdc28 consensus sites (Longhese et al., 1997).

A second activator of Mec1/ATR turned is the replication protein Dpb11, the budding yeast homologue of *S. pombe* Cut5/Rad4 and human TopBP1. Dpb11 and its homologs are essential for the initiation of DNA replication in eukaryotic organisms. Dpb11 was first identified as a multicopy suppressor of conditional lethal mutation in DNA polymerase ε (Araki et al., 1995). Later, it was shown that Dpb11 and DNA polymerase ε are mutually dependent for the association with pre-replicative complexes (Masumoto et al., 2000). The C-terminal domain of Dpb11 has been shown to interact with Ddc2 leading to Mec1 activation *in vitro* (Navadgi-Patil and Burgers, 2008; Mordes et al., 2008b). Moreover, Mec1-dependent phosphorylation of Dpb11 on Thr731 further enhances the ability of Dpb11 to amplify Mec1-Ddc2 activity (Mordes et al., 2008b). Analogously, it has been demonstrated that activation of human and Xenopus ATR requires TopBP1 too; this function can be restricted to a small region of the protein termed AAD, ATR

Activation Domain (Kumagai et al., 2006). More recently, it has also been shown that ATRIP promotes the association of ATR and TopBP1 (Mordes et al., 2008a).

Data obtained from *S. pombe* and human cells demonstrated that Rad9 (Corresponding to scDdc1) recruits TopBP1/Cut5 via an interaction between one of its phosphorylated residues and a BRCT domain of TopBP1. (Furuya et al., 2004; Delacroix et al., 2007; Lee et al., 2007). This suggested that phosphorylated 9-1-1 may recruit TopBP1/Cut5 to damaged DNA, leading to ATR activation (Furuya et al., 2004; Delacroix et al., 2007).

In budding yeast the dynamics seems more complicated. As mentioned before, Dpb11 can stimulate Mec1 kinase activity, but also the checkpoint clamp, in particular by the Ddc1 subunit, has been reported to be competent for this function (Majka et al., 2006; Navadgi-Patil and Burgers, 2008). At what stages of the cell cycle these proteins activate Mec1? Do they act independently or in synergy? The questions have been addressed in a recent paper, which suggests that in G1 Mec1 activation is achieved by the Ddc1 subunit of the 9-1-1 clamp, while Dpb11 is dispensable. On the other hand, in G2, 9-1-1 activates Mec1 by two distinct mechanism; one involves the direct activation of Mec1 by Ddc1, while the second relies on the Dpb11 recruitment via Ddc1T602 phosphorylation (Navadgi-Patil and Burgers, 2009).

RAD9 AND SIGNAL TRANSDUCTION

After Mec1-Ddc2/ATR-ATRIP and 9-1-1 complexes have been recruited to the proximity of the lesion and the kinase activity of Mec1 has been activated, the DNA damage signal is transmitted to the effector checkpoint kinases thanks to the so called "adaptor" proteins. In budding yeast a crucial role is played by Rad9; its fission yeast homologue is Crb2, whereas in mammalian cells there are three proteins with similar functions: 53BP1, MDC1 and BRCA1.

RAD9 has been the first checkpoint gene to be isolated (Weinert and Hartwell, 1988). It encodes for a 148 kDa protein, characterized by a modular structure with numerous highly conserved functional domains (Fig. I8). From the N to the C terminus we recognize a CAD (Chk1 Activating Domain), the Serine Cluster Domain (SCD), a Tudor domain and a tandem repeat of the BRCT (BRCA1 C-terminus) motif, required for Rad9 oligomerization and function (Soulier and Lowndes, 1999; Hammet et al., 2007; Nnakwe et al., 2009).

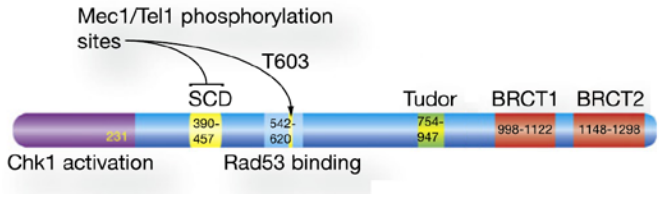

Fig.I 8 Functional domains of Rad9 (from Usui et al., 2009)

RAD9 was 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 this protein is phosphorylated after DNA damage in a manner that depends on Mec1, Tel1 and the Rad24 epistasis group (*MEC3-DDC1-RAD17-RAD24*) (Emili, 1998; Vialard et al., 1998), suggesting that the sensor factors act upstream of Rad9 in the cascade and are necessary for its functional activation.

It is generally assumed that once phosphorylated by Mec1, Rad9 dimerizes through its BRCT domains, generating a docking site for Rad53, which binds the phospho-sites near the SCD of Rad9, using its FHA (Fork Head Associated) domains (Gilbert et al., 2001). This binding facilitates the Mec1-dependent phosphorylation of Rad53, required for the activation of Rad53 kinase activity (see below) (Vialard et al., 1998; Soulier and Lowndes, 1999).

Rad9 is required also for the activation of Chk1, which acts in a pathway parallel to that of Rad53 (Sanchez et al., 1999). The Rad9 domains required for Rad53 activation are distinct from the one required for Chk1 regulation, the so called Chk1 Activation Domain (CAD) which includes the first 231 aminoacids at the N-terminus of this protein (Blankley and Lydall., 2004).

In the last few years it became evident that chromatin remodeling activities and post-translational modifications of chromatin components , including histones, influence DNA damage checkpoint signalling and repair in all eukaryotic cells (see below). In particular, it was shown that different post-translational modifications of histones, including H2A serine 129 phosphorylation and H3 lysine 79 methylation, play important roles in the localization of Rad9 onto chromatin. In budding yeast, phosphorylation of H2A on Ser129 by Mec1/Tel1 is one of the earlier events in response to DSBs; a similar modification take place also on histone H2AX Ser139 in mammalian cells (see below). The phosphorylated form of histone H2A(X), called γ-H2A(X) contributes to DNA repair and is required, both in yeast and mammalian cells, for survival to DNA damage treatments and to checkpoint activation (see below). It has been observed that this histone modification is required for the efficient recruitment of Rad9 (and its orthologues, Crb2 and 53BP1) onto the chromatin after DNA damage, through physical interaction among Rad9/Crb2/53BP1 BRCT domains and γ-H2A(X) (Ward et al., 2003; Nakamura et al., 2004; Du et al., 2006; Javaheri et al., 2006; Toh et al., 2006; Ward et al., 2006; Kilkenny et al., 2008). Rad9 mutations in a conserved region of the first BRCT motif affect binding to γ-H2A, thus altering the G1 checkpoint signalling in response to DSBs (Javaheri et al., 2006; Hammet et al., 2007) and the G2/M response to uncapped telomeres (Nnakwe et al., 2009)

Recent works demonstrated also that histone H2B ubiquitination, carried out by Rad6-Bre1 and the subsequent methylation of histone H3 on lysine 79, performed by Dot1, contribute to Rad9 recruitment to chromatin, even in the absence of DNA damage (Giannattasio et al., 2005; Toh et al., 2006; Grenon et al., 2007; Hammet et al., 2007). This pathway depends on an interaction

between methylated H3K79 and the Tudor domain of Rad9. Loss of these histone modifications or mutation of the Rad9 Tudor domain prevents Rad9 and Rad53 phosphorylation in G1- arrested cells and abolishes the G1/S arrest following DNA damage (Giannattasio et al., 2005). A similar mechanism has been described also in fission yeast and in higher eukaryotes, where it was report that Rad9 orthologues need to interact with H4K20me to be properly localized onto the chromatin, although human 53BP1 may also be recruited to chromatin through interactions with H3K79me (Sanders et al., 2004; Huyen et al., 2004; Botuyan et al., 2006; Du et al., 2006).

Surprisingly, in budding yeast G2/M- arrested cells, deletion of *DOT1* is not sufficient to completely eliminate the checkpoint function. *dot1Δ* cells irradiated in M, display only a partial defect in Rad9 and Rad53 phosphorylation. These evidences suggest that the pathways involved in the recruitment of Rad9 to chromatin are cell cycle specific, and in G2/M cells another mechanism, partially redundant with the histone modification pathway, must be active to obtain Rad9 phosphorylation and effective checkpoint activation.

As previously anticipated, the role of Rad9 in response to DNA damage in S phase and to replicative stress is only partial; in these conditions a second adapter comes into play, Mrc1 (Claspin in vertebrates) (Kumagai and Dunphy, 2000; Tanaja and Russel, 2001). *mrc1Δ* strain exhibit only a minor defect in Rad53 phosphorylation after hydorxyurea treatment because, in the absence of Mrc1, secondary 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.

RAD53, CHK1 AND THE EFFECTORS

As previously mentioned, once recruited to the proximity of the lesion, Rad9 oligomerizes, likely through a physical interaction between its BRCT and SCD domain, creating a scaffold which alloes the recruitment of Rad53 (Pellicioli and Foiani, 2005; Usui et al., 2009). In particular, this association involves the Rad53 FHA (Fork Head Associated) domains and some sites present in the hyperphosphorylated form of Rad9 (Schwarts et al., 2002). Once recruited to the proximity of the lesion, Rad53 is phosphorylated by Mec1, which stimulates Rad53 autophosphorylation (Gilbert et al., 2001; Pellicioli and Foiani., 2005; Sweeney et al., 2005). This autophosphorylation is likely facilitated by a local increase of Rad53 concentration, due to its binding to Rad9, that, in this sense, act as a solid-phase catalyst (Gilbert et al., 2001). Autophosphorylation of Rad53 determines its release from Rad9; in particular, after its activation, Rad53 phosphorylates the Rad9 BRCT domain, breaking up the oligomer and facilitating the release from the Rad9 platform

(Stern, 2009; Usui et al. 2009). Once released, Rad53 can phosphorylate and activate the final effectors (Gilbert et al., 2001).

The level of Rad53 phosphorylation is thus linked to the kinase activity of the protein. This modification can be visualized as a slower form of the protein in a SDS-PAGE, which is thus used to monitor the activation of the cascade.

Chk1 is the second effector kinase of the DNA damage checkpoint pathway and it acts in parallel with Rad53 in the G2/M DNA damage response. In this phase, deletion of *RAD53* or *CHK1* causes a partial defect in checkpoint activiation, suggesting that at the G2/M transition these proteins are partially redundant in signalling the presence of a DNA damage (Sanchez et al., 1999).

THE RESPONSES CONTROLLED BY THE DNA DAMAGE CHECKPOINT

The DNA damage checkpoint induces numerous of cellular responses; among which are the cell cycle arrest, histones modifications, transcriptional changes, post-translational modifications of proteins involved in DNA repair. In this occasion I will not analyze the S-phase specific responses, which are activated by the S-phase DNA damage checkpoints.

Cell cycle arrest

This response varies with the cell-cycle phase where lesions are recognized.

Yeast cell do not exhibit a strong G1 arrest, as seen in mammalian cell, but only a a DNA damage checkpoint-dependent delay of the G1/S transitio. In particular, active Rad53 phosphorylates Swi6, which inhibits the transcription of the *CLN1* and *CLN2* genes and the formation of the Cdc28-Cln1/2 complexes, required for the G1/S transition. (Sidorova and Breeden, 1997). In the absence of the Cdc28-Cln1/2 complexes, Sic1 is stabilized and this contributes to maintaining the G1 arrest by inhibiting the Cdc28-cyclin B complexes (Wysocki et al., 2006).

The most evident arrest in budding yeast concerns the metaphase/anaphase transition and therefore the exit from mitosis. During an unperturbed cell cycle, Pds1 securin is ubiquitinated by APC/Cdc20 and is degraded to allow entry into anaphase. Pds1 is an inhibitor of the

endoprotease Esp1, also called separase, which regulates sister chromatid cohesion. Pds1 degradation activates Esp1 that promotes chromatid separation, allowing anaphase. (Ciosk et al., 1998). In the presence of DNA damage, Pds1 is phosphorylated in a Mec1-, Rad9- and Chk1-dependent, but Rad53-independent manner; this event prevents its ubiquitin-dependent degradation, whereas Rad53 inhibits the interaction between Pds1 and Cdc20. The molecular mechanism is still unknown, but Cdc20 has been identified as a likely substrate of Rad53 phosphorylation (Sanchez et al., 1999; O'Neill et al., 2002; Agarwal et al., 2003). This samage-induced stabilization of Pds1 therefore prevents anaphase entry.

Rad53 also inhibits mitotic exit. This checkpoint kinase is required to maintain elevated CDK activity during checkpoint arrest and acts by inhibiting Cdc5 (Cheng qt al., 1998; Sanchez et al., 1999). Cdc5 in turn inhibits the Bub2/Bfa1 complex, blocking the mitotic exit network (MEN) and the progression through mitosis. (Hu et al., 2001; 2002; Geymonat et al., 2003).

Despite its importance, little is known about Rad53 targets in cell cycle control and also the molecular details of the dynamics of action of Cdc5, Cdc20 and perhaps Pds1 are still under investigation.

Histones modifications

Activation of the DNA damage checkpoint leads to phosphorylation of the histone variant H2A(X) at serine 139 in mammals and of serine 129 of H2A in budding yeast cells. Phosphorylated H2A(X) is detected very soon after DNA damage and it has been shown to contribute to DNA repair and to be required for full viability of yeast and animal cells in the presence of DNA damaging agent (see next chapter).

The DNA damage checkpoint also promotes the maintenance of the acetylation of histone H3 Lys56 in *S. cerevisiae*, which occurs independently of the checkpoint activation in newly synthesized histones during the S phase. It has been speculated that the persistence of H3 Lys56 acetylation facilitates DNA repair, since its absence causes spontaneous DNA damage and chromosome loss (Masumoto et al., 2005; Celic et al., 2006).

Transcriptional Response

In response to DNA damage, nine genes are specifically induced in a Mec1-dependent manner. Among these there are *RNR2* and *RNR4,* which encode subunits of the ribonucleotide reductase (RNR) (Gasch et al. 2001). RNR plays a role in controlling the levels of deoxyribonucleoside triphosphates (dNTPs), required for DNA replication and DNA repair. In response to DNA damage, several mechanisms act in concert to upregulate RNR activity, leading to a significant

increase in dNTPs concentration, which likely facilitate the DNA polymerase activities involved in DNA repair (Chabes et al., 2003).

Mec1 also promotes the so called "environmental stress response" (ESR), which involves more than 900 genes whose expression is similarly altered in response to diverse environmental stress (Gasch et al., 2000). Many of the genes repressed in this program are involved in protein synthesis and metabolism, so it is likely that their repression in response to stressful environment represents a way to conserve energy in the cell, whereas genes induced in the ESR may protect critical features of cell homeostasis like protein folding (Gasch et al., 2000).

Post-translational modifications of proteins involved in DNA repair

After DNA damage, Several DNA repair proteins are phosphorylated in budding yeast after in a checkpoint-dependent way; these include Rad55-Rad57, Rad51, the members of the MRX complex, RPA and Mus81 (Herzberg et al., 2006), but the physiological significance of these phosphorylation is still unknown. It has been reported that the checkpoint-dependent phosphorylation of Rad55 is required for efficient recombination after replication fork stalling (Herzberg et al., 2006), but the precise biochemical effects of this phosphorylation event are unknown.

CHECKPOINT INACTIVATION: RECOVERY AND ADAPTATION

The DNA damage checkpoint, blocking cell-cycle progression, allows more time to repair the DNA lesions. Once the repair is completed, cells can resume cell cycle progression and continue their physiological programme; this return to homeostasis is usually called recovery.

However, data from *S. cerevisiae* and *Xenopus laevis* have suggested that there may be an alternative route to re-enter cell cycle progression, even in the presence of unrepaired DNA damage, namely through a process termed "checkpoint adaptation". This process was originally defined in *S. cerevisiae* as the ability to divide in the presence of unrepairable DNA breaks, following a checkpoint-imposed cell cycle arrest. (Sandell and Zakian, 1993; Toczyski et al., 1997).

During recovery, the repair of DNA lesions brings switches off most upstream components of the DNA damage checkpoint, which therefore revert to their inactive form. Adaptation is a process whereby cells decrease their responses to a stimulus after exposition for a prolonged period. One possibility is that the DNA structures that first trigger the checkpoint cascade are subsequently metabolized into non-signaling DNA lesions; alternatively, the sensors that detect DNA lesion could be downregulated.

Both the two mechanisms have been amply studied in budding yeast, using HO-induced DSBs (Vaze et al., 2002; Keogh et al., 2006), but they have been observed also in the presence of other types of DNA lesion such as stalled replication fork in higher eukaryotes (O'Neill et al., 2007; Syljuasen, 2007).

Many components of the DNA damage checkpoint are phosphorylated upon DNA damage; it was thus reasonable to presume that phosphatases could play a critical role in the checkpoint inactivation. Indeed, it has been shown that in budding yeast, the PP2C-family phosphatases Ptc2 and Ptc3 work at the level of Rad53 to extinguish the checkpoint signal, playing an important role both in recovery and in adaptation (Leroy et al., 2003). Ptc2 interacts constitutively with Rad53 phosphopeptide binding domain FHA1 through a specific threonine, Thr376, which is phosphorylated by the CKII kinase (Guillemain et al., 2007). Another phosphatase, the PP2A-like phosphatase Pph3, forms a complex with the regulatory subunit Psy2 and dephosphorylates Rad53 activated upon MMS treatment (O'Neill et al., 2007).

Dephosphorylation of γH2A(X) also influences the duration of the checkpoint. Studies in yeast have identified an evolutionary conserved PP4C phosphatase complex, consisting of Pph3 phosphatase and two regulatory subunits Psy2 and Ybl046w, which promotes H2A dephosphorylation *in vitro* and *in vivo* (Keogh et al., 2006). In budding yeast cells lacking the Pph3 subunit, DNA repair is not defective, but the persistence of phosphorylated H2A prolongs the checkpoint signal, impairing the recovery process (Keogh et al., 2006).

The absence of some proteins involved in DNA or chromatin metabolism also affect the inactivation of the DNA damage checkpoint. These proteins include the Yku70 and Yku80 subunits of the Ku complex, which binds DSB ends (Lee et al., 1998), the HR component Rad51, the Srs2 helicase (Lee et al., 2000) and Sae2 (Clerici et al., 2006). It is commonly thought that the absence or mutation of these proteins alters the metabolism of DSB ends, which increases or attenuates the signal sensed by the checkpoint components, thus compromising or promoting its inactivation (Clemenson et al., 2009).

Finally, an important role in regulating recovery and adaptation in budding yeast is played by the Polo-like kinase Cdc5. Cdc5 is responsible for the turning off of Rad53; in a *cdc5-ad* mutant strain Rad53 activity cannot be downregulated following the induction of a single DSB and therefore checkpoint inactivation is impaired (Pellicioli et al., 2001). Studies in higher eukaryotes

provide supporting evidence that polo kinase can inhibit the checkpoint response after DNA damage. The Xenopus homolog of Cdc5, Plx1, affects Chk1 activity by promoting the dissociation of the replication-checkpoint adaptor Claspin from chromatin (Yoo et al., 2004). Similarly, during recovery after DNA damage, human Plk1 phosphorylates Claspin to promote its degradation, which in turn prevents further Chk1 activation (Mailand et al., 2006).

While the physiological significance of recovery seems obvious – the cell resumes the cell cycle after DNA repair is completed, downregulating the checkpoint activation -, the meaning of adaptation is not so evident. In unicellular organisms, the termination of the checkpoint responses in the presence of a persistent lesion may facilitate cell survival by avoiding death induced by permanent cell cycle arrest at the expense of potential mutation; it has been also speculated that the adaptation process may provide opportunities for the cell to repair the DNA damage in a subsequent cell cycle. (Galgoczy and Toczyski, 2001; Clemenson et al., 2009). In pluricellular organisms checkpoint inactivation may not be related to increased viability and could facilitate apoptosis in subsequent cell cycle (Yoo et al., 2004)

4

CHROMATIN DYNAMICS COUPLED WITH DNA REPAIR

The accommodation of genomic DNA into the small nucleus of an eukaryotic cell is made possible through its organization into a highly condensed structure, known as chromatin. The basic unit of chromatin is the nucleosome, which consists of 146 base pairs of DNA wrapped in nearly two left-handed superhelical turns, around an octamer of histone proteins, containing a tetramer of histone H3 and H4, flanked by two H2A-H2B dimers (Kornberg, 1977). Nucleosomes with linker DNAs, each about 20-60 bp, form an approximately 10 nm diameter "beads-on-a-string" structure, where a linkers histone H1 contacts the exit and entry of DNA strand on the nucleosome. The "beads-on-a-string" structure then coils into a 30 nm diameter helical structure known as the 30 nm filament (fig. I9). Further levels of chromatin condensation, which culminate with the compaction of DNA in metaphase chromosomes, are less well understood, but are facilitated by the linker histone H1 and condensins.

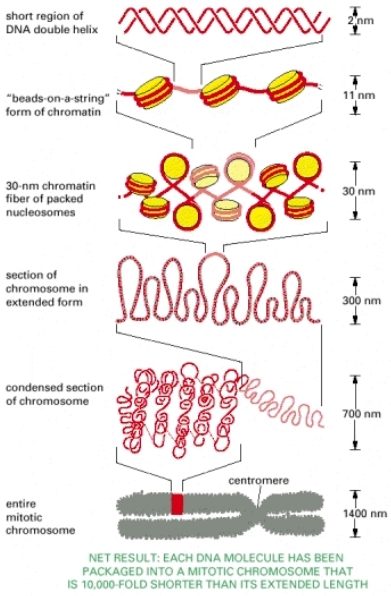

Fig.I 9 The many levels of chromatin packing which give rise to the highly condensed mitotic chromosome. (Alberts et al. Molecular Biology of the cell, 4th ed., 2002)

The arrangement of DNA into chromatin is not only important for resolving problems of spatial accommodation and organization, but it is also essential for the functional utilization of DNA and the proper coordination of its metabolic activities. Indeed, by organizing DNA, histones and non-histone proteins generate a structural barrier to thousands of DNA-binding factors and DNA enzymes, whose uncontrolled access would compromise the activity and function of the DNA molecule. On the other hand, the packaging of the eukaryotic genome into chromatin provides a formidable obstacle to the machineries that mediate genomic process such as transcription, repair and replication. Therefore, any process that requires intimate contact with the DNA would necessitate at least transient modifications of chromatin structure, which could allow enzymes involved in DNA metabolism to access to the DNA.

First of all, chromatin can be modified by post-translational modification of histone tails by enzymes that covalently attach various chemical groups to modifiable aminoacids. Histones are basic proteins particularly rich in lysine, arginine, serine and threonine residues. Given the polar nature of these aminoacids, they are generally located on the external surface of the histone octamer, where they're easily accessible for enzymes able to bring about post translational modifications. Some modifications, such as the acetylation of lysine residues or the phosphorylation of serines and threonines, affect the charge of histone tails, neutralizing the positive charge in the case of lysine acetylation and adding a negative charge in the case of phosphorylation. Other modifications (mono-,di-, trimethylation of lysines/arginines or ubiquitination and sumoylation of lysines) do not change the charge of the histone tail, but create novel recognition sites that promotes or prevents binding of other proteins.

A different kind of chromatin modification, generally used by a cell to change the accessibility of DNA to proteins is nucleosome remodeling. In contrast to what was initially thought, nucleosomes are dynamic structures that can be altered by their replacement, repositioning or, in some cases, eviction by chromatin remodeling complexes that use the energy derived from ATP hydrolysis to alter histone-DNA interactions (Saha et al., 2006; Osley et al., 2007).

Albeit modifications of chromatin structure has been extensively studied in the context of transcriptional regulation, there is now wide evidence that they play a central role also in the regulation of DNA repair. Here I will review the most important steps of chromatin remodeling, which occur during the DNA damage response.

CHANGES IN CHROMATIN STATE DURING THE EARLIEST STEPS OF DDR

Chromatin remodeling activities

DNA damage affects chromatin condensation; indeed it has been observed, both in yeast and mammalian cells, that chromatin becomes relaxed in the vicinity of a DSB (Tsukuda et al., 2005; Kruhlak et al., 2006). This local expansion occurs independently of ATM (ScTel1) and H2AX phosphorylation, one of the earliest event of the DDR, but it requires the hydrolysis of ATP (Tsukuda et al., 2005; Kruhlak et al., 2006). Moreover, it has been speculated that this phenomenon might be a signal for ATM activation; indeed, chromatin decondensation induced by chloroquine or by treatment with deacetylases inhibitors can trigger ATM activation (Kanu et al., 2007). In addition to local chromatin relaxation, DSB triggers also a global chromatin relaxation process whose role in the DNA damage response is still unknown. So far, the only protein found to regulate this process, is hKAP-1 which has been shown to spread rapidly through damaged chromatin, leading to chromatin relaxation. According to this model, KAP-1 deficient cells are sensitive to DSB-inducing agents, suggesting that chromatin relaxation is crucial for an effective DNA damage response (Ziv et al., 2006). Since KAP-1 is not an ATP-dependent chromatin remodeler, it remains still obscure how it mediates chromatin relaxation.

Many ATP-dependent chromatin remodeling factors are involved in the earlier steps of DDR, but it is not known whether they are responsible for the local chromatin relaxation that occurs after induction of the lesion. Among these there is the ScRSC complex, a member of the SWI/SNF chromatin-remodeling subfamily. There is a mutual dependency between RSC and ScMRX recruitment to DSBs: the accumulation of MRX and Ku70 to a DSB site is dependent on Sth1, an ATPase subunit of the RSC complex (Shim et al., 2007), but, at the same time, both Ku70 and MRX are required for RSC recruitment to DSBs, which occurs through interactions with its Rsc1 and Rsc2 subunits (Shim et al., 2005). Moreover, RSC is required for the recruitment of Mec1 and Tel1 to the break site and for ensuring full levels of H2A phosphorylation (Liang et al., 2007). The requirement of SWI/SNF complex for the efficient H2AX phosphorylation has been observed also in mammalian cells (Park et al., 2006).

Histones post translational modifications

H3/H4 methylation

Methylation of lysine 20 of histone H4 (H4K20me) and methylation of lysine 79 of histone H3 (H3K79me) are very important histone modifications for the DNA Damage Response, since they act as docking sites for recruiting the *Sp*Crb2/h53BP1/*Sc*Rad9 adaptors. It is important to note that these methylations are not DNA damage-induced, but are constitutive. Since they are but buried in the chromatin, it has been speculated that the damage-induced passive relaxation of higher order chromatin structure, or chromatin conformational changes during the DNA damage response, might expose the methylated residues.

Budding yeast methylation of H3K79 is promoted by the methyltransferase Dot1, a protein conserved from yeast to human cells (Feng et al. 2002). Dot1 is not only responsible for monomethylation of H3K79, but also for its dymethylation and trimethylation. These higher methylation states depend on previous H2BK123 ubiquitylation (K120 in humans), by the ubiquitin-conjugatin enzyme Rad6 in complex with the ubiquitin ligase Bre1, in a histone cross-talk pathway (Feng et al., 2002; Ng et al., 2002; Van Leeuwen et al., 2002).

During the last years, it has been shown that H3K79me is crucial for the recruitment of ScRad9 and h53BP1 in the proximity of the lesion. By pulldown assays, Huyen and colleagues were the first to demonstrate that 53BP1 and Rad9 bind H3K79me via conserved hydrophobic residues in their tandem Tudor domain. They also showed that 293 cells lacking Dot1 exhibited reduced 53BP1 foci formation after irradiation (Huyen et al., 2004). Further investigations pointed out that deletion of *DOT1* or mutation of the Rad9 Tudor domain completely prevent Rad9 function in the G1- and intra S- phase checkpoints, but not in the G2/M checkpoint (Giannattasio et al., 2005; Wysocki et al., 2005; Grenon et al., 2007). The H3K79 mediated chromatin binding of Rad9 is not only required for maintaining the integrity of the signalling cascade, but it also controls the amount of resection which generates the ssDNA that activates the cascade (Lazzaro et al., 2008). Recent evidence suggested that Rad9 may be bound to methylated H3 also in the absence of DNA damage. Constitutive chromatin binding could modulate better Rad9 functions after damage occurs, enhancing the speed and efficiency of the DNA damage response (Hammet et al., 2007).

Analogously, in *S.pombe* H4K20 methylation, promoted by the Set9 methyltransferase, is necessary for Crb2 foci formation at sites of DSBs induced by IR and for its subsequent phosphorylation (Sanders et al., 2004). Crb2 IRIF formation is mediated by its Tudor domain, which recognizes H4K20 methylation. Further analysis has demonstrated that dymethylated H4K20 contributes also to the relocation of h53BP1 to sites of DNA DSB (Botuyan et al., 2006).

CHROMATIN REMODELING ACTIVITIES DURING DNA REPAIR

During the DNA Damage Response, chromatin structure must be modified in order to make it permissive for access and accumulation of repair and signaling proteins. Different histone modifications occur, often in a sequential and interdependent fashion, resulting in a sort of "guide code" for an efficient DNA repair.

Histones post translational modifications

H2A(X) phosphorylation

One of the most extensively studied repair-specific modifications is the phosphorylation of histone H2A in yeast, or histone variant H2AX (which constitutes ~10% of nuclear H2A) in mammals. Phosphorylation occurs rapidly in response to DNA damage on a serine residue near the C terminus of these proteins (S129 in yeast H2A and S139 in mammalian H2AX). This phosphorylation mark is commonly referred to as γH2A(X) and it is dependent on the action of Mec1/Tel1 in budding yeast (Downs et al., 2000) and ATM/ATR/DNA PK in mammalian cells. In particular, ATM and DNA-PK function redundantly to phosphorylate H2AX after DSBs (Stiff et al., 2004), while ATR phosphorylates H2AX in response to single-strand breaks and stalled replication forks (Cimprich and Cortez, 2008).

In yeast, phosphorylated H2A can be detected as far as 50 kb on either side of a double strand break, but little H2A phosphorylation is detected in the 1-2kb region immediately adjacent to the break (Shroff et al., 2004). In human cells, γH2AX involves approximately 2 Mb DNA region and forms foci that are easily detectable by immunofluorescence microscopy (Rogakou et al., 1998).

The first evidence for a function of H2A phosphorylation in DNA damage repair came from studies in yeast. Mutation of the C-terminal S129 causes a moderate sensitivity to DNA-damaging agents and influences efficient repair of DSBs during replication (Redon et al., 2003). The impact of H2AX phosphorylation has also been examined in mammalian cells. Mouse embryonic stem (ES) cells deficient for H2AX were shown to be hypersensitive to the induction of DSBs by IR, and exhibited genomic instability (Celeste et al., 2002; Celeste et al., 2003), suggesting that phosphorylated H2A(X) might facilitate the recruitment of DNA repair proteins to the site of damage. Indeed, indirect immunofluorescence and live fluorescence microscopy studies show that in the absence of H2A(X) phosphorylation, the formation of DSB-induced foci of DNA repair and checkpoint proteins, such as NBS1, BRCA1, 53BP1 is compromised (Celeste et al., 2003). Further

studies revealed that the stable accumulation, rather that the initial recruitment of these proteins, is strictly connected to a ubiquitin-dependent pathway activated by phosphorylated H2A(X) (see later).

H2A(X) phosphorylation seems to have also a role in the recruitment of ATP-dependent chromatin remodeling complexes. Downs and co-workers showed that a peptide corresponding to the histone H2A C terminus, containing the phosphorylated serine, interacts with the NuA4 histone acetyltransferase complex *in vitro*. This interaction depends on Arp4, a subunit of NuA4 and the ATP-dependent chromatin remodeling complexes INO80 and SWR1 (Shen et al., 2000, Mizuguchi et al., 2004. Downs et al., 2004), which are also implicated in DNA repair (see later).

Finally, phosphorylation of histone H2A is also required for cohesin loading at a DSB. (Unal et al., 2004). Cohesin consistss of Scc1, Scc3 and two structural maintenance of chromosome (Smc) proteins, Smc1 and Smc3, which physically link sister chromatids during S phase. The establishment of cohesion is crucial for accurate chromosome segregation in mitosis. By ChIP experiments, it has been shown that cohesins are also recruited in a ~50 kb domain around the DSB (Unal et al., 2004). This cohesin-rich domain showed extensive overlap with the region that contained phospho-H2A and it is dependent upon phosphorylation of histone H2A by Mec1 and Tel1, and the presence of the DNA repair protein Mre11 (Unal et al., 2004). This damage-linked spread of cohesins was shown to facilitate repair, presumably by maintaining sister chromatids in close proximity for post-replicative recombination (Unal et al., 2004; Strom et al., 2004).

Histones acetylation

A transient acetylation of H2A, H3 and H4 in their amino-terminal tails has been found to occur at DSBs in both mammalian and yeast cells (van Attikum and Gasser, 2005). Acetylation promotes chromatin relaxation, thus making chromatin more accessible for DNA repair and checkpoint factors. The acetylation is mediated by TRRAP/TIP60 complex in mammals or its homologue NuA4 in yeast.

NuA4 is recruited to DSB sites by interacting with phosphorylated H2A (Downs et al., 2004). Once recruited in the proximity of the lesion, it acetylates H2A and H4. Consistently, mutation in H4 N-terminal tail or NuA4 subunits render the cells hypersensitive to genotoxic treatment (Bird et al., 2002)

The TRRAP/TIP60 complex acetylates several lysine residues (H3K14, K23, H4 K5, K8, K12 and K14) of core histones in vitro (Kimura and Horikoshi, 1998). Mutations of lysine target residues in H4 confer sensitivity to DSB-generating agents (Bird et al., 2002; Tamburini and Tyler, 2005; Murr et al., 2006) Mouse TRRAP deficient cells show defects in the recruitment of 53BP1, Rad51 and BRCA1 at DSB sites and consequent impaired HR repair (Murr et al., 2006). It is not yet

known how histone acetylation by the TIP60 complex regulates chromatin organization. More recently, TIP60 was found to promote ubiquitination of γH2AX (Ikura et al., 2007). It has been therefore suggested that acetylation may be a prerequisite for ubiquitination of γH2AX. Furthermore, acetylation leads to release of H2AX from chromatin; thus sequential acetylation and ubiquitination of H2AX may promote histone dynamics at DSBs (Ikura et al., 2007).

Histones ubiquitination

Evidence collected in the last few years demonstrated that the damage-dependent ubiquitination of histones H2A and H2A(X) is an important step in the DNA Damage Response in mammalian cells.

The carboxy-terminal part of γH2A is recognized by the tandem BRCT domain of MDC1, a scaffold protein critical for mediating downstream events (Stucki et al., 2005). Initial recruitment of MDC1 to the DSB site leads to the subsequent loading of Ubc13/Rnf8 ubiquitin ligase complex to the proximity of the lesion (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Wang and Elledge, 2007). Such enzyme ubiquitinate γH2AX and H2A at the DSBs. The ubiquitination is initiated by the Ubc13/Rnf8 complex and it is then amplified by another ubiquitin ligase, Rnf168 (Doil et al., 2009).

Histone ubiquitination participates in the remodeling of chromatin, facilitating accumulation of DNA repair proteins in response to DNA damage. Ubiquitinated γH2AX and H2A create docking sites for the Rap80/Abraxas/Brca1/Brcc36 complex (Wang and Eleldge, 2007) and for 53BP1 (Huen et al., 2007; Kolas et al., 2007). Not surprisingly, in the absence of Rnf8, the G2/M checkpoint is impaired, cells become sensitive to low doses of IR and are compromised for DSB repair by HR (Huen et al., 2007; Kolas et al., 2007).

Besides γH2AX, H2B is also a substrate of Rnf8 (Wu et al., 2009), however, it is not know whether H2B ubiquitination contributes to DDR.

H2A ubiquitination by Ucb13/Rnf8 occurs also at the sites of UV-induced DNA damage (Marteijn et al., 2009). Depletion of these enzymes confers UV sensitivity. Similar to what has observed for DSBs, Rnf8 is recruited to the sites of UV damage in a MDC1-dependent manner, but requires ATR as well as NER-generated single-stranded repair intermediates.

In a recent work, Wang and coworkers found that the CUL4-DDB-ROC1 complex ubiquitinates H3 and H4 (Wang et al., 2006). Further biochemical studies indicated that the H3/H4 ubiquitination weakens the interaction between histones and DNA, suggesting that it might play a crucial role for chromatin disassembly at the sites of UV lesion, which is functional for the recruitment of NER factors (Wang et al., 2006).

ATP-dependent chromatin remodeling activities involved in DNA repair

Increasing evidence suggests that an effective DNA repair requires histones exchange and nucleosome release by ATP-dependent chromatin remodeling activities.

The multi-subunit Ino80 complex is perhaps the most intensely studied ATP-dependent remodeling factors involved in DNA repair. INO80 is recruited to the proximity of the DSB through a direct interaction of its Arp4 and/or Nhp10 subunits with γH2AX (Downs et al., 2004; Morrison et al., 2004; van Attikum et al., 2004). Once there, it might remove γH2AX and the other core histones (van Attikum et al., 2007), enabling access to DNA repair factors, in particular end-processing enzymes such as the MRX complex. This is confirmed by the observation that mutation in INO80-specific subunits Arp8 and Nhp10 impair the binding of Mre11, Ku80 and Mec1 at the DSB, resulting in defective end-processing and reduced checkpoint activation (van Attikum et al., 2007).

Another important ATP-dependent chromatin remodeling factor involved in DDR is the RSC complex. RSC is essential in yeast and has homologues in other eukaryotes. It interacts with Mre11 and is recruited to DSB during the early steps of the DNA damage response (Shim et al., 2005). As mentioned before, it is commonly thought that the RSC complex participates in chromatin remodeling and in increasing the ability of MRX to bind and mediate resection of DNA ends (Shim et al., 2007)

The SWI/SNF chromatin remodeling complexes are also recruited to DSB sites during the DSB repair, just before strand invasion, where they promote the release of nucleosomes surrounding the breaks, thus facilitating the search for homology (Chai et al., 2005). Like RSC, they are also recruited to the homologous donor sequences, suggesting that on these sequences nucleosomes have to be evicted by these remodeling factors in order to expose homologous DNA to the homology-searching complex (Chai et al., 2005). Finally, several lines of evidence link SWI/SNF activity to the repair of UV-induced lesions: it has been shown that, *in vitro*, SWI/SNF enhances the incision and excision steps by purified NER proteins in reconstituded nucleosomes with UV-damaged DNA (Hara and Sancar, 2002; Gailard et al., 2003). SWI/SNF also appears to act also *in vivo* during NER, where it is at least partially responsible for increasing DNA accessibility following UV treatment. (Yu et al., 2005; Teng et al., 2005).

RESTORING CHROMATIN STRUCTURE AFTER DNA REPAIR

After successful completion of DNA repair, the DNA damage signalling must be turned off and normal chromatin structure must be restored. This step is essential to maintain a functional genome, and it is a process tightly coordinated with DNA repair.

Clearance of chromatin modifications following DNA repair

H2A(X) dephosphorylation

The elimination of the repair-specific isoform γH2A(X) is necessary for restoring chromatin structure following DNA repair. In particular, dephosphorylation of γH2A(X) appears to be crucial for reverting the chromatin configuration to one that is less permissive to the access of DNA damage responsive proteins (Heo et al., 2008).

In mammalian cells, PP2A and PP4C are involved in dephosphorylation of γH2AX (Chowdhury et al., 2005; Nakada et al., 2008), and PP4C seems to be the principal participant in γH2AX dephosphorylation at IR-induced DSBs (Nakada et al., 2008). It is not clear whether the dephosphorylation takes place *in situ* or whether it requires removal of γH2A(X) from chromatin. In this regard, the partial colocalization of γH2A(X) with PP2A after DNA damage is compatible with *in situ* dephosphorylation. On the other hand, in yeast, γH2A is first removed and it is subsequently dephosphorylated by the histone H2A phosphatase complex (HTP-C), whose active subunit, Pph3, is 60% identical to PP2A (Keogh et al., 2006). As a result of this, yeast *pph3* null mutants and siRNA PP4C-depleted human cells contain high steady-state levels of γH2A(X) and are defective in checkpoint extinction (Keogh et al., 2006; Nakada et al., 2008).

Histone deacetylation

As described above, various HAT complexes are recruited during the DNA damage response and generate a transient increase in histone acetylation. Likewise, several histone deacetylases (HDACs) have been implicated in the DNA damage response. Yeast Rpd3, Sir2 and Hst1 seem to act late in the DNA damage response reducing histone acetylation once repair has been completed, allowing the recovery of the higher-order structure of the chromatin (Jazayeri et al., 2004; Tamburini and Tyler, 2005; Utley et al., 2005). Moreover, chromatin compaction upon

deacetylation contributes to the termination of the associated checkpoint activity (Murr et al., 2006).

At a late stage of the damage response, serine 1 of histone H4 is phosphorylated by casein kinase 2 (CK2) (Utley et al., 2005). Moreover, CK2 associates with the HDAC Sin3-Rpd3 complex, which promotes histone deacetylation at DSBs. Interestingly, phosphorylation of H4S1 inhibits H4 acetylation by NuA4, suggesting that histone phosphorylation and deacetylation regulate chromatin restoration after the completion of DNA repair.

Chromatin reassembly after DNA repair

During chromatin restoration, histones that have been evicted from the sequences closer to the DNA breaks, have to be redeposited on newly repaired DNA, by a process known as chromatin assembly.

Genetic and biochemical studies performed in budding yeast demonstrated that both Chromatin Assembly Factor 1 (CAF-1) and Anti-Silencing Factor 1 (Asf1) are implicated in chromatin assembly following the repair of different DNA lesions. Indeed, deletion of CAF-1 or Asf1 makes yeast cells sensitive to different DNA-damaging agents, such as UV and gamma radiation, radiomimetics and alkylating compounds (Qin and Parthun, 2002; Linger and Tyler, 2007).

CAF-1 is recruited to UV-damaged sites by interacting with the sliding clamp PCNA. Its main role is to deposit new histone H3.1 (the major H3 variant) in a post-repair step (Polo et al., 2006). It has been also demonstrated that CAF-1 promotes incorporation of new histone H3 at DSBs site, contributing to DSB repair (Nabatiyan et al., 2007).

During DNA replication, NER and DSB repair CAF-1 cooperates functionally with the chromatin assembly and disassembly factor Asf1 (Linger and Tyler, 2007). Asf1 and CAF-1 also physically interact and this could be the means by which Asf1 is recruited to the DNA damage sites (Mello et al., 2002). Asf1 stimulates Rtt109 to acetylate free H3K56 after DNA repair. It seems that stretches of chromatin bearing acetylated H3K56 signals that DNA repair is complete and therefore drive chromatin reassembly and recovery from the DNA damage response (Chen et al., 2008).

PART II –

THESIS

PROJECT

I

AIM OF THE PROJECT

Eukaryotic cells respond to DNA damage by activating a variety of DNA repair pathways and by triggering the DNA damage checkpoint, a surveillance mechanism required to control cell cycle progression in response to genotoxic stress (Elledge, 1996).

A considerable amount of information is now available relative to the key protein factors involved in the DNA damage checkpoint. In particular, the molecular details of the signaling pathway in fission and budding yeasts have been mostly worked out by ANALYZING the phosphorylation of critical kinase substrates (Longhese et al., 1998; Carr, 2002).

In *S. cerevisiae,* the first biochemical event detectable in the signal transduction cascade is the Mec1-dependent phosphorylation of its interacting partner Ddc2 (Paciotti et al., 2000, Rouse and Jackson, 2000). Other critical Mec1 targets are histone H2A, the Ddc1 subunit of the 9-1-1 complex and the Rad9 mediator, the orthologue of human 53BP1 and fission yeast Crb2. Phosphorylation of Rad9, followed by its oligomerization is necessary for the recruitment and activation of the main effector kinase Rad53.

Interfering with Rad9 recruitment to the proximity of the lesion and its phosphorylation impairs the signal transduction cascade, the consequent Rad53 activation and thus, the checkpoint response.

It has been previously shown that histones modifications play a significant role in Rad9 recruitment. Indeed, the ubiquitylation of histone H2B by the Rad6/Bre1 complex and the subsequent methylation of histone H3 on the K79 residue, mediated by Dot1, are prerequisites for a

functional DNA damage reponse (Giannattasio et al., 2005; Wysocki et al., 2005; Grenon et al., 2007). Recruitment of the Rad9 orthologues to methylated residues exposed at sites of DNA damage seems to be a highly conserved mechanism for efficient checkpoint signalling. In fact, also 53BP1 and Crb2 recognize H4 methylated at lysine 20 (H4-K20me), although human 53BP1 may be recruited to chromatin also through interactions with H3K79me (Huyen et al., 2004; Sanders et al., 2004;Du et al., 2006;Bouyan et al., 2006; Schotta et al., 2008) The recognition of methylated histones occurs through the tandem Tudor domain of "Rad9-like" family of proteins, which accommodates the lysine methylated in its pocket, at the interface of the two Tudor motif.

In *S. cerevisiae*, the impairment of this pathway, that is, the abolition of H3K79 methylation or the mutation of the Rad9 Tudor domain, prevents the Rad9 and Rad53 phosphorylation in G1-arrested cells following DNA damage (Giannattasio et al., 2005; Wysocki et al., 2005). In these conditions, Rad9 cannot be loaded onto DNA and therefore, the cells 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, in M-arrested cells, deletion of *DOT1* is not sufficient to eliminate the checkpoint function. *dot1Δ* mutant cells treated with zeocin or UV light in the G2/M phase of the cell cycle display a residual Rad9 and Rad53 phosphorylation and moreover, an apparently normal cell cycle arrest (Giannattasio et al., 2005). These observations suggests that a different mechanism of Rad9 recruitment can compensate for the loss of the histone-dependent pathway.

At the beginning of my PhD work I tried to define the nature of this pathway, looking for the factor/s involved in the G2/M checkpoint activation in the absence of the histone H3 methyltransferase. In particular, I focused my attention on the mechanism involved in Rad9 recruitment to the proximity of the apical kinase Mec1.

After having unveiled the second mechanism which permits the DNA damage checkpoint activation in the G2/M phase of the cell cycle, I tried to explain why this mechanism is solely active in this phase of the cell cycle, investigating the possible role of the CDK in the control of this mechanism.

The data collected in these 3 years were published in the papers which follows:

- Puddu F*, **Granata M***, Di Nola L, Balestrini A, Piergiovanni G, Lazzaro F, Giannattasio M, Plevani P, Muzi-Falconi M.(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.* Aug;28(15):4782-93.

  *= co-first authors

- **Granata M**, Lazzaro F, Novarina D, Panigada D, Puddu F, Abreu CM, Kumar R, Grenon M, Lowndes NF, Plevani P, Muzi-Falconi M. (2010) **Dynamics of Rad9 chromatin binding and checkpoint function are mediated by its dimerization and are cell cycle-regulated by CDK1 activity.** *PLoS Genet.* Aug 5;6(8)

During my PhD I also contributed to the writing of a review about the crosstalk between DNA damage checkpoint and DNA repair pathways:

- Lazzaro F, Giannattasio M, Puddu F, **Granata M**, Pellicioli A, Plevani P, Muzi-Falconi M. (2009) **Checkpoint mechanisms at the intersection between DNA damage and repair.** *DNA Repair (Amst)*. Sep 2;8(9):1055-67.

All these papers are attached to the following section.

2

PUBLICATIONS

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

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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▽†

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Following genotoxic insults, eukaryotic cells trigger a signal transduction cascade known as the DNA damage checkpoint response, which involves the loading onto DNA of an apical kinase and several downstream factors. Chromatin modifications play an important role in recruiting checkpoint proteins. In budding yeast, methylated H3-K79 is bound by the checkpoint factor Rad9. Loss of Dot1 prevents H3-K79 methylation, leading to a checkpoint defect in the G_1 phase of the cell cycle and to a reduction of checkpoint activation in mitosis, suggesting that another pathway contributes to Rad9 recruitment in M phase. We found that the replication factor Dpb11 is the keystone of this second pathway. *dot1Δ dpb11-1* mutant cells are sensitive to UV or Zeocin treatment and cannot activate Rad53 if irradiated in M phase. Our data suggest that Dpb11 is held in proximity to damaged DNA through an interaction with the phosphorylated 9-1-1 complex, leading to Mec1-dependent phosphorylation of Rad9. Dpb11 is also phosphorylated after DNA damage, and this modification is lost in a nonphosphorylatable *ddc1-T602A* mutant. Finally, we show that, in vivo, Dpb11 cooperates with Dot1 in promoting Rad9 phosphorylation but also contributes to the full activation of Mec1 kinase.

The cellular response to DNA damage is based on signal transduction mechanisms that are essential for the maintenance of genome integrity. The molecules involved and the organization of the pathway are generally conserved in all eukaryotes (2, 29, 30, 42). A major output of this response is a controlled delay in cell cycle progression that regulates the G_1-S transition (G_1 checkpoint) or the G_2-M transition (G_2/M checkpoint; in budding yeast, this response does not regulate the passage from G_2 to M but prevents the anaphase-to-metaphase transition). This is achieved by regulating Cdk kinase or anaphase-promoting complex activities. The current model predicts that genotoxin treatments activate the DNA damage checkpoint response through the recruitment of the ATM and ATR phosphoinositide 3-kinase-related kinases to damaged chromatin (42, 51). The molecular details of the DNA damage signaling pathway in fission and budding yeasts have been mostly worked out by following the phosphorylation of critical kinase substrates in appropriately mutated genetic backgrounds (5, 25). In budding yeast, the prevalent apical kinase is represented by Mec1, which is associated with a Ddc2 subunit. Processing of DNA lesions by repair mechanisms generates single-stranded DNA (ssDNA) filaments that are rapidly coated by replication protein A (RPA). This structure seems to be responsible for the recruitment of Mec1-Ddc2 (24, 38, 51). The first biochemical event in the signal transduction cascade seems to be the direct phosphorylation of Ddc2 (33, 37). A heterotrimeric complex (9-1-1) composed of Rad17, Mec3, and Ddc1 is loaded onto damaged DNA by a replication factor C-like complex and is itself phosphorylated by Mec1 on the Ddc1 subunit (25, 28, 34). Another Mec1 target is checkpoint factor Rad9, the orthologue of human 53BP1 and fission yeast Crb2. Phosphorylation of Rad9, followed by its oligomerization, allows the recruitment of Rad53 kinase and its activation; it can be visualized as a hyperphosphorylated slower-mobility form by Western blotting (12, 36, 40, 43). Interfering with Rad9 recruitment prevents the activation of Rad53 and cell cycle arrest after DNA damage. Recent work demonstrated that histone H2B ubiquitylation, carried out by Rad6-Bre1, and histone H3 methylation on lysine 79 (H3-K79), performed by Dot1, contribute to Rad9 recruitment to chromatin (11, 13, 14, 48). This pathway depends on an interaction between methylated H3-K79 and the Tudor domain of Rad9. Loss of these histone modifications (e.g., in *dot1Δ* mutant cells) or mutation of the Rad9 Tudor domain prevents Rad9 and Rad53 phosphorylation in G_1-arrested cells and abolishes the G_1-S arrest following DNA damage (11, 13, 48). The current model predicts that Rad9 bound to histone H3 can be phosphorylated by Mec1 and then binds to phosphorylated H2A (14). Surprisingly, in M cells, deletion of *DOT1* is not sufficient to eliminate checkpoint function. *dot1Δ* mutant cells are not particularly sensitive to Zeocin or UV and, when irradiated in M, display an apparently normal cell cycle arrest, despite a lower level of Rad53 phosphorylation, mirrored by a slightly reduced modification of Rad9 (11). These observations suggest that the

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† Supplemental material for this article may be found at http://mcb.asm.org/.

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pathways involved in the recruitment of Rad9 to chromatin are somehow cell cycle specific; in M cells, another mechanism, partially redundant with the histone modification pathway, must be active to obtain Rad9 phosphorylation and effective checkpoint activation. In the last few years, results obtained with fission yeast, *Xenopus laevis* extracts, and human cells revealed that a new player involved in the DNA damage response is a factor called Rad4/Cut5 in *Schizosaccharomyces pombe*, TopBP1 in higher eukaryotes, and Dpb11 in budding yeast (6–8, 21, 35). These proteins share the presence of BRCT domains, which are involved in protein-protein interactions. The general picture that is starting to emerge is that this factor interacts with phosphoinositide 3-kinase-related kinases, possibly controlling their activity; it is recruited to DNA by interacting with the 9-1-1 complex and facilitates downstream signaling by interacting with Crb2/53BP1 (3, 9). The role played by Dpb11 in the DNA damage response in budding yeast has not been described, and here we show that it is an essential component of this new G_2/M pathway which allows Rad9 recruitment and checkpoint activation in the absence of histone H3 methylation. We provide evidence suggesting that, in M-phase cells, Rad9 can be phosphorylated by Mec1 through H3-K79 methylation or through an interaction with Dpb11. We also show that the functional interaction between Dpb11 and the Ddc1 subunit of the 9-1-1 complex is regulated by a Mec1-dependent phosphorylation of a specific Ddc1 C-terminal threonine, which likely allows the recruitment of Dpb11 to damaged chromatin and its phosphorylation by Mec1. Finally, we provide in vivo evidence that in budding yeast, Dpb11 is involved in directly regulating the apical kinase Mec1.

MATERIALS AND METHODS

Strains and plasmids. All of the strains used in this work are derivatives of W303 (K699 [*MATa ade2-1 trp1-1 can1-100 leu2-3,12 his3-11,15 ura3*]), except histone H4 mutants. The K20R and K59R histone H4 mutants were obtained by plasmid shuffling with pFL17.5 and pFL19.5, respectively, in strain UCC1111 (20). These last plasmids were obtained by PCR over pMP3 (20) with mutagenic oligonucleotides.

The YFP20 (*dpb11-1*) and YMAG6 (*dpb11-1dot1*Δ) strains were obtained by PstI-directed integration of YIplac211-*dpb11-1* (1) into K699 and YFL234, respectively (11). Plasmid pop-out events were selected on 5-fluoroorotic acid plates, and the presence of the *dpb11-1* allele was confirmed by checking the temperature-sensitive phenotype and by PCR analysis to confirm the presence of the mutation. All of the other *DPB11* mutant strains were obtained by crossing; myc-tagged *DPB11* mutant strains were obtained by using the one-step PCR system (27) to allow detection by Western blotting; however, tagged Dpb11 cannot be immunoprecipitated, likely because the tag is hidden in the native protein.

DDC1 site-specific mutations were obtained by PCR with mutagenic oligonucleotides by using the pML89 plasmid (26). Multiple round of mutagenesis over these plasmids allowed the construction of the pLD12, pLD26, and pLD31 plasmids, carrying the *ddc1-M3* (*S413A*, *S436A*, *T444A*), *ddc1-M8* (*T342A*, *S469A*, *S471A*, *S495A*, *T529A*, *S532A*, *S580A*, *T602A*), and *ddc1-M11* (containing a combination of all of the above-mentioned point mutations) alleles, respectively. All of these plasmids were transformed into *ddc1*Δ mutant strain YLL244 (26) to obtain *ddc1* mutant yeast strains. Plasmid pFP9 carrying the *ddc1-T602S* mutation was obtained by PCR with mutagenic oligonucleotides by using the pML89 plasmid as the template.

Strains carrying a Dpb11 degron tag, YJT70 (*dpb11^{td}*) and Y1812 (*dpb11^{td} DPB11*), were a kind gift from J. F. X. Diffley.

All of the strains used in this study are described in Table 1.

DNA damage sensitivity assay. In order to assess cell survival after UV irradiation, yeast strains were cultured overnight to stationary phase. Cells were then diluted, and approximately 200 cells were plated on petri dishes, which were irradiated with different UV doses. After 3 days, the total number of colonies that formed on each plate was determined. Alternatively, overnight cultures were diluted to 1×10^6 cells/ml and then 10-fold serial dilutions were prepared and 10-μl volumes of the suspensions were spotted onto plates, which were either UV irradiated or mock treated. To assess survival of Zeocin treatment, exponentially growing cells were treated for 30 min with different concentrations of the drug. After Zeocin removal, cultures were diluted to 1×10^6 cells/ml and then 10-fold serial dilutions were prepared and 10-μl volumes of the suspensions were spotted onto YPD plates (31). Images were taken 3 days later.

SDS-PAGE and Western blotting. Protein extracts obtained with trichloroacetic acid (31) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% acrylamide gels; for analysis of Rad9 phosphorylation, NuPAGE Tris-acetate 3 to 8% gels were used by following the manufacturer's instructions. Western blotting was performed with anti-Rad53, anti-myc (9E10), antihemagglutinin (anti-HA; 12CA5), anti-Ddc1, and anti-Rad9 antibodies by using standard techniques. For more efficient detection of phosphorylated Dpb11 isoforms, 7.5% acrylamide gels supplemented with Phos tag-conjugated acrylamide were used according to the manufacturer's instructions (NARD Institute Ltd.).

Cell cycle blocks and DNA damage treatment. Cells were grown in YPD medium at 28°C (25°C in the experiments with strains harboring the *dpb11-1* mutation) to a concentration of 5×10^6/ml and arrested with nocodazole (20 μg/ml). Fifty-milliliter volumes of cultures were spun, resuspended in 500 μl of fresh YPD plus nocodazole, and plated on a petri dish (14-cm diameter). Plates were quickly irradiated at 75 J/m^2, and cells were resuspended in 50 ml of YPD plus nocodazole. A 25-ml sample was taken immediately and processed for protein extract preparation, while a second 25-ml sample was taken 30 min afterward. For analysis of the double-strand break (DSB) checkpoint response, nocodazole-arrested cells were treated with 100 μg/ml Zeocin. Samples were taken from the culture every 15 min and processed for protein extraction.

G_2/M checkpoint assay. Yeast cells were synchronized in M by treating exponentially growing cultures with 5 μg/ml nocodazole. UV treatment was performed as described previously (10), except that 6 μg/ml α-factor was added to the resuspension medium. Cells were then stained with 4′,6′-diamidino-2-phenylindole (DAPI), and nuclear division was monitored by microscopic analysis.

Use of the *dpb11^{td}* allele. As previously described (44, 49), the *dpb11^{td}* mutant strain (YJT70) contains the Dpb11-td fusion under the control of tTA and the *tetO2* promoter, the E3 ubiquitin ligase gene *UBR1* under the control of the inducible *GAL1* promoter, and three copies of pCM244 harboring a mutated Tet repressor-SSN6 fusion (*tetR′-SSN6*) gene integrated at the *LEU2* locus. Y1812 (*dpb11^{td} DPB11*) is isogenic to strain YJT70, but it also contains a copy of the *DPB11* gene under the control of its own promoter. YMAG78/4b and YMAG82/15a are derivatives of YJT70 and Y1812, respectively, carrying an HA-tagged version of Ddc2.

These strains were grown in YP plus raffinose at 28°C to a concentration of 5×10^6 cells/ml and arrested with nocodazole. Twenty-five milliliters of arrested cells was immediately processed for protein extraction with trichloroacetic acid. The rest of the culture was shifted to 37°C in the presence of galactose (2%) and tetracycline (50 μg/ml) for 2.5 h. This treatment leads to Dpb11-td degradation and represses *dpb11^{td}* transcription, inducing the *dpb11*-encoded phenotype. A 150-ml volume of cells was spun, resuspended in 1.5 ml of the same medium, and UV irradiated as described previously. After treatment, cultures were shifted to 28°C. A 25-ml sample was taken immediately and processed for protein extract preparation, and a second 25-ml sample was taken 30 min later.

RESULTS

We have 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 G_1 phase of the *Saccharomyces cerevisiae* cell cycle (11). 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 G_1 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 (11, 13, 14, 18, 48). Surprisingly, if *dot1*Δ mutant cells are treated with Zeocin or UV light in the

TABLE 1. Strains used in this study

Strain	Relevant genotype	Source
K699	*MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100*	K. Nasmyth
K700	*MAT*α *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3 can1-100*	K. Nasmyth
YFL448/1a	K700 *dot1::kanMX6 tel1::HIS3*	This work
YFL438	K699 *dot1::kanMX6 mec1-1 sml1*	This work
YFL499/3d	K699 *dot1::kanMX6 chk1::kanMX6*	This work
YFL224	K699 *bre1::kanMX6*	M. Giannattasio
YFL236	K699 *set2::kanMX6*	This work
YMG203	K699 *bre1::kanMX6 set2::kanMX6*	This work
UCC1111	*MAT*α *ade2::his3Δ200 leu2Δ0 lys2Δ0 met15Δ trp1Δ63 ura3Δ0 adh4::URA3-TEL (VII-L) hhf2-hht2::MET15 hhf1-hht1::LEU2*(pRS412–*ADE2 CEN ARS–HHF2-HHT2*)	D. E. Gottschling
YFL287	UCC1111(pMP3)	This work
YFL288	UCC1111(pFL17.5 [H4-K20R])	This work
YFL290	UCC1111(pFL19.5 [H4-K59R])	This work
YFL292	UCC1111 *dot1::kanMX6*(pMP3)	This work
YFL294	UCC1111 *dot1::kanMX6*(pFL17.5 [H4-K20R])	This work
YFL296	UCC1111 *dot1::kanMX6*(pFL19.5 [H4-K59R])	This work
YFP20	K699 *dpb11-1*	This work
YFL234	K699 *dot1::kanMX6*	M. Giannattasio
YMAG6	K699 *dot1::kanMX6 dpb11-1*	This work
YMIC4F6	K699 *mec3::TRP1 rad9::URA3*	This work
YLL683.8/3B	K699 *ddc2::DDC2-3HA:URA3*	M. P. Longhese
YFP24/6b	K699 *dpb11-1 ddc2::DDC2-3HA:URA3*	This work
YFL403/10b	K699 *dot1::kanMX6 ddc2::DDC2-3HA:URA3*	F. Lazzaro
YFL687/2b	K699 *dot1::kanMX6 dpb11-1 ddc2::DDC2-3HA:URA3*	This work
YFL211/3a	K699 *RAD9-13myc:TRP1 ddc1::DDC1-HA:LEU2*	This work
YMAG48/5b	K700 *dpb11-1 RAD9-13myc:TRP1 ddc1::DDC1-HA:LEU2*	This work
YMAG34/4a	K699 *dot1::kanMX6 RAD9-13myc:TRP1 ddc1::DDC1-HA:LEU2*	This work
YMAG52/3d	K699 *dot1::kanMX6 dpb11-1 RAD9-13myc:TRP1 ddc1::DDC1-HA:LEU2*	This work
YMIC4E8	K699 *rad9::URA3*	F. Lazzaro
YMAG149/7B	K699 *hta1-htb1::LEU2 hta2-htb2::TRP1*(pSAB6 [*HTA1-HTB1*])	This work
YMAG168	K699 *hta1-htb1::LEU2 hta2-htb2::TRP1*(pJD151 [*hta1-S129A-HTB1*])	This work
YMAG150/4A	K699 *dot1::kanMX6 hta1-htb1::LEU2 hta2-htb2::TRP1*(pSAB6 [*HTA1-HTB1*])	This work
YMAG170	K699 *dot1::kanMX6 hta1-htb1::LEU2 hta2-htb2::TRP1*(pJD151 [*hta1-S129A-HTB1*])	This work
YLDN25	K699 *ddc1::kanMX4*(pML89)	This work
YLDN17	K699 *ddc1::kanMX4*(pLD12)	This work
YLDN23	K699 *ddc1::kanMX4*(pLD26)	This work
YLDN24	K699 *ddc1::kanMX4*(pLD31)	This work
YFP27	K699 *ddc1::kanMX4 dot1::HIS3*(pML89)	This work
YFP28	K699 *ddc1::kanMX4 dot1::HIS3*(pLD12)	This work
YFP29	K699 *ddc1::kanMX4 dot1::HIS3*(pLD26)	This work
YFP30	K699 *ddc1::kanMX4 dot1::HIS3*(pLD31)	This work
YLDN9	K699 *ddc1::kanMX4*(pLD9)	This work
YFP37	K699 *ddc1::kanMX4 dot1::HIS3*(pLD9)	This work
YFP148	K699 *ddc1::kanMX6*(pFP9)	This work
YFP149	K699 *ddc1::kanMX6 dot1::HIS3*(pFP9)	This work
YFP38	K699 *dpb11::DPB11-13myc:HIS3*	This work
YFP48/3a	K699 *dpb11::DPB11-13myc:HIS3 mec1-1 sml1-1*	This work
YFP49/1d	K699 *dpb11::DPB11-13myc:HIS3 rad53::kanMX6 sml1::HIS3*	This work
YFP55/6c	K699 *ddc1::kanMX6 dpb11::DPB11-13myc:HIS3*	This work
YFP56	K699 *ddc1::kanMX6 dpb11::DPB11-13myc:HIS3*(pML89)	This work
YFP57	K699 *ddc1::kanMX6 dpb11::DPB11-13myc:HIS3*(pLD9)	This work
YFP63	K699 *ddc1::kanMX6*(pML89)	This work
YFP64	K699 *ddc1::kanMX6*(pLD9)	This work
YFP65	K699 *ddc1::kanMX6 dpb11-1*(pML89)	This work
YFP66	K699 *ddc1::kanMX6 dpb11-1*(pLD9)	This work
YFP 152	K699 *ddc1::kanMX6* [Ycplac111)	This work
YFP 142	K699 *dot1::HIS3 dpb11-1 ddc1::kanMX6*(pML89)	This work
YFP 144	K699 *dot1::HIS3 dpb11-1 ddc1::kanMX6*(pLD9)	This work
YFP50	EGY48(pSH18.34/pFP1/pFP2)	This work
YFP52	EGY48(pSH18.34/pFP1/pFP4)	This work
YFP113	K699 *mec1-1 sml1*(pSH18.34/pFP1/pFP2)	This work
YFP114	K699 *mec1-1 sml1*(pSH18.34/pFP1/pFP4)	This work
YFP86	EGY48(pSH18.34/pJG4-5/pFP2)	This work
YFP54	EGY48(pSH18.34/pFP1/pEG202)	This work
YFP153	EGY48(pSH18.34/pFP1/pFP10)	This work
YMAG78/4b	Y1812 *ddc2HA-URA3*	This work
YMAG82/15a	YJT70 *ddc2HA-URA3*	This work

M phase of the cell cycle, residual phosphorylation of Rad53 can be observed and the G_2/M checkpoint response is partially proficient, allowing *dot1*Δ mutant cells to survive the treatment (11). 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., *dot1*Δ mutant cells) was due to the unscheduled activation of a pathway dependent upon the apical kinase Tel1 and/or Chk1. *dot1*Δ, *dot1*Δ *tel1*Δ, *dot1*Δ *chk1*Δ, and *dot1*Δ *mec1-1* 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 (see Fig. S1A in the supplemental material; data not shown).

Rad53 phosphorylation correlates with Rad9 phosphorylation also in the absence of methylated H3-K79 (11); we thus tested whether other histone modifications known to be somehow involved in the DNA damage response might be redundant with H3-K79 methylation and cooperate in Rad9 recruitment. The Set1 and Set2 histone methyltransferases are required for H3-K4 and H3-K36 methylation, respectively (22). Moreover, Set1 has been suggested to play a partial role in the intra-S DNA damage checkpoint (11). Abolishing the function of Set1 and Set2 did not affect Rad53 phosphorylation in wild-type (WT) cells, nor did it reduce the residual Rad53 activation detected when *dot1*Δ mutant cells were UV irradiated in M phase (see Fig. S1B in the supplemental material) (13). In the structure of the nucleosome, H3-K79 is very close to H4-K59 (50), and in *S. pombe*, methylated H4-K20 binds Crb2, the Rad9 orthologue (39). We thus tested the contribution of these residues by analyzing Rad53 phosphorylation in cells carrying H4-K20R or H4-K59R mutations in a *dot1*Δ mutant background. When these strains were treated with UV in M phase, they displayed the same level of Rad53 phosphorylation as the isogenic *dot1*Δ mutant cells (see Fig. S1C in the supplemental material); similar results were obtained when the deletion of *DOT1* was combined with a point mutation in the histone H2A tail, preventing the damaged-induced phosphorylation of serine 129 (see Fig. S1D in the supplemental material). These observations suggested the existence of a different, histone-independent, pathway involved in Rad9 recruitment.

In *S. pombe*, Crb2 can be recruited to chromatin through an interaction with Cut5/Rad4 to fulfill its function in the checkpoint response (7). We analyzed 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 *dot1*Δ mutant M-phase cells.

In order to address this question, we generated strains carrying a temperature-sensitive *dpb11-1* mutation in a *dot1*Δ mutant background and monitored the cellular response to UV. The *dpb11-1* mutant at permissive temperature grows normally (1). Under our experimental conditions, when exposed to different levels of UV light, the *dpb11-1* and *dot1*Δ mutant strains are slightly more sensitive than WT cells. Interestingly, the *dot1*Δ and *dpb11-1* mutations exhibit synergistic effects on sensitivity to UV; indeed, the *dot1*Δ *dpb11-1* double mutant is noticeably more sensitive than either one of the single mutants and closely resembles a *rad9*Δ mutant strain (Fig. 1A). 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 Fig. 1B, UV-treated *dpb11-1* and *dot1*Δ 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 *mec3*Δ *rad9*Δ mutant, checkpoint-null control cells.

We then analyzed the phosphorylation cascade that is triggered by UV, monitoring the phosphorylation state of the Ddc2, Rad9, and Rad53 factors, which act sequentially in the checkpoint cascade. Figure 1C shows that in M phase, *dot1*Δ 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 in the *dot1*Δ *dpb11-1* double mutant, while Mec1 activity, as measured by Ddc2 phosphorylation, does not seem to be significantly reduced. 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. If UV irradiated in G_1, *dot1*Δ mutant cells are unable to delay entry into S phase and budding, and Rad53 phosphorylation is grossly defective (11). Under these conditions, a minor phosphorylation of Rad53 can be detected in *dot1*Δ mutant cells only if cultures are held in G_1 for at least 30 min after the genotoxic treatment, and this residual checkpoint activity is *DPB11* dependent, being lost in *dot1*Δ *dbp11-1* mutant cells (Fig. 1D).

We then analyzed whether this mechanism is UV specific or is also involved in the response to DSBs. Nocodazole-arrested cells were treated with the DSB-inducing agent Zeocin; survival and Rad53 activation were then monitored in WT and *dot1*Δ, *dpb11-1*, and *dot1*Δ *dpb11-1* mutant cells. Even in response to DSBs, a mutation in *DPB11* is synthetic with the loss of H3-K79 methylation; in fact, the *dot1*Δ *dpb11-1* double mutant is more sensitive than either single mutant (Fig. 2A) and Rad53 phosphorylation is grossly defective in double-mutant cells (Fig. 2B).

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 phosphoprotein binding motif (47). Since Ddc1 is subject to cell cycle-dependent and DNA damage-dependent phosphorylation (26, 34), we tested whether Ddc1 phosphorylation plays any role in controlling this Dpb11-dependent pathway. The deduced protein se-

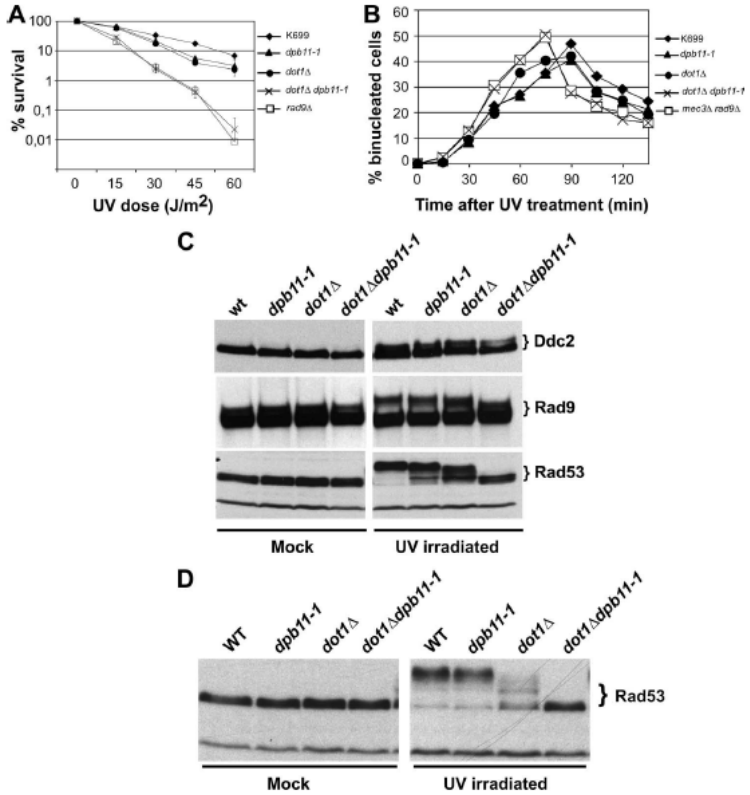

FIG. 1. Dpb11 function is required for the Dot1-independent checkpoint activation pathway in response to UV irradiation. (A) UV survival assay. Strains K699 (WT), YMIC4E8 (*rad9*Δ), YFP20 (*dpb11-1*), YFL234 (*dot1*Δ), and YMAG6 (*dot1*Δ *dpb11-1*) were grown overnight to stationary phase and then diluted and plated on YPD plates, which were irradiated with the indicated UV doses. Survival was assayed by determining the number of colonies that formed on the plates after 3 days. (B) UV checkpoint assay. Yeast strains K699 (WT), YFP20 (*dpb11-1*), YFL234 (*dot1*Δ), YMAG6 (*dot1*Δ *dpb11-1*), and YMIC4F6 (*mec3*Δ *rad9*Δ) were synchronized in M phase with nocodazole, UV irradiated at 40 J/m^2, and released in YPD plus α-factor. Every 15 min, samples were taken and scored for the presence of binucleated cells. (C) Analysis of the phosphorylation of checkpoint factors. WT and *dpb11-1*, *dot1*Δ, and *dot1*Δ *dpb11-1* mutant cells carrying Ddc2-HA and Rad9-myc were arrested with nocodazole and either mock or UV irradiated (75 J/m^2); 30 min after irradiation, Ddc2, Rad9, and Rad53 phosphorylations were analyzed by SDS-PAGE and Western blotting. (D) Strain K699 (WT), YFP20 (*dpb11-1*), YFL234 (*dot1*Δ), and YMAG6 (*dot1*Δ *dpb11-1*) cells were cultured to mid-log phase, arrested in G_1 with 20 μg/ml α-factor, and either mock or UV irradiated (75 J/m^2); 30 min after irradiation, Rad53 phosphorylation was analyzed by SDS-PAGE and Western blotting.

quence of Ddc1 reveals the presence of three consensus phosphorylation sites for cyclin-dependent kinases and eight putative target sites for Mec1. By site-specific mutagenesis, we converted the phosphorylatable residues to alanine and constructed the *ddc1-M3* allele, lacking the putative Cdk target sites; the *ddc1-M8* allele, lacking the Mec1 target sites; and the *ddc1-M11* allele, lacking all sites (Fig. 3A). In order to determine the contribution of these phosphorylation sites to DNA damage-induced Ddc1 phosphorylation, the phosphorylation state of these mutant proteins was tested after treatment with UV light. While mutations of 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 (Fig. 3B). The role of these phosphorylation sites in the downstream events in the DNA damage checkpoint cascade was further investigated by analyzing 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 *dot1*Δ mutation; both *ddc1-M8 dot1*Δ and *ddc1-M11 dot1*Δ mutant strains lose the ability to hyperphosphorylate Rad9 and Rad53 (Fig. 4A and data not shown) and acquire a UV hypersensitivity similarly to what we ob-

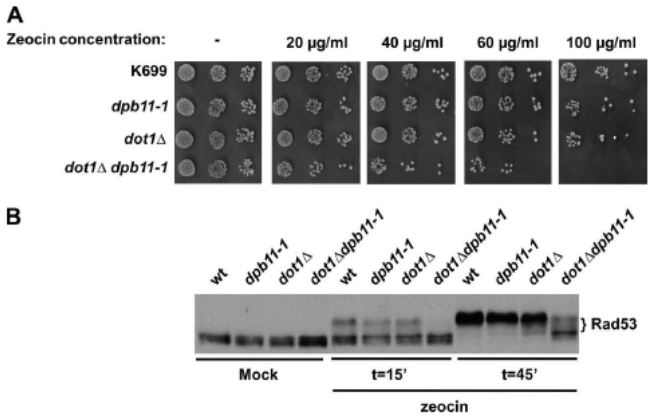

FIG. 2. Dpb11 and Dot1 cooperate in the checkpoint activation pathway in response to DSB-inducing agents. (A) DSB survival assay. Strains K699 (WT), YFP20 (*dpb11-1*), YFL234 (*dot1*Δ), and YMAG6 (*dot1*Δ *dpb11-1*) were grown to mid-log phase and then treated for 30 min with Zeocin at the indicated concentrations. Serial dilutions were then spotted onto YPD plates and incubated for 3 days. (B) Analysis of checkpoint activation after treatment with DSB-inducing agents. Strains K699 (WT), YFP20 (*dpb11-1*), YFL234 (*dot1*Δ), and YMAG6 (*dot1*Δ *dpb11-1*) were cultured to mid-log phase and either mock treated or treated with 100 μg/ml Zeocin. At 15 and 45 min after drug addition, samples were taken and processed for the analysis of Rad53 phosphorylation.

served in *dot1*Δ *dpb11-1* mutant cells (Fig. 4B 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 (6, 8, 23).

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 *dot1*Δ with *ddc1* 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 determined that T602 is the critical residue for the function of this pathway. In fact, Fig. 5A shows that *ddc1-T602A* has the same synthetic effect, in combination with *dot1*Δ, 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 *dot1*Δ

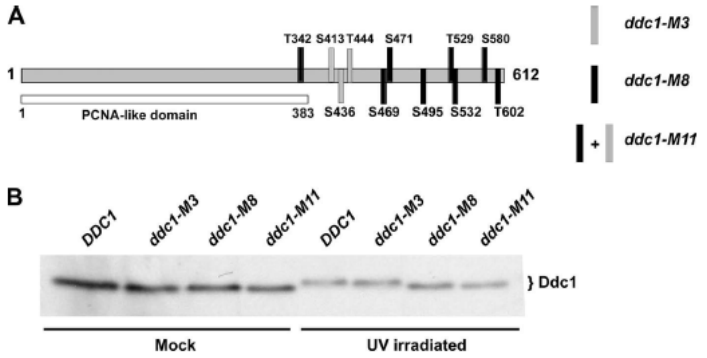

FIG. 3. UV-induced Ddc1 phosphorylation depends upon the presence of Mec1 kinase consensus sites. (A) Outline of the Cdc28 (gray) and Mec1 (black) putative phosphorylation target sites in Ddc1. Cdc28 and Mec1 target sites were mutated to alanine in *ddc1-M3* and *ddc1-M8* mutant strains, respectively. 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/m^2) or mock treated. Protein extracts prepared immediately after UV treatment were separated by SDS-PAGE and analyzed with anti-Ddc1 antibodies.

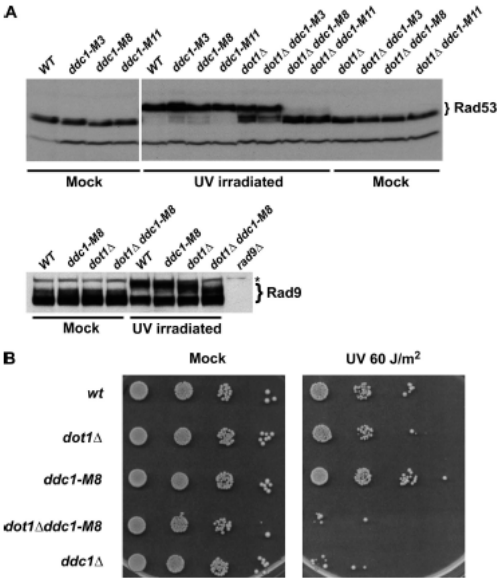

FIG. 4. Phosphorylation of Ddc1 and DOT1 are required for the establishment of an effective UV response. (A) Strains YLDN25 (WT), YLDN17 (*ddc1-M3*), YLDN23 (*ddc1-M8*), YLDN24 (*ddc1-M11*) YFP27 (*dot1*Δ), YFP28 (*dot1*Δ *ddc1-M3*), YFP29 (*dot1*Δ*ddc1-M8*), and YFP30 (*dot1*Δ *ddc1-M11*) were arrested with nocodazole and either UV irradiated (75 J/m^2) or mock treated. Rad9 and Rad53 phosphorylations were analyzed 30 min after irradiation. A protein extract from YMIC4E8 (*rad9*Δ) was loaded onto the same gel in order to identify the anti-Rad9 cross-reacting band, indicated by an asterisk. (B) In order to measure sensitivity to UV irradiation, 10-fold serial dilutions of overnight cultures of the strains from panel A and strain YFP152 (*ddc1*Δ) 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.

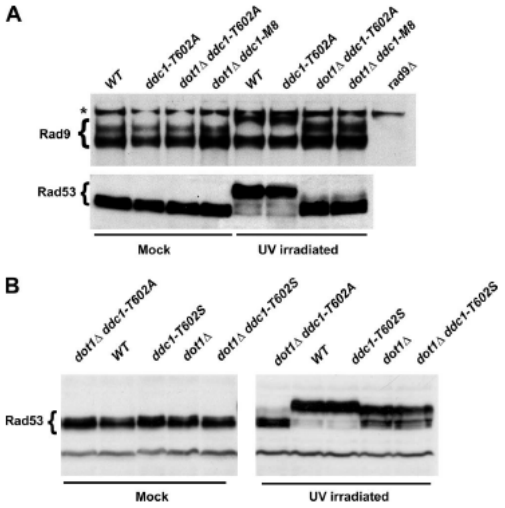

FIG. 5. Phosphorylation of Ddc1 T602 is required for Rad53 and Rad9 phosphorylation in the absence of *DOT1*. (A) Strains YLDN25 (WT), YLDN9 (*ddc1-T602A*), YFP37 (*dot1*Δ *ddc1-T602A*), and YFP29 (*dot1*Δ *ddc1-M8*) were arrested with nocodazole and subjected to UV irradiation (75 J/m^2) or mock treated. Rad53 phosphorylation was analyzed 30 min after UV treatment. A protein extract from strain YMIC4E8 (*rad9*Δ) was loaded onto the same gel in order to identify the anti-Rad9 cross-reacting band, indicated by an asterisk. (B) Strains YFP37 (*dot1*Δ *ddc1-T602A*), YLDN25 (WT), YFP148 (*ddc1-T602S*), YFP27(*dot1*Δ), and YFP149 (*dot1*Δ *ddc1-T602S*) were arrested in M phase with nocodazole and either UV irradiated (75 J/m^2) or mock treated. Rad53 phosphorylation was analyzed 30 min after treatment.

mutant cell (Fig. 5A and data not shown). To support the hypothesis that the synthetic effect observed when we combined *dot1*Δ with *ddc1-T602A* is due to a loss of Ddc1 phosphorylation, we show that this phenotype is almost completely rescued by a *ddc1-T602S* mutation, which restores a different phosphorylatable residue (Fig. 5B). These observations 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 supported by the fact that *ddc1-T602A* and *dpb11-1* are in the same epistasis group for DNA damage-induced Rad53 activation and sensitivity to UV irradiation. In fact, combining the *ddc1-T602A* and *dpb11-1* mutations does not cause defective Rad53 phosphorylation (Fig. 6A). Moreover, the *ddc1-T602A dpb11-1* double mutant is as sensitive to UV irradiation as either single mutant, while a combination of *dot1*Δ with either *ddc1-T602A* or *dpb11-1* is more sensitive than any single mutant and as sensitive as the *dot1*Δ *ddc1-T602A dpb11-1* triple mutant (Fig. 6B).

Phospho-Ddc1 may be involved in recruiting Dpb11 to the lesion, bringing it close to the checkpoint kinases. We 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 (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 (Fig. 7A). The data presented in Fig. 7A 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 (Fig. 7B and C). 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 coimmunoprecipitation (47). We confirmed these findings and

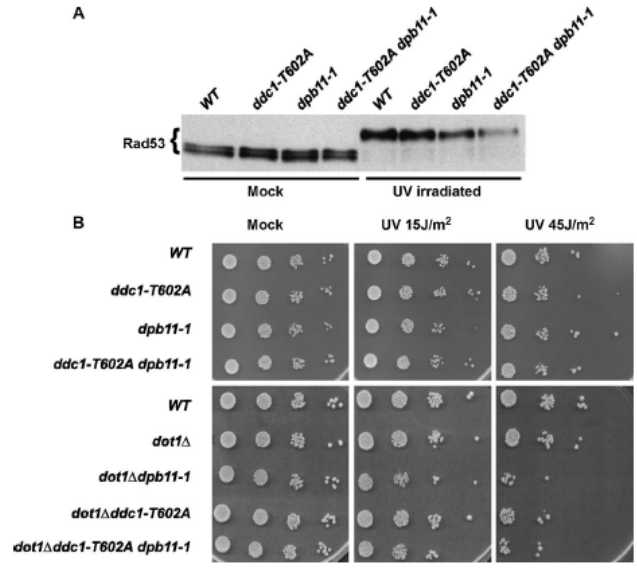

FIG. 6. *ddc1-T602A* and *dpb11-1* mutations are epistatic for UV sensitivity and effect on Rad53 phosphorylation. (A) Strain YFP63 (WT), YFP64 (*ddc1-T602A*), YFP65 (*dpb11-1*), and YFP66 (*dpb11-1 ddc1-T602A*) cells were arrested with nocodazole and UV irradiated. Rad53 phosphorylation was assayed 30 min after treatment. (B) Strains in panel A, YFP27 (*dot1*Δ), YFP142 (*dot1*Δ *dpb11-1*), YFP37 (*dot1*Δ *ddc1-T602A*), and YFP144 (*dot1*Δ *ddc1-T602A dpb11-1*) 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.

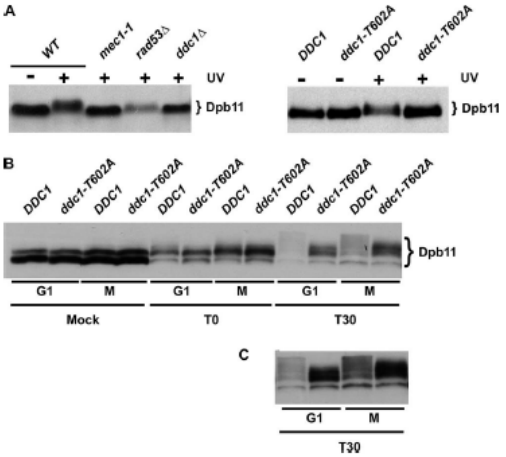

FIG. 7. UV-induced Dpb11 phosphorylation is mediated by Ddc1-T602 and requires Mec1 kinase. (A) Strains YFP38 (WT), YFP48/3a (*mec1-1*), YFP49/1d (*rad53*Δ), YFP55/6c (*ddc1*Δ), YFP63 (*DDC1*), and YFP64 (*ddc1-T602A*), all expressing a myc-tagged Dpb11 protein, were blocked in nocodazole and UV irradiated (75 J/m^2). 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 (G_1) or nocodazole (M) and UV treated. Protein extracts prepared immediately (T0) or 30 min (T30) after UV irradiation were separated on Phos tag-conjugated acrylamide gels as described in Materials and Methods. (C) Overexposure of the T30 samples from panel B.

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 to 612). Figure 8A 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 8B shows that under these conditions, the interaction is greatly 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. Another possibility is that, even in the absence of Ddc1 phosphorylation, the highly expressed bait and prey can produce enough hybrid molecules to activate the reporter genes.

As shown in Fig. 1C, a *dpb11-1* temperature-sensitive mutant did not exhibit a significant effect on Ddc2 phosphorylation at permissive temperature. Under these experimental conditions, the Dpb11 protein, albeit missing its C-terminal part, is still present in the cells and is likely to be partially functional. In order to determine whether Dpb11 had a possible role in activating Mec1 kinase, we took advantage of degron technology (49). Briefly, a Dpb11 fu-

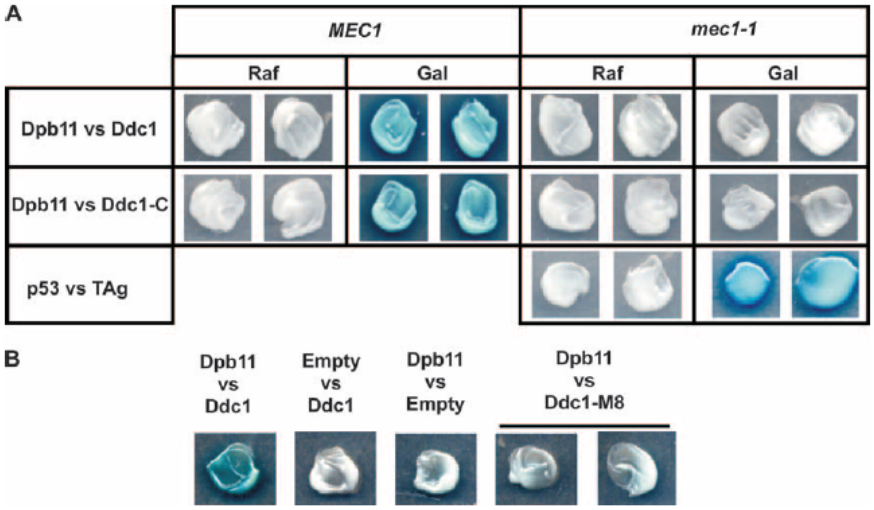

FIG. 8. The interaction between Dpb11 and Ddc1 requires a functional Mec1 kinase. Plasmids pFP1 (pJG4-5-*DPB11*) and pFP2 (pEG202-*DDC1*) were cotransformed with pSH18-34, a β-galactosidase reporter plasmid, in either *MEC1* or *mec1-1* mutant yeast cells. A similar strategy was adopted for pFP4 (pEG202-*ddc1-C*), which carries only the C-terminal fragment (nucleotides 309 to 612) of *DDC1*, containing the 11 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 A are YFP50 (*MEC1*, top), YFP52 (*MEC1*, bottom), YFP113 (*mec1-1*, top), and YFP114 (*mec1-1*, bottom). A positive control in the *mec1-1* mutant strain (p53 versus large T antigen [TAg]) was also used. The strains in panel B are, from left to right, YFP50, YFP86, YFP54, and YFP153.

sion protein carrying a temperature-sensitive degron tag (*dpb11^{td}*) is expressed in yeast cells. At 28°C, this construct complements the complete deletion of *DPB11* and does not exhibit any *dpb11*-encoded phenotype (49; data not shown). Once the *dpb11^{td}* culture is shifted to 37°C, the degron tag unfolds and drives the whole fusion protein to rapid degradation via the ubiquitin-mediated pathway (Fig. 9A) (49), allowing us to monitor the effect of a complete loss of the Dpb11 protein. Cells expressing *dpb11^{td}* were grown at 28°C and arrested with nocodazole. Cultures were shifted to 37°C to obtain the complete depletion of Dpb11, shifted back to 28°C, UV irradiated, and analyzed for DNA damage-induced Mec1 activation. Figure 9B shows that depletion of Dpb11 before UV irradiation greatly impairs Mec1-dependent phosphorylation of Ddc2. An isogenic strain, which also expresses WT *DPB11*, responds to UV irradiation with normal Ddc2 phosphorylation. These observations suggest that, after UV irradiation, Dpb11 may also have a more direct function in the robust activation of Mec1, possibly by strengthening its kinase activity, and are supported by similar results obtained with multicellular eukaryotes. A physical interaction between TopBP1 (orthologue of Dpb11) and ATR (orthologue of budding yeast Mec1) has been described in *Xenopus*, and it has been linked to a role for TopBP1 in the checkpoint response, specifically in the activation of ATR itself (21).

DISCUSSION

Loss of genome integrity is a hallmark of cancer cells, and maintenance of genome stability is fundamental to the prevention of tumor development (19). Eukaryotic cells possess a set of complex pathways devoted to monitoring the presence of different kinds of genomic lesions and signaling their presence to downstream effectors. The output of these checkpoint path-

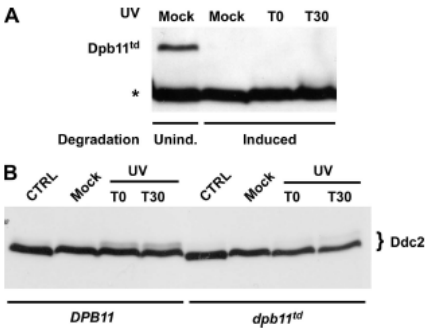

FIG. 9. Dpb11 is required for the full activation of Mec1. (A) Protein extracts from strain YMAG78/4B (*dpb11^{td}*) were prepared under different conditions to assess the presence of the Dbp11-degron protein. Cells were cultured at 28°C in YP plus raffinose (Mock Unind.) and then shifted to the degradation medium at 37°C (Mock Induced), UV irradiated, and shifted back to 25°C (T0, sample taken immediately; T30, sample taken 30 min later). The presence of Dpb11-degron was assessed by using anti-HA antibodies. (B) The experiment was repeated under the same conditions in parallel with strains YMAG82/15a (*DPB11*) and YMAG78/4B (*dpb11^{td}*). Ddc2 hyperphosphorylation was monitored by SDS-PAGE and Western blotting. CTRL, control.

ways is cell cycle arrest, DNA repair, modifications of the transcriptional program, and apoptosis (29, 41). The DNA damage checkpoint pathways are triggered by the activity of apical phosphoinositide 3-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) seems to be recruited by RPA-covered ssDNA filaments generated after nucleolytic processing of damaged DNA (51). 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 (5, 25). In budding yeast, once the 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 downstream kinase (25, 30). 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 G_1 phase of the cell cycle, while they have only a partial role in the G_2/M checkpoint response, which in budding yeast arrests the cell cycle in M phase. 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 aids in bringing Rad9 into proximity to the active Mec1 kinase (11, 13, 14, 48). A similar pathway has been described in fission yeast and in higher eukaryotes (4, 7, 16, 39). Given the facts that the G_2/M checkpoint response is still functional in cells lacking Dot1, the histone H3-K79 methyltransferase, and that Rad9 is still highly hyperphosphorylated after UV irradiation of M-phase-arrested cells (11, 48), a parallel, partially redundant pathway leading to the recruitment of Rad9 to damaged chromatin must exist in later stages of the cell cycle. We analyzed in more detail the signaling after UV irradiation of M-phase-arrested *dot1*Δ mutant cells and showed that the residual phosphorylation of Rad9 and Rad53 in these cells 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. The analysis of the nucleosomal structure reveals that H3-K79 is in close proximity to H4-K59, and mutation of this residue leads to silencing defects, similarly to mutations in *DOT1* (17, 50). Moreover, in *S. pombe*, Crb2 is recruited through interaction with methylated H4-K20 (39). Our results show that these residues do not seem to be redundant with H3-K79 methylation in the G_2/M checkpoint pathway leading to Rad9 activation; in fact, when mutations in H4-K59 or H4-K20 were combined with *dot1*Δ, we could not detect any synthetic effect on checkpoint activation. We obtained a similarly negative response when we tested strains combining *dot1*Δ with the deletion of the *SET1* or *SET2* histone methyltransferase coding gene. We then tested the contribution of histone H2A phosphorylation on serine 129, which has been shown to be relevant for Rad9 phosphorylation in G_1 cells (14), and we confirmed that in G_2 this histone modification plays a minor role (14, 18, 46).

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 reference 9), particularly in DNA replication. Recent work showed that TopBP1 in *Xenopus* and mammalian cells can activate the ATR kinase in vitro and this function is mediated by a specific protein domain, which seems to be missing in the fungal orthologues of TopBP1 (15, 21). Moreover, TopBP1 can also interact with the 9-1-1 checkpoint clamp (6, 23). 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 (7, 8). We combined a *dpb11-1* allele with a deletion of *DOT1* and analyzed the DNA damage checkpoint response after UV irradiation and Zeocin treatment 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 G_2/M checkpoint arrest. On the other hand, when *dpb11-1* is combined with a *dot1*Δ allele, the G_2/M checkpoint is not functional and cells become quite sensitive to UV irradiation and DSB-inducing agents, 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 through a Dpb11-dependent pathway. Another possible interpretation is that loss of Rad9 phosphorylation may be due to a combination of a reduction of Mec1 kinase activity and a reduction of Rad9 recruitment. In G_1-arrested cells, the importance of Dpb11 for the response to UV is minor; indeed, *dot1*Δ mutant cells cannot arrest at the G_1/S transition and a *dpb11-1* mutation does not worsen this phenotype. Close monitoring of Rad53 phosphorylation in these cells shows that Dpb11 contributes only marginally.

How does Dpb11 mediate Rad9 hyperphosphorylation? In fission yeast, the interaction between the two orthologous factors depends upon the activity of Cdk1 (7), possibly explaining why this pathway is predominant in G_2-M cells. Moreover, Dpb11 contains four BRCT domains and has been reported to interact with the Ddc1 subunit of the 9-1-1 complex (32, 47). In order to investigate the molecular details of this pathway, we analyzed a collection of *DDC1* mutants. Ddc1 sequence analysis revealed the presence of eight consensus sites for Mec1-dependent phosphorylation and three consensus sites for Cdk1-dependent phosphorylation; accordingly, Ddc1 has been reported to be phosphorylated in a cell cycle- and DNA damage-dependent manner (26, 34). 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 *dot1*Δ,

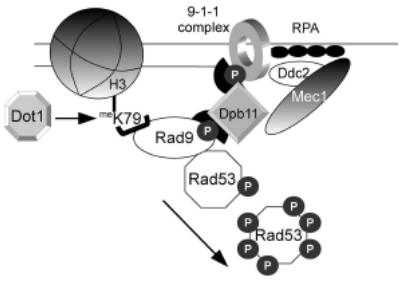

FIG. 10. Possible model of Dpb11 function in the UV-induced DNA damage checkpoint. Dpb11 is recruited to sites of DNA damage through the interaction of its C-terminal BRCTs with the 9-1-1 subunit Ddc1, phosphorylated by Mec1 on T602. Once recruited, it plays a double role in checkpoint activation. First, it enhances Mec1 kinase activity, and second, in G_2/M, it participates with H3-K79 in Rad9 recruitment, likely through an interaction of its N-terminal BRCTs with a Cdc28-phosphorylated site on Rad9. Full Mec1 activity and tight Rad9 recruitment allow rapid and full phosphorylation of Rad53, which correlates with checkpoint activation.

these mutants also eliminate the UV-induced phosphorylation of Rad9 and Rad53 and displayed a synthetic lethality after UV irradiation. This phenotype can be recapitulated by the single *ddc1T602A* mutation and strongly resembles the *dpb11-1*-encoded phenotype described above. Moreover, *ddc1T602A* and *dpb11-1* appear to be in the same epistasis group, which is consistent with the notions 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. We showed that Dpb11 is phosphorylated in a DNA damage-dependent and *MEC1*-dependent manner and that this modification appears to be greatly reduced in a *ddc1-T602A* mutant strain, but the functional significance of this modification of Dpb11 is still not clear and will be approached in future work.

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* and mammalian cell extracts. This could be due to a TopBP1 function which is specific for higher eukaryotes, but recent evidence suggested that an interaction between Rad4/Cut5 and the checkpoint sensor kinase Rad3-Rad26 also exists in *S. pombe* (8, 45). We thus exploited a temperature-sensitive degron version of Dpb11 (*dpb11^{td}*), which can be conditionally eliminated from cells by a combination of transcriptional repression and ubiquitin-dependent degradation (44, 49), to evaluate a possible role for Dpb11 in controlling Mec1 kinase activity in vivo. After cells had been depleted of Dpb11 and irradiated with UV light, we detected a noticeable defect in Mec1 activation, as measured by the phosphorylation of its Ddc2 subunit, suggesting that, in budding yeast, Dpb11 can regulate Mec1 by strengthening its kinase activity, even though there is no sequence conservation with the TopBP1 domain required to activate ATR in higher eukaryotes.

Altogether, our data support a model (Fig. 10) in which UV-induced lesions activate the checkpoint cascade to a basal level, likely by bringing Mec1 to damaged DNA via a Ddc2-RPA interaction; full activation of Mec1 seems to be supported by the presence of Dpb11. Mec1-induced phosphorylation of Ddc1 allows binding of Dpb11, which may cooperate with modified histones in the recruitment of Rad9 to damaged chromatin, allowing signal amplification and a complete response to DNA damage.

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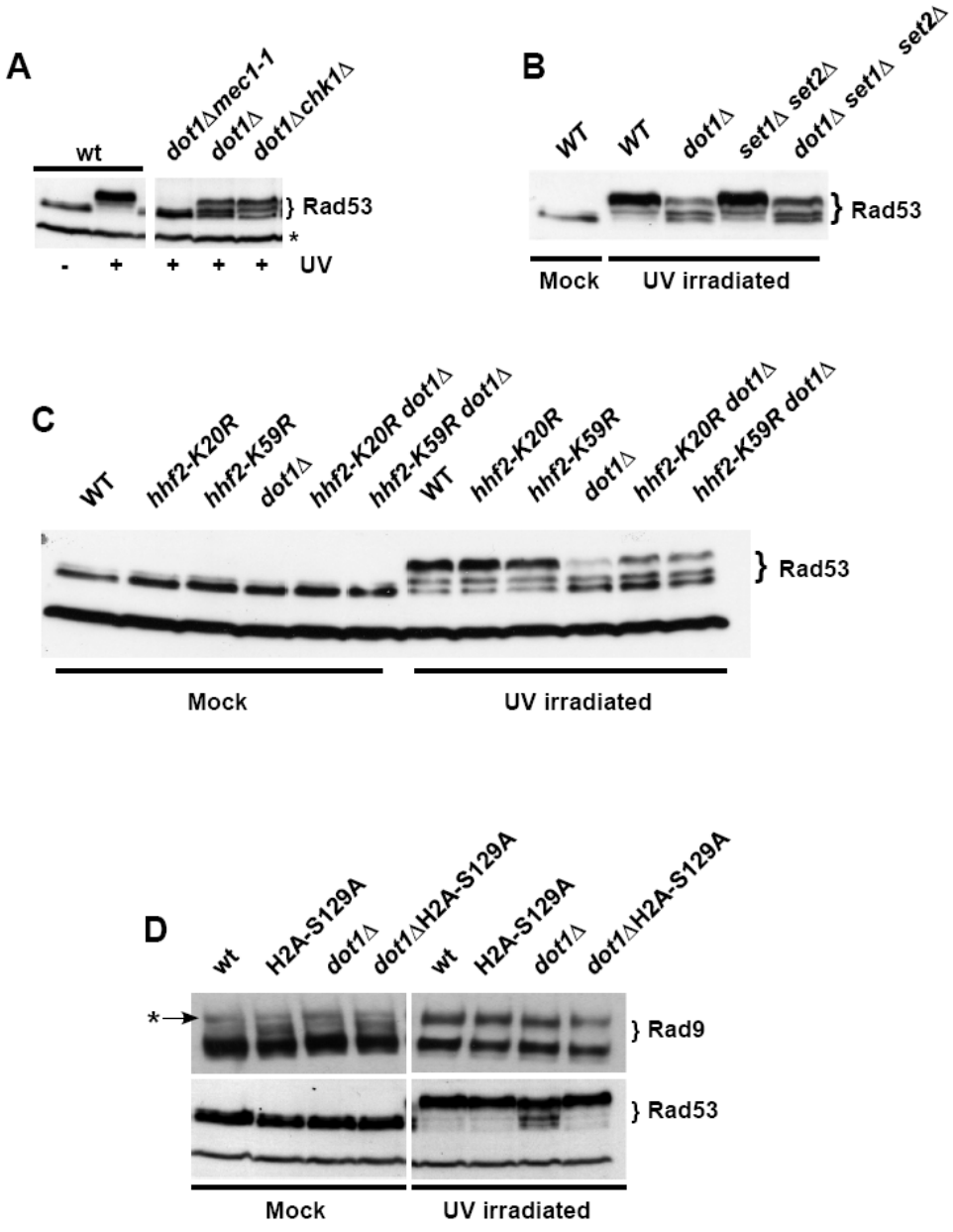
Supplementary Figure 1.

(A) WT (K699), YFL234 (*dot1Δ*), YFL499/3d (*dot1Δchk1Δ*) and YFL438 (*dot1Δmec1-1*) cells were held arrested in M phase with nocodazole and either mock or UV irradiated (75 J/m^2). Analysis of Rad53 phosphorylation, 30 minutes after UV irradiation, was performed by monitoring the mobility shift in SDS-PAGE. (B) WT, *dot1Δ*, *set1Δset2Δ* and *dot1Δset1Δset2Δ* cells were held arrested in M phase with nocodazole and either mock or UV irradiated. Analysis of Rad53 phosphorylation, 30 minutes after UV irradiation, was performed by monitoring the mobility shift in SDS-PAGE. (C) WT, *hhf2-K20R, hhf2-K59R, dot1Δ, hhf2-K20Rdot1Δ, hhf2-K59Rdot1Δ* cells were arrested in nocodazole in M-phase and either mock or UV irradiated. Analysis of checkpoint activation, 30 minutes after UV irradiation, was performed by monitoring the mobility shift of Rad53 in SDS-PAGE. (D) YMAG149/7b (WT), YMAG168 (H2A-S129A), YMAG150/4A (*dot1Δ*) and YMAG170 (*dot1Δ*H2A-S129A) were arrested with nocodazole and either mock or UV irradiated (75 J/m^2); 30 minutes after irradiation, Rad53 and Rad9 proteins were analyzed by SDS PAGE and western blotting.

Puddu et al. Supplementary Figure 1



DYNAMICS OF RAD9 CHROMATIN BINDING AND CHECKPOINT FUNCTION ARE MEDIATED BY ITS DIMERIZATION AND ARE CELL CYCLE-REGULATED BY CDK1 ACTIVITY

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Dynamics of Rad9 Chromatin Binding and Checkpoint Function Are Mediated by Its Dimerization and Are Cell Cycle–Regulated by CDK1 Activity

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Abstract

Saccharomyces cerevisiae Rad9 is required for an effective DNA damage response throughout the cell cycle. Assembly of Rad9 on chromatin after DNA damage is promoted by histone modifications that create docking sites for Rad9 recruitment, allowing checkpoint activation. Rad53 phosphorylation is also dependent upon BRCT-directed Rad9 oligomerization; however, the crosstalk between these molecular determinants and their functional significance are poorly understood. Here we report that, in the G1 and M phases of the cell cycle, both constitutive and DNA damage-dependent Rad9 chromatin association require its BRCT domains. In G1 cells, GST or FKBP dimerization motifs can substitute to the BRCT domains for Rad9 chromatin binding and checkpoint function. Conversely, forced Rad9 dimerization in M phase fails to promote its recruitment onto DNA, although it supports Rad9 checkpoint function. In fact, a parallel pathway, independent on histone modifications and governed by CDK1 activity, allows checkpoint activation in the absence of Rad9 chromatin binding. CDK1-dependent phosphorylation of Rad9 on Ser11 leads to specific interaction with Dpb11, allowing Rad53 activation and bypassing the requirement for the histone branch.

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Introduction

The DNA damage checkpoint coordinates cell cycle progression, DNA repair, replication, recombination, apoptosis and senescence in response to genotoxic stress. Defects in this surveillance mechanism lead to increased genomic instability, cancer susceptibility, ageing and several human pathologies [1,2]. The checkpoint is organized as a signal transduction cascade, whose players have been conserved throughout evolution [3,4]. When DNA is damaged, cells are able to sense and process the lesions generating a series of phosphorylation events, which are then amplified and propagated to specific targets [3,4]. Critical checkpoint factors are phosphorylated in response to DNA damage and their order of functions in the cascade has been mainly inferred by monitoring their phosphorylation state [5]. The apical kinases in the pathway are members of a family of phosphatidylinositol 3′ kinase-like kinases (PIKKs), which includes Mec1 and Tel1 from budding yeast, as well as mammalian ATM, ATR and DNA-PK [6]. In the yeast *Saccharomyces cerevisiae* the first biochemical event in response to checkpoint activation is the Mec1-dependent phosphorylation of its interacting subunit Ddc2 [7–9]. Other critical Mec1 targets are histone H2A, the 9-1-1 complex and the Rad9 mediator which is necessary for the recruitment and activation of the main effector kinase Rad53 [10–16]. Rad53 phosphorylation is a key step in the signal transduction cascade and it is generally used as a marker to monitor full checkpoint activation [17].

In a pioneering study, *RAD9* was the first DNA damage checkpoint gene identified in yeast and it is required for proper DNA damage response in all cell cycle phases and in response to a variety of genotoxins [18–20]. Rad9 is a large protein of 148 kDa containing a tandem repeat of the BRCT (BRCA1 C-terminus) motif, which is required for Rad9 oligomerization and function [21–23]. Until recently the biochemical role of the *RAD9* gene product remained obscure. Gilbert et al., were the first to purify Rad9 complexes from undamaged and UV-treated cells; structural characterization of such complexes led to the proposal that Rad9 recruits and catalyzes the activation of Rad53, by acting as a scaffold protein bringing Rad53 molecules in close proximity, thus facilitating the Rad53 autophosphorylation reaction [14].

The Rad9 protein contains several potential target sites for CDK1/Cdc28 kinase and PIKK-directed phosphorylation [24].

Author Summary

In response to DNA damage all eukaryotic cells activate a surveillance mechanism, known as the DNA damage checkpoint, which delays cell cycle progression and modulates DNA repair. Yeast *RAD9* was the first DNA damage checkpoint gene identified. The genetic tools available in this model system allow to address relevant questions to understand the molecular mechanisms underlying the Rad9 biological function. By chromatin-binding and domain-swapping experiments, we found that Rad9 is recruited into DNA both in unperturbed and in DNA–damaging conditions, and we identified the molecular determinants required for such interaction. Moreover, the extent of chromatin-bound Rad9 is regulated during the cell cycle and influences its role in checkpoint activation. In fact, the checkpoint function of Rad9 in G1 cells is solely mediated by its interaction with modified histones, while in M phase it occurs through an additional scaffold protein, named Dpb11. Productive Rad9-Dpb11 interaction in M phase requires Rad9 phosphorylation by CDK1, and we identified the Ser11 residue as the major CDK1 target. The model of Rad9 action that we are presenting can be extended to other eukaryotic organisms, since Rad9 and Dpb11 have been conserved through evolution from yeast to mammalian cells.

Rad9 is phosphorylated in an unperturbed cell cycle and it is hyper-phosphorylated in a Mec1- and/or Tel1-dependent manner after genotoxic treatments [12,13]. This hyper-phosphorylation is a pre-requisite for Rad9-Rad53 association, which is mediated by the two forkhead associated (FHA) Rad53 domains and specific Rad9 amino acid residues that are modified in the hyper-phosphorylated Rad9 form [12,13,15,16,25–27]. Recent data confirmed that the Rad9 BRCT domains mediate Rad9 oligomerization, and these interactions are also modulated by Mec1/Tel1-dependent phosphorylation of a SQ/TQ cluster domain (SCD) in Rad9. Rad9 oligomerization is required to maintain checkpoint signaling through a feedback loop involving Rad53-dependent phosphorylation of the Rad9 BRCT domains, which attenuates BRCT-SCD interactions [27].

Despite the fundamental nature of the cellular response to DNA damage, Rad9 and its *Schizosaccharomyces pombe* and metazoan orthologs Crb2 and 53BP1 show a modest level of amino acid sequence conservation. Dimerization mediated by the BRCT domains has been shown to be essential for the biological function of both Rad9 and Crb2 [21,28], however, 53BP1 oligomerization occurs in a BRCT-independent manner [29,30]. Recent structural analysis showed that an equivalent surface is conserved to a certain degree also in 53BP1 and it provides the binding site for p53. It was thus suggested that a functional requirement for dimerization of a checkpoint mediator may have been conserved in the evolution, but in metazoan organisms it may be delivered via a second protein rather than through homotypic interactions [31].

In the last few years it became evident that chromatin remodelling activities and post-translational modifications of chromatin components, including histones, influence DNA damage checkpoint signalling and repair in all eukaryotic cells (see [32] for a recent review). Moreover, it has been recently suggested that Rad9 may also be chromatin-bound in the absence of DNA damage [22]. This dynamic interaction with chromatin appears to require the Tudor domain of Rad9 and methylated lysine 79 of histone H3 (H3-K79me). Furthermore, this interaction modulates Rad9 functions after DNA damage [22,23,33–35]. However, the Crb2 and 53BP1 orthologues of Rad9 both recognize H4 methylated at lysine 20 (H4-K20me), although human 53BP1 may also be recruited to chromatin through interactions with H3-K79me [34,36–39].

For the Rad9/Crb2/53BP1 mediator proteins, efficient recruitment seems to require additional molecular interactions. Rad9 and Crb2 interact via their BRCT domains with H2A phosphorylated at serine 129 (γH2A) at sites of DNA damage [22,31,37,40–42]. 53BP1 binding to DSBs is facilitated by phosphorylation of serine 139 of the histone variant H2AX (γH2AX) [29,43–45]. It has been reported that various oligomerization domains in 53BP1 facilitate its recruitment to damaged DNA sites [30]. Moreover, 53BP1 recruitment to chromatin is facilitated by ubiquitination of H2A and H2AX by RNF8 through a yet unidentified mechanism [46–48].

Recently, it has been shown that Dpb11 in *S. cerevisiae* and its *S. pombe* and metazoan orthologs, termed Rad4/Cut5 and TopBP1, respectively, are required for full PIKK-dependent checkpoint activation in response to DNA damage [49,50]. Moreover it has been suggested that Dpb11 orthologs may modulate checkpoint activation through interaction with mediator/adaptor proteins [37,51]. To explore the functional role and the relationship between the BRCT domains and Rad9 ability to bind chromatin, we have analyzed both Rad9 chromatin recruitment and checkpoint activation in cells engineered to express various forms of Rad9 harboring mutated BRCT domains, including point mutations, deletion and substitutions with heterologous dimerization domains. We found that the requirements for Rad9 binding to chromatin are different in G1 or in M phase cells and in damaging versus unperturbed conditions. Moreover, we tested the requirements for Rad9 chromatin binding in yeast mutants defective in either the histone-dependent and/or histone-independent pathways essential for full checkpoint activation in M phase. Importantly, we found that CDK1-dependent Rad9 phosphorylation on Ser11 modulates the Dpb11-dependent branch in the M phase of the cell cycle in a chromatin-independent manner.

Results

Rad9 BRCT domains are required for its binding to chromatin in unperturbed and DNA damaging conditions

The Rad9 checkpoint mediator protein contains a tandem repeat of the BRCT motif at its C-terminus. Previous experiments have shown that the BRCT domains are critical for the activation of the DNA damage checkpoint and two-hybrid and GST pull-down analysis indicated that the BRCT domains modulate Rad9-Rad9 interactions [21]. More recently, it has been shown that Rad9 mutations in a conserved region of the first BRCT motif affect binding to γH2A, thus altering the G1 checkpoint signaling in response to DSBs [22,40] and the G2/M response to uncapped telomeres [23]. However, the mutations analyzed did not influence Rad9 chromatin binding in unperturbed conditions [22].

The *rad9-F1104L* or the *rad9-W1280L* mutations substitute the most highly conserved amino acid residues in the two BRCT motifs and each mutation affects productive Rad9-Rad9 interactions [21]. We tested whether such *rad9* mutations impair Rad9 recruitment to chromatin both in unperturbed and DNA damaging conditions. As expected, a proportion of wild-type Rad9 migrated much more slowly under our gel running conditions after UV treatment, consistent with hyper-phosphorylation of Rad9 (Figure 1A). A relevant fraction of Rad9 was found associated to chromatin in the absence of DNA damage, both in G1- and in M-arrested cells, confirming previous observations [22]. Control experiments were routinely performed to verify the distribution of standard protein markers in the soluble and

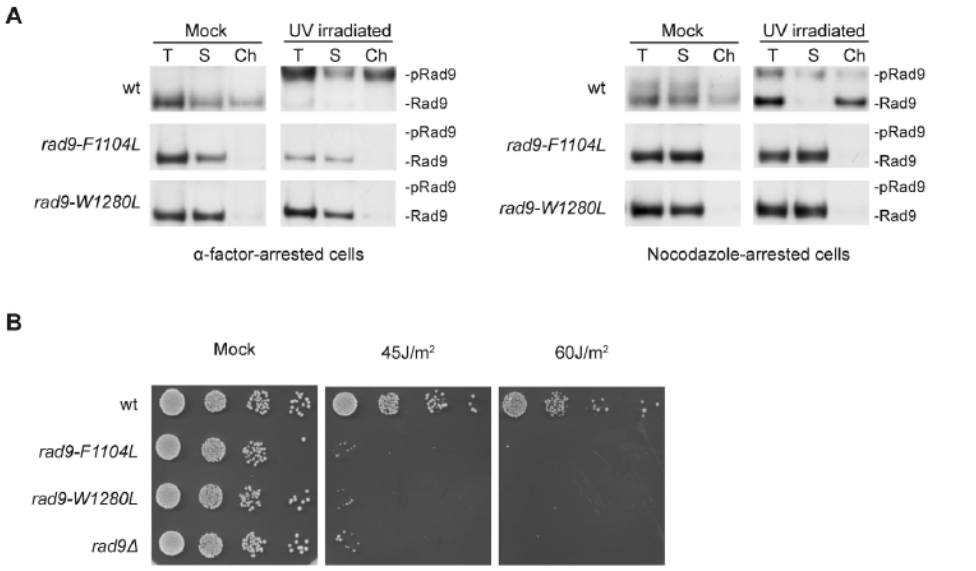

Figure 1. Rad9 chromatin binding requires an intact BRCT domains in UV–treated and in unperturbed conditions. (A) wt (K699), *rad9-F1104L* (YNOV15), *rad9-W1280L* (YNOV31) strains were arrested in G1 with α-factor or in M with nocodazole and either mock or UV irradiated (75 J/m^2). 10 min after irradiation, samples were collected and analyzed in their total (T), soluble (S) and chromatin-enriched (Ch) fractions. Blots were probed with anti-Rad9 antibodies and, after staining, the blots were cut to eliminate the Rad9-unrelated protein species migrating adjacent to the hyper-phosphorylated Rad9 isoform (Figure S1A). The positions of Rad9 and its hyper-phosphorylated isoform (pRad9) are indicated. (B) The same yeast strains analyzed in A and a *rad9Δ* strain (YMAG88) were grown overnight to log phase and serial dilutions were spotted onto YPD plates, which were then irradiated at the indicated UV doses and incubated for 3 days.
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chromatin fractions (Figure S1B). In various experiments we consistently found that the ratio of hyper- to hypo-phosphorylated Rad9 was approximately constant in both the soluble and chromatin fractions in G1 cells. Interestingly, in M phase cells, hyper-phosphorylated Rad9 was mostly present in the soluble fraction, while chromatin was enriched in the hypo-phosphorylated form (Western blot quantitation are shown in Figure S1C). As shown in Figure 1A, any of the two BRCT mutations abolished Rad9 phosphorylation and recruitment to chromatin in G1- or M-arrested cells. As expected [21], *rad9-F1104L* and *rad9-W1280L* mutant cells were highly sensitive to UV treatments (Figure 1B).

These results indicate that BRCT domains influence not only Rad9 binding to chromatin by modulating its interaction with γH2A after DNA damage [22], but they also control Rad9 recruitment to chromatin in unperturbed conditions.

A heterologous dimerization domain restores Rad9 binding to chromatin in G1-arrested, but not M-arrested, cells

To further evaluate the relevance of Rad9-Rad9 interactions in chromatin binding, we generated a set of yeast strains in which the C-terminal region of Rad9, containing the BRCT motifs, was substituted with either a 13-MYC epitope or a GST tag (see Materials and Methods). The latter has been shown to act as a heterologous constitutive dimerization domain [28,52,53].

As shown in Figure 2A, the GST tag was capable of driving, albeit somewhat less efficiently, Rad9 chromatin binding in G1-arrested cells, both in the absence or presence of DNA damage. Importantly, Rad9ΔBRCT::GST recruitment to chromatin still occurs through its interaction with H3-K79me, as it was drastically reduced in a *dot1Δ* background, lacking the specific H3-K79 histone methyl-transferase. Rad9 dimerization through the GST tag also significantly recovered Rad9 hyper-phosphorylation after UV irradiation and full checkpoint function (Figure 2A and data not shown).

It must be underlined that addition of the GST tag to Rad9ΔBRCT, allowing Rad9 dimerization, reconstitutes chromatin binding even though Rad9ΔBRCT::GST lacks the BRCT tandem repeats and is, therefore, unable to interact with γH2A [22]. These authors suggested that, after DNA damage, Rad9 shifts from H3-K79me to phosphorylated H2A-S129, and this translocation would be deficient in *rad9ΔBRCT::GST* cells. As a consequence of its defective interaction with γH2A, binding of Rad9ΔBRCT::GST to chromatin is probably much less stable. This hypothesis may explain the finding that in the *rad9ΔBRCT::GST* strain the majority of phosphorylated Rad9 after UV irradiation in G1 is found in the soluble fraction (Figure 2A).

To further support the role of Rad9 dimerization in its chromatin binding in G1-arrested cells solely by inducing Rad9-Rad9 interactions, we tested the possibility to direct a Rad9ΔBRCT isoform to chromatin by adding to the truncated protein a FKBP

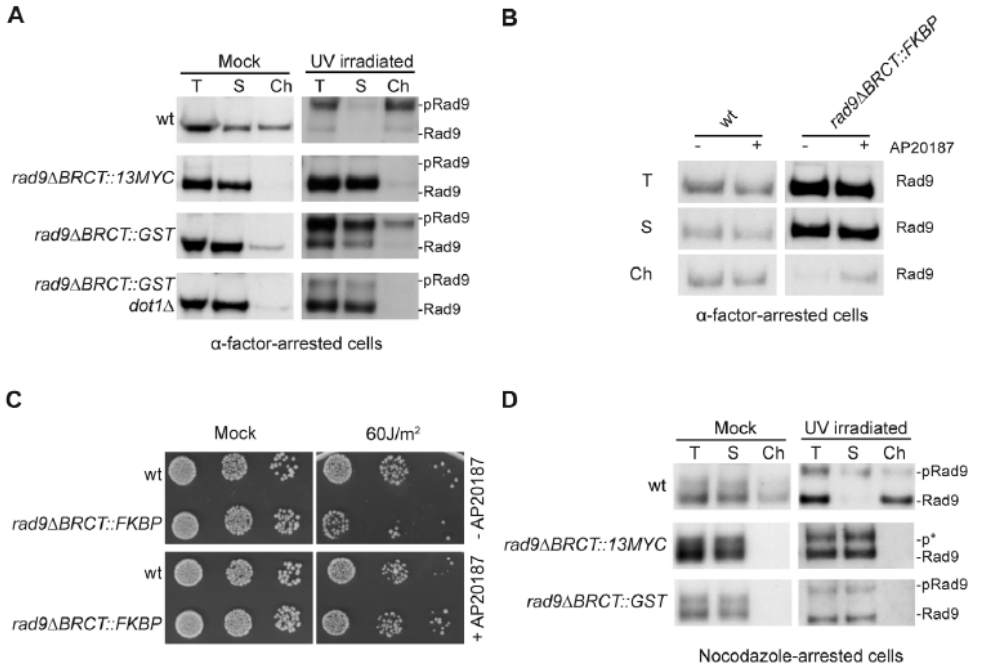

Figure 2. GST-driven Rad9 dimerization recovers its binding to chromatin in G1, but not in M phase. (A) wt (K699), *rad9ΔBRCT::13MYC* (YFL696/1b), *rad9ΔBRCT::GST* (YMAG74) and *rad9ΔBRCT::GST dot1Δ* (YFL773/2c) cells were arrested in G1 with α-factor and either mock or UV irradiated (75 J/m^2). After 10 min, samples were collected and analyzed in their total (T), soluble (S) and chromatin-enriched (Ch) fractions. Blots were probed with anti Rad9 antibodies as in the legend to Figure 1A. (B) wt (K699) and *rad9ΔBRCT::FKBP* (YFL901) cells were incubated for 6 h in the presence or in the absence of the dimerization-inducing molecule AP20187, blocked in G1 with α-factor and analyzed in their total (T), soluble (S) and chromatin-enriched (Ch) fractions. Blots were probed with anti Rad9 antibodies. (C) The same strains as in B were grown overnight to log phase and incubated for 6 h in the presence or in the absence of the dimerization-inducing molecule AP20187. Serial dilutions were spotted onto YPD plates, which were then irradiated at the indicated UV doses and incubated for 3 days. (D) Western blot analysis of the total, soluble and chromatin-enriched fractions from wt (K699), *rad9ΔBRCT::13MYC* (YFL696/1b) and *rad9ΔBRCT::GST* (YMAG74) cells arrested in M phase and either mock or UV irradiated (75 J/m^2). In all panels, the positions of Rad9 and its hyper-phosphorylated isoform (pRad9) are indicated. p* marks a partially phosphorylated Rad9 species.
doi:10.1371/journal.pgen.1001047.g002

tag, which can dimerize only in the presence of the small inducing molecule AP20187 [54]. Indeed, the presence of the FKBP tag partially rescued Rad9 chromatin binding in G1-arrested cells, but only in the presence of inducing AP20187 (Figure 2B). Importantly, addition of the dimerization inducing molecule fully recovered the UV sensitivity of *rad9DBRCT* cells (Figure 2C).

Contrary to our observations in G1-arrested cells, the heterologous GST dimerization domain did not rescue Rad9 binding to chromatin in nocodazole-arrested cells, although it restored checkpoint activation after DNA damage (Figure 2D, Figure 3A). Rad9 missing the BRCT domains only exhibits partial phosphorylation; this form can be distinguished from the hyper-phosphorylated isoform due to different electrophoretic mobility and its incapacity to activate Rad53 (see Figure 3A).

Altogether, the findings reported above indicate that dimerization is required for Rad9 to bind H3-K79me in G1-arrested cells, both with and without an exogenous DNA damaging agent. However, this is not the case in M phase-arrested cells, where GST-directed Rad9 dimerization partially recovers genotoxin-induced Rad9 hyper-phosphorylation, but fails to rescue its binding to chromatin. This may suggest that, at least in M phase, Rad9 chromatin binding is not directly linked to Rad9 hyper-phosphorylation.

GST-driven Rad9 dimerization rescues checkpoint activation and UV–sensitivity, despite undetectable chromatin binding

Although the addition of a heterologous dimerization domain to truncated Rad9ΔBRCT was not able to allow Rad9 chromatin binding in M phase-arrested cells, it rescues Rad53 activation after UV irradiation. In fact, as shown in Figure 3A, the phosphorylation state of the effector checkpoint kinase, Rad53, was found to be very different after UV-irradiation of *rad9ΔBRCT::GST* or *rad9ΔBRCT::13MYC* cells arrested with nocodazole. The hyper-phosphorylated form of Rad53 is absent in UV treated *rad9ΔBRCT::13MYC* cells, while it is clearly detectable in *rad9ΔBRCT::GST* cells. Although the extent of

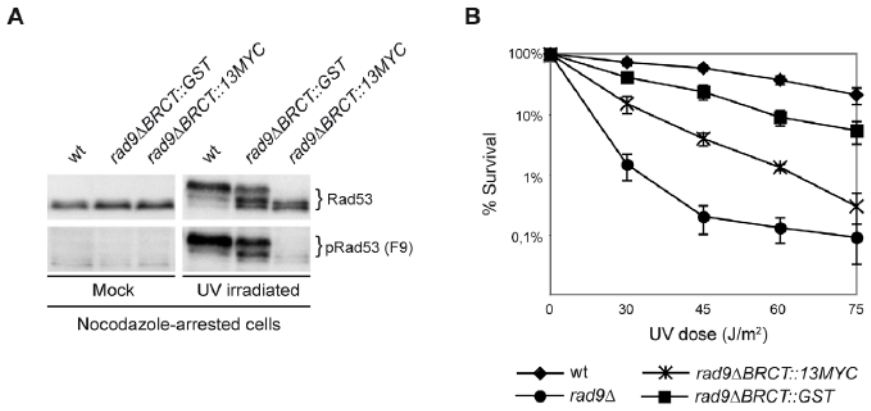

Figure 3. GST-driven Rad9 dimerization allows M checkpoint function regardless of Rad9 chromatin binding. (A) wt (K699), *rad9ΔBRCT::13MYC* (YFL696/1b), *rad9ΔBRCT::GST* (YMAG74) cells were cultured to mid-log phase, arrested in M with nocodazole, and either mock or UV irradiated (75 J/m^2); 10 min after irradiation, Rad53 phosphorylation was analyzed by SDS-PAGE and Western blotting with polyclonal Rad53 antibodies and with the F9 monoclonal antibody (Mab) recognizing only the hyper-phosphorylated active form of Rad53 to monitor checkpoint activation. (B) The same cells analyzed in A and a *rad9Δ* control strain (YMAG88) were cultured overnight, diluted and plated on YPD plates, which were irradiated with the indicated UV doses. Cell survival was assayed by determining the number of colonies grown on plates after 2 days; error bars were obtained from 3 independent experiments.
doi:10.1371/journal.pgen.1001047.g003

Rad53 phosphorylation was reduced in *rad9ΔBRCT::GST* relative to wild-type cells, the presence of the heterologous GST dimerization domain recovers the Rad9 checkpoint function, as confirmed by a direct checkpoint assay (data not shown). This conclusion is also supported by the observation that addition of the GST tag significantly rescued, although not completely, the UV sensitivity of the *rad9ΔBRCT::13MYC* strain (Figure 3B), and these findings are in agreement with previous experiments in *S.pombe* [28].

Thus far our data indicate that dimerization of Rad9 directed by an heterologous domain confers activation of the DNA damage checkpoint cascade, as well as significant resistance to UV in M phase-arrested cells, despite undetectable binding of Rad9 to chromatin (see Figure 2D).

Checkpoint activation in M phase requires CDK1 activity and is driven by Rad9–Dpb11 interaction

We have recently demonstrated that in the M phase of the cell cycle, full activation of the DNA damage checkpoint in response to various genotoxic stress is dependent upon Dpb11 [50]. Our data suggested that Dpb11 facilitates the recruitment of Rad9 proximally to DNA lesions through a mechanism independent of histone modifications. Indeed, as shown in Figure 4A, checkpoint activation after UV irradiation of nocodazole-arrested cells is only partially affected either in *dot1Δ* or in *dpb11ΔCT* cells. On the other hand, *dot1Δ dpb11ΔCT* double mutant cells are dramatically deficient in Rad53 phosphorylation since both the histone-dependent and histone-independent pathways for checkpoint activation are not functional. This finding can be interpreted by hypothesizing that when Rad9 cannot bind to chromatin via histone marks, Dpb11 may act as a platform for Rad9 recruitment in a histone-independent manner. Moreover, because the Dpb11-dependent pathway is particularly relevant in the G2 to M phases of the cell cycle [50], it was tempting to hypothesize that the proposed interaction between Rad9 and Dpb11 might be regulated by cell cycle-dependent control mechanisms [55]. Initially, we monitored this interaction using two-hybrid analysis performed at different cell cycle stages (see Materials and Methods). As shown in Figure 4B, a strong Rad9-Dpb11 interaction was observed in nocodazole-arrested cells. Several independent two-hybrid experiments showed that Rad9-Dpb11 interaction was more evident in M- rather than in G1-arrested cells. Experiments performed with a bait and a prey already known to interact by two-hybrid, indicate that the M/G1 ratio of Rad9-Dpb11 interaction was significantly higher than that found in the controls, suggesting a cell cycle-specific effect (Figure S2A). The Rad9-Dpb11 interaction was further confirmed biochemically (see below).

Since the interaction between Rad9 and Dpb11 appears to be induced in M phase, we reasoned that the Dpb11-dependent branch of the DNA damage checkpoint in M phase might be related to the increasing level of CDK1 kinase activity as cells move through the S, G2 and M phases of the cell cycle. To address this issue, we took advantage of the *cdc28-as1* mutant (in which only the Cdc28 kinase is specifically sensitive to bulky ATP analogues, such as 1NMPP1 [56]) to conditionally inactivate CDK1 in nocodazole-treated cells. Cdc28 kinase activity was inhibited or not with 1NMPP1 in nocodazole arrested cells and mitotic cells were then mock- or UV irradiated to induce DNA damage. Western blot analysis of Rad53 revealed that CDK1 inhibition abolished phosphorylation of Rad53 in the absence of the histone-dependent pathway, while no effect was observed in *DOT1* cells (Figure 4C). A similar experiment was performed by tethering checkpoint factors to DNA in the absence of damage [57]. The difference between our result and that reported by Bonilla, may be explained if, in their experimental conditions, without the addition of genotoxic agents, checkpoint activation is independent upon the Dpb11 branch.

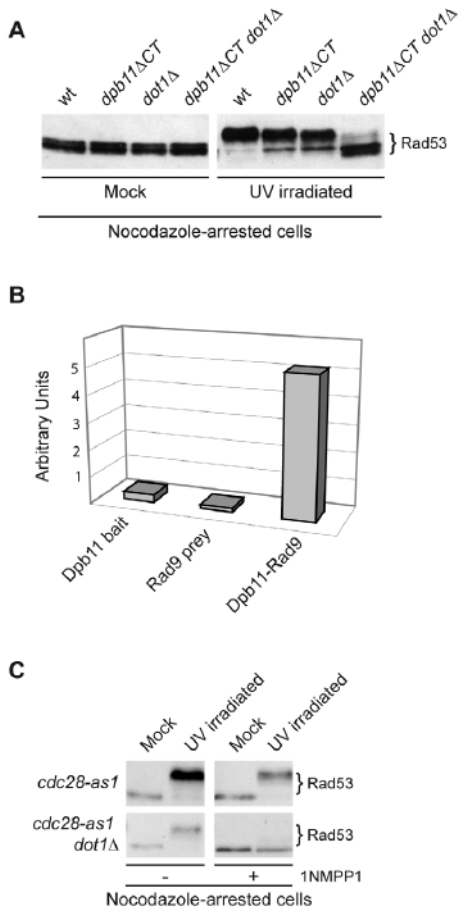

Figure 4. A cell cycle–dependent interaction between Dpb11 and Rad9 may regulate the Dpb11-dependent pathway. (A) wt (YMAG149/7B), *dpb11ΔCT* (YMAG145/20C), *dot1Δ* (YMAG150/4A) and *dpb11ΔCT dot1Δ* (YMAG148) strains were arrested in M with nocodazole and mock or UV irradiated (75 J/m^2). 10 min after irradiation, samples were taken and protein extracts were separated by SDS-PAGE. Blots were analyzed with anti Rad53 antibodies. (B) EGY42 cells, containing the pSH18-34 β-galactosidase reporter plasmid, were transformed with the Rad9 prey plasmid pMAG11.1 (pJG4-5-*RAD9*) and/or with the Dpb11 bait plasmid pFP15 (pEG202-*DPB11*). Strains were cultured overnight in -His, -Trp, -Ura medium plus raffinose and arrested in M phase by nocodazole treatment. Galactose was then added to the medium to induce bait expression. A modified version of ONPG yeast two-hybrid assay was used to determine the β-galactosidase activity in each strain, expressed in relative units. (C) *cdc28-as1* (JAU01) and *cdc28-as1 dot1Δ* (YNOV4) strains were arrested in M with nocodazole and, after incubation for 2 h in the absence or in the presence of 5 μM 1NMPP1, were either mock or UV irradiated (75 J/m^2). After 10 min, samples were collected and protein extracts were separated by SDS-PAGE. Blots were analyzed with anti-Rad53 antibodies.
doi:10.1371/journal.pgen.1001047.g004

Altogether, our results indicate that CDK1 activity is required for the function of the histone-independent branch necessary for Rad53 phosphorylation in cells arrested in mitosis.

CDK1-dependent phosphorylation of serine 11 of Rad9 modulates the Dpb11-dependent branch in M phase cells

Rad9 contains 20 potential (SP or TP) target sites for CDK-dependent phosphorylation, 9 of which conform to the canonical CDK phosphorylation site (S/T-P-x-K/R) (Figure S2B). We hypothesized that Rad9 could be a relevant CDK1 target in the histone-independent branch of the DNA damage checkpoint in M phase cells. Initially, we tested a *rad9ΔNT* mutant strain, in which the first 231 amino acids, including 9 S/T-P sites, of Rad9 are missing (Materials and Methods and [58]). As shown in Figure 5A, Rad53 phosphorylation was partially defective in both *dot1D* and *rad9DNT* mutants and essentially abolished in a *rad9ΔNT dot1Δ* double mutant strain.

All 9 potential Cdc28 phosphorylation sites in the Rad9 N-terminal region were individually mutagenized and different mutant combinations tested (Materials and Methods and data not shown). *rad9-S11A* cells displayed a detectable defect in cell cycle-regulated Rad9 phosphorylation (Figure S2C). Moreover, the *rad9-S11A* mutation recapitulates the phenotype we observed in *rad9ΔNT* cells, namely, severe loss of DNA damage-dependent Rad53 phosphorylation when combined with *dot1D* (Figure 5B). Consistently, the *rad9-S11A* mutation alone did not confer a strong sensitivity to UV irradiation (Figure 5C), while a *rad9-S11A dot1Δ* double mutant strain was synthetically sensitive to genotoxic treatment. On the other hand, a *rad9-S11A dpb11ΔCT* double mutant strain did not exhibit an increased sensitivity to UV irradiation when compared to strains harboring the single mutations, indicating that Dpb11 and Rad9-S11 phosphorylation act in the same pathway (data not shown). Phosphorylation of Rad9S11 has been reported *in vivo* [59]. In order to verify the relevance of S11 phosphorylation in our experimental conditions, we reverted the S11A mutation to Thr, another phosphorylatable residue. Figure 5D shows that Rad9 carrying a Thr at position 11 rescues the phenotype imparted by the S11A mutation, since checkpoint activation in the *rad9-S11T dot1Δ* strain is identical to that found in *dot1Δ* cells.

Interestingly, Rad9-Dpb11 interaction by two-hybrid analysis was reduced when the Rad9NT isoform, lacking the 9 potential CDK1 phosphorylation sites, was used as a prey in a wild-type background, or when Cdc28 activity was inhibited by 1NMPP1 addition in the *cdc28-as1* strain (Figure 6A). The *in vivo* interaction between Rad9 and Dpb11 was also confirmed by co-immunoprecipitation of the endogenous proteins after genotoxic treatment. As shown in Figure 6B, immunoprecipitation of MYC-tagged Dpb11 recovers the hyper-phosphorylated isoform of Rad9, and this interaction is virtually lost in the *rad9-S11A* mutant strain (Figure 6C). We also noticed that the Rad9-S11A mutant protein has slighlty less gel-mobility than its wild type counterpart, as shown in Figure 6C. This observation can be explained by either a mild defect in Mec1/Tel1-dependent hyperphosphorylation of the Rad9-S11A protein, due to the loss of Rad9-Dpb11 interaction, or a direct effect of the S11A mutation which, affecting CDK1-dependent phosphorylation of Rad9, may directly modify its migration in SDS PAGE.

Altogether, the above findings indicate that the Ser11 CDK1-consensus site on Rad9 is a relevant target to modulate Rad9-Dpb11 interaction and the CDK1-dependent checkpoint response in M phase cells.

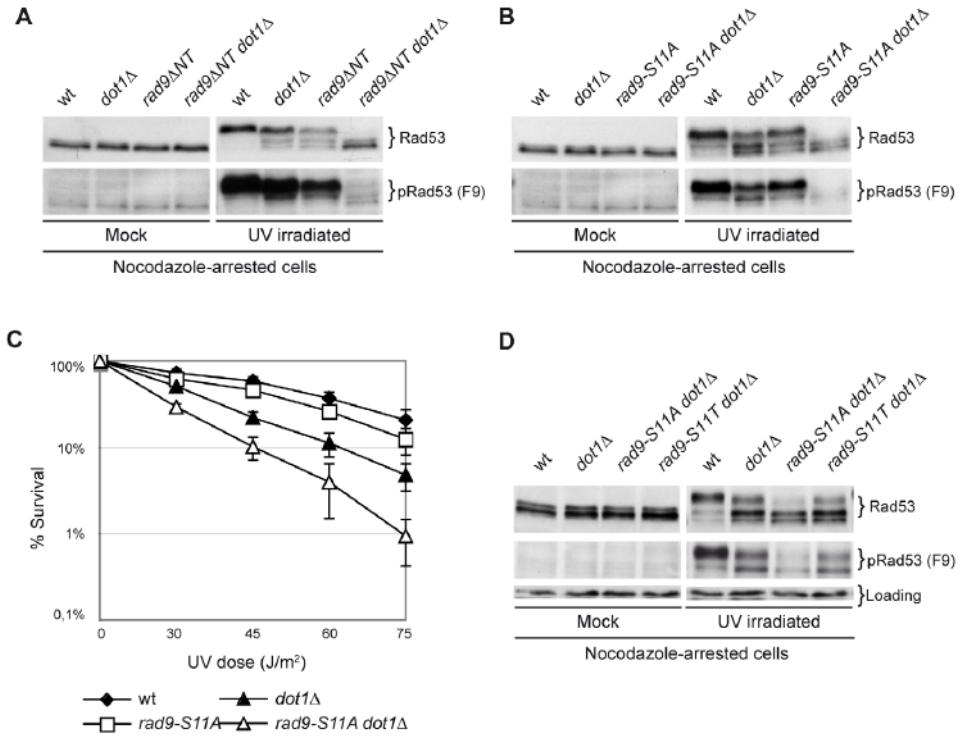

Figure 5. Phosphorylation of Rad9S11 by CDK1 is required for the establishment of an effective UV response in the absence of Dot1. (A) wt (K699), *dot1Δ* (YFL234), *rad9ΔNT* (DLY2236) and *rad9ΔNT dot1Δ* (YFP91) strains were arrested with nocodazole and either mock or UV irradiated (75 J/m^2). After 10 min samples were collected and protein extracts were separated by SDS-PAGE. Blots were analyzed with anti-Rad53 or with the F9 Mab to monitor checkpoint activation. (B) wt (K699), *dot1Δ* (YFL234), *rad9-S11A* (YMAG162) and *rad9-S11A dot1Δ* (YMAG164) strains were arrested in M, irradiated and Rad53 was detected by Western blotting as describe in panel A. (C) The same strains analyzed in B were cultured overnight, diluted and plated on YPD plates, which were irradiated with the indicated UV doses. Cell survival was assayed as described in the legend of Figure 3. (D) wt (K699), *dot1Δ* (YFL234), *rad9-S11A dot1Δ* (YMAG164) and *rad9-S11T dot1Δ* (YNOV52) strains were arrested with nocodazole and either mock or UV irradiated (75 J/m^2). After 10 min samples were collected and protein extracts were separated by SDS-PAGE. Blots were analyzed with anti-Rad53 or with the F9 Mab to monitor checkpoint activation.
doi:10.1371/journal.pgen.1001047.g005

The Dpb11-dependent branch in M phase modulates checkpoint activation in a chromatin-independent manner

To gain further insights into the mechanisms involving Rad9 and the Dpb11-dependent branch of the DNA damage checkpoint operating in nocodazole-arrested cultures, cell extracts were fractionated into soluble and chromatin fractions. Specifically, we monitored Rad9 chromatin binding and Rad53 phosphorylation in strains harbouring defects in the different branches known to regulate Rad9 checkpoint functions during M phase.

As shown in Figure 7, following DNA damage, the Dpb11 C-terminal region carrying the BRCT domain does not appear to be required for Rad9 binding to chromatin, as *dpb11DCT* cells behave as wild type. However, as expected, Rad9 chromatin recruitment is defective in *dot1Δ* and *H2A-S129A* mutant cells, as binding of Rad9 is dependent upon H3-K79me and γH2A, via its Tudor and BRCT domains respectively [22,34,60]. Checkpoint activation, as determined by Rad53 phosphorylation, was abolished in any double or triple mutant combinations carrying the *dpb11ΔCT* mutation (Figure 7). Intriguingly, even when detectable Rad9 binding to chromatin is abrogated (as in the single *dot1Δ* and *H2A-S129A* or in the double *dot1Δ H2A-S129A* mutant strains) Rad53 can be fully phosphorylated. Similar genetic dependencies were found when the various single, double and triple mutant strains were tested for checkpoint activation in response to zeocin treatment, which is known to cause DSBs (Figure S3 and data not shown).

Dpb11 is responsible for checkpoint activation in M phase cells when the Rad9 BRCT domains are replaced with a heterologous dimerization domain

We have determined (Figure 3A) that in nocodazole-arrested cells defective checkpoint activation due to the absence of the Rad9 BRCT domain can be partially rescued by adding the GST

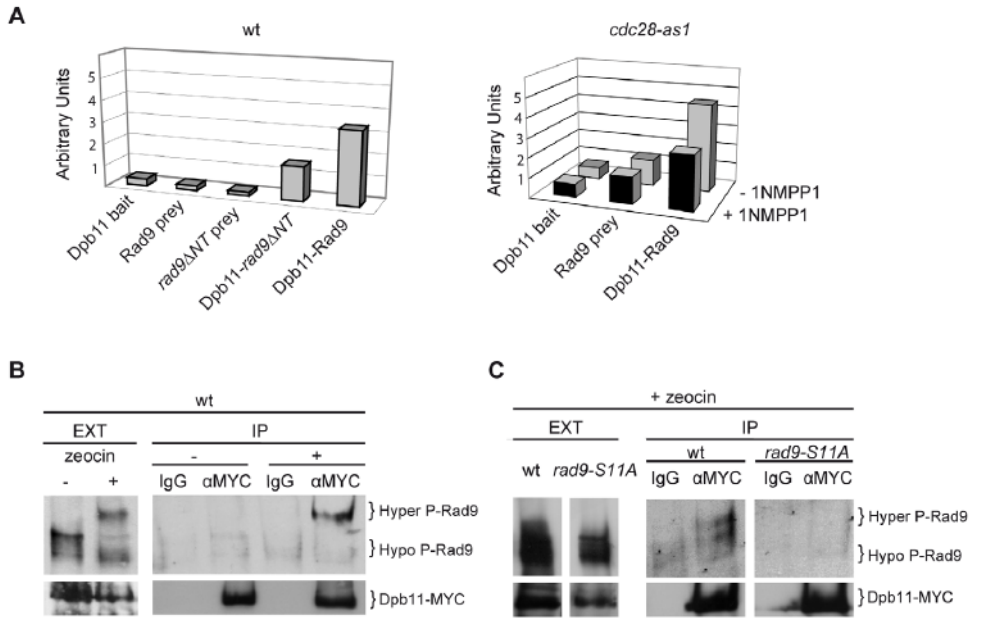

Figure 6. CDK1-dependent phosphorylation of S11-Rad9 modulates Rad9-Dpb11 interaction. (A) Two-hybrid interaction between Dpb11 and Rad9 was tested in a wt (K699) (left) or in a *cdc28-as1* (JAU01) (right) genetic background with the indicated bait and prey plasmids. Where specified 5 μM 1NMPP1 was added to the media for 1 h before bait induction and extracts preparation. (B) The *Dpb11-myc* (YFP38) strain was arrested with nocodazole and either mock treated or treated with 150 μg/ml of zeocin for 30 min. Whole cell protein extract was prepared and tagged Dpb11-MYC was immunoprecipitated either with anti-MYC antibodies or unspecific mouse IgG as described in Materials and Methods. The presence of Rad9 in the IPs was detected by Western blot analysis of the immunoprecipitates with specific anti-Rad9 antibodies. (C) Immunoprecipitations with anti-MYC antibodies were performed on extracts from nocodazole arrested cells, treated with 150 μg/ml of zeocin for 30 min, expressing Dpb11-MYC in a *RAD9* (YFP38) or *rad9S11A* (YMAG281) background. The presence of Rad9 was detected by Western blot analysis of the immunoprecipitates with specific anti-Rad9 antibodies. Lower exposure of the crude extracts lanes are shown to allow visualization of both Rad9 and Dpb11 specific bands.
doi:10.1371/journal.pgen.1001047.g006

dimerization domain. Moreover, we demonstrated that the M phase-specific DNA damage checkpoint contains a pathway based on Rad9-Dpb11 interactions and modulated via phosphorylation of the Ser11 residue of Rad9 by CDK1 (Figure 4, Figure 5, and Figure 6). As a consequence, we tested whether, in nocodazole-arrested cells, checkpoint activation supported by the heterologous dimerization motif in the *rad9ΔBRCT::GST* mutant strain was dependent upon Dpb11. To address this question, we introduced the *S11A* point mutation in the *rad9ΔBRCT::GST* strain (*rad9-S11AΔBRCT::GST)*. Whilst either single mutant strain was only partially defective in Rad53 phosphorylation, in *rad9-S11AΔBRCT::GST* cells, checkpoint activation was severely impaired (Figure 8A). This result indicates that in *rad9ΔBRCT::GST* cells residual checkpoint activation depends upon an active Dpb11 branch acting through a potential CDK1 site (S11) in the amino terminus of Rad9. As expected, *rad9-S11ADBRCT::GST* cells, in which the sole Rad9 expressed contains both the point mutation and the domain swap, are more sensitive to UV irradiation than either single mutant (Figure 8B).

In conclusion, our data are consistent with the hypothesis that Rad9 plays two independent roles in checkpoint activation: the first mediated by its dimerization and binding to modified histones, the second, which involves its phosphorylation by CDK1 and interaction with Dpb11 (Figure 9).

Discussion

RAD9 was the first DNA damage checkpoint gene identified in yeast [18]; however, the precise molecular details regarding the role of the corresponding gene product, its function and regulation remain far from being fully understood. In budding yeast, Rad9 seems to act as an adaptor protein in the signal transduction checkpoint cascade, mediating the transmission of the signal from the apical PIKKs to the main primary transducer kinase, Rad53 [27,61]. Rad9 phosphorylation, mediated by Mec1, is an early event in the signal transduction cascade and this modification in G1 is mainly influenced by histone H3 methylation [22,33,60,62]. In M phase, Rad9 phosphorylation also requires Dpb11, whose role as an alternative scaffold for Rad9 activation has been unveiled only recently [50]. The dynamics of Rad9 recruitment at various cell cycle stages and the genetic dependencies controlling Rad9 interaction with DNA/chromatin and other proteins are largely unknown.

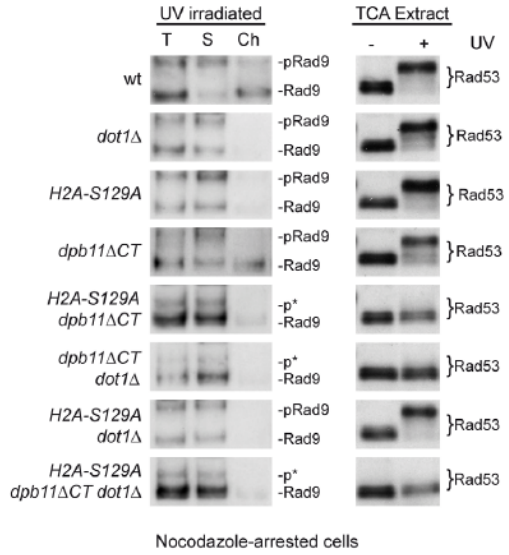

Figure 7. The Dpb11-dependent pathway in M phase modulates Rad53 activation in a chromatin-independent manner. wt (YMAG149/7B), *H2A-S129A* (YMAG168), *dpb11ΔCT* (YMAG145/20C), *dot1Δ* (YMAG150/4A), *H2A-S129A dpb11ΔCT* (YMAG155), *H2A-S129A dot1Δ* (YMAG170), *dpb11ΔCT dot1Δ* (YMAG148) and *H2A-S129A dpb11ΔCT dot1Δ* (YMAG157) strains were arrested in M with nocodazole and UV irradiated (75 J/m^2). After 10 min, samples were collected and analyzed in their total (T), soluble (S) and chromatin-enriched (Ch) fractions; blots were probed with anti-Rad9 antibodies (left panel). Protein extracts were also prepared from mock and UV treated samples and analyzed by SDS-PAGE and Western blotting with anti-Rad53 antibodies to monitor checkpoint activation (right panel). The positions of Rad9 and its hyper-phosphorylated isoform (pRad9) are indicated. p* marks partially phosphorylated Rad9 species.
doi:10.1371/journal.pgen.1001047.g007

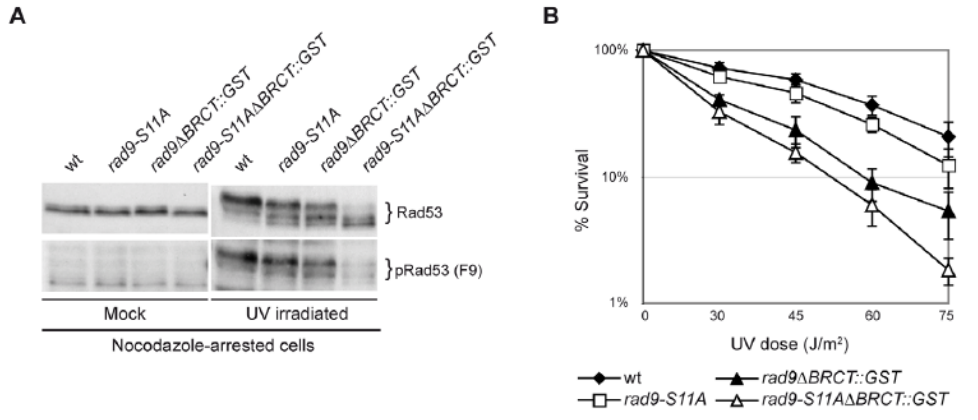

Figure 8. Partial checkpoint activation after forced Rad9 dimerization in M phase acts through the Dpb11-dependent checkpoint pathway. (A) wt (K699), *rad9-S11A* (YMAG162), *rad9ΔBRCT::GST* (YMAG74) and *rad9-S11AΔBRCT::GST* (YFL1177) strains were arrested with nocodazole and mock or UV irradiated (75 J/m^2). After 10 min, samples were collected and protein extracts were separated by SDS-PAGE. Blots were analyzed either with anti-Rad53 antibodies or with the F9 Mab to monitor checkpoint activation. (B) UV survival assay. The same strains as in A were cultured overnight and then diluted and plated on YPD plates, which were irradiated with the indicated UV doses. Cell survival was assayed as described in the legend to Figure 3.
doi:10.1371/journal.pgen.1001047.g008

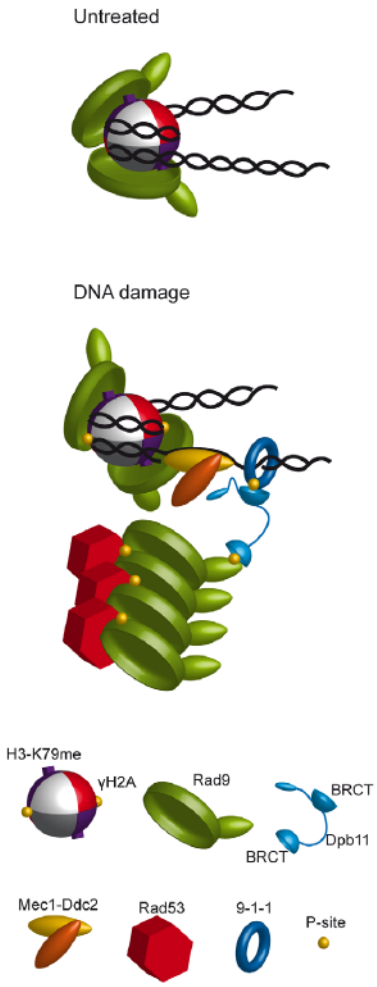

Figure 9. Possible model of the dynamics of Rad9 chromatin binding and its interaction with Dpb11 to modulate checkpoint activation in M phase. Under untreated conditions, Rad9 is chromatin bound through the interaction of its Tudor domain with H3-K79me and its BRCT-mediated dimerization. After DNA damage, activated Rad9 may change its conformation, interacting also with γ-H2A. In M-phase an alternative means of Rad9 recruitment near DNA lesions involves its interaction with Dpb11. This factor is brought near the Mec1-Ddc2 complex via its interaction with the 9-1-1 clamp, and it binds the phosphorylated N-terminal portion of Rad9 leading to full checkpoint activation.
doi:10.1371/journal.pgen.1001047.g009

Here, we show that a significant proportion of Rad9 is already chromatin-bound in unperturbed conditions throughout the cell cycle, confirming previous suggestions [22] and supporting our earlier model [14]. According to the current view, Rad9-chromatin association is controlled by interaction between its Tudor domain and H3-K79me. Constitutive, dynamic recruitment of Rad9 to chromatin may facilitate the efficiency and speed of the Rad9-dependent response to genotoxins. After DNA damage, Rad9 binding to chromatin is further strengthened through its BRCT domain, which is required to productively interact with γH2A [22,23]. In this study we found that the BRCT domain of Rad9, in addition to promoting interaction with γH2A, has a more general function in modulating Rad9 recruitment. In fact, the *rad9-F1104L* and *rad9-W1280L* mutations, affecting the folding of the whole BRCT domain [21], alter binding to chromatin also in the absence of any genotoxic treatment. The observation that *rad9-K1088M* cells are defective in Rad9 chromatin recruitment only after γ-irradiation may be explained if such mutation only prevents Rad9-γH2A interaction [22].

In G1 cells, Rad9 binding to chromatin can be achieved by substituting the BRCT repeats with a heterologous dimerization domain; such recruitment requires the activity of Dot1 histone methyl-transferase, indicating that BRCT-mediated dimerization may be a pre-requisite for constitutive interaction between the Rad9 Tudor domain and H3-K79me. Given the symmetrical structure of the histone octamer within the nucleosome core, dimerization might facilitate the correct orientation and positioning of two Rad9 molecules on the nucleosome, allowing productive interactions with modified histones (Figure 9). Such hypothesis is supported by structural modeling of a dimeric *S. pombe* Crb2 complex on a single nucleosome, where all the interactions with H4-K20me and γH2A are satisfied without changing the conformation of the histone core [31].

It is worth noting that dimerization forced by replacement of the Rad9 BRCT domains with the heterologous GST tag only restores Rad9 binding to chromatin in G1-, and not in M-arrested cells. In fact, in cells arrested with nocodazole, we observed that GST-induced dimerization can rescue Rad9 hyper-phosphorylation and DNA damage checkpoint activation, but not its stable recruitment to chromatin. It is possible that in mitosis cell cycle-dependent phosphorylation of Rad9 may interfere with the chromatin association of this artificial Rad9 dimer. Alternatively, in nocodazole-arrested cells the Rad9 BRCT motifs may play additional roles in modulating Rad9-chromatin interactions.

Several findings indicate that the cellular response to DNA damage, including the repair mechanisms themselves, are regulated differently in distinct cell cycle stages. Multiple layers of cell cycle regulation may modulate the recruitment of critical checkpoint and repair factors to damaged DNA, as well as facilitate their reciprocal cross-talk [63–67]. We have previously shown that Dpb11 is essential for full DNA damage checkpoint activation in M-arrested cells [50]. Dpb11 is held in proximity to damaged DNA through its interaction with phosphorylated 9-1-1 complex, leading to Mec1-dependent Rad9 phosphorylation. Taking advantage of the *cdc28-as1* mutation, which allows conditional turn off of CDK1 kinase activity, we have demonstrated that CDK1, targeting Rad9, is required for the function of the Dpb11-dependent branch of the checkpoint response. Indeed, yeast cells carrying a truncated Rad9 version lacking 9 putative Cdc28 phosphorylation sites in the N-terminal region, are checkpoint-defective in M phase, in the absence of the histone-dependent branch. The Ser11 residue in the Rad9 N-terminal region is the most relevant Cdc28 target site, since a *rad9-S11A* mutation recapitulates the phenotypes observed in *rad9ΔNT* cells.

By two-hybrid analysis we showed that Rad9 and Dpb11 specifically interact in M-phase arrested cells, even in the absence of DNA damage, and this interaction is stimulated by CDK1-dependent Rad9 phosphorylation. Co-immunoprecipitation exper-

iments confirmed that Rad9-Dpb11 interaction requires phosphorylation of Rad9-S11 and revealed that it depends upon genotoxic treatment, although we cannot exclude a weak/transient interaction in untreated conditions. This finding can be explained if activation of Mec1 by DNA damage facilitates or controls this interaction, e.g phosphorylating Dpb11 [50], exposing phospho-S11 or stimulating Rad9-S11 modification by CDK1. The overexpression conditions typical of the two hybrid system can easily explain why a weak interaction can be detected also in the absence of DNA damage. Interestingly, the functional interactions between Dpb11 and Rad9 in budding yeast are reminiscent of similar findings in the distantly related *S. pombe*, where histone-independent checkpoint activation is also modulated by CDK1 [37].

The Dpb11-dependent pathway does not require the histone modifications modulating Rad9 recruitment to chromatin. We found that a truncated C-terminal version of Dpb11 does not affect Rad9 recruitment to chromatin, which is instead abolished when the histone-dependent pathway is defective. Surprisingly, in a *dot1Δ H2A-S129A* double mutant strain checkpoint activation in M phase is virtually undistinguishable from that found in wild type cells, although Rad9 is not stably bound to chromatin. Only when the *dpb11ΔCT* mutation is combined with the *dot1Δ* or *H2A-S129A* mutation the checkpoint response is turned off. The working model presented in Figure 9, suggests that Dpb11 may act in M-phase as an alternative means of Rad9 recruitment. Dpb11 is located close to sites of DNA damage through its interaction with the Mec1-phosphorylated 9-1-1 complex; DNA damage leads to Mec1-dependent phosphorylation of Dpb11 [50], which interacts with S11-phosphorylated Rad9 (Figure 9). This Dpb11-dependent localization of Rad9 to sites of DNA damage allows rapid Rad9 hyper-phosphorylation by PIKKs, as suggested by the observation that the interaction between Rad9 and Dpb11 is induced by genotoxic agents and hyper-phosphorylated Rad9 is enriched in the Dpb11-bound population. Subsequently, Rad53 recruitment via its FHA domains leads to full activation of the checkpoint response. Unlike Rad9 bound via histone marks, Rad9 complexed with Dpb11 does not appear to be tightly linked to chromatin, explaining why the Dpb11-dependent branch for checkpoint activation seems to act in a chromatin-independent manner. However, we cannot rule out the possibility that the Rad9-Dpb11 complex can transiently or weakly bind to chromatin.

The model suggested here is in agreement with similar findings in the distantly related *S.pombe* fission yeast [37] as well as with recent *in vitro* data describing Dpb11 role in checkpoint activation [68], suggesting that the proposed mechanism can be extended to other eukaryotic organisms.

Materials and Methods

Strains and plasmids

All of the strains used in this work are derivatives of W303 [*MATa ade2-1 trp1-1 can1-100 leu2-3,12 his3-11,15 ura3 rad5-535*]; only strains YFP91 and DLY2236 (provided by D. Lydall), are *RAD5*$^{+}$. All the strains used in this study are listed in Table S1 and further information regarding strains and plasmids is available upon request.

Plasmids pMAG11.1 and pFP15 are, respectively, the Rad9 prey and Dpb11 bait plasmids used for the yeast two-hybrid analysis. They were obtained by amplifying the relevant coding sequences from genomic DNA and by ligating the resulting fragments into pJG4-5 and pEG202 [69], respectively.

The plasmid pMAG9, which encodes the Rad9ΔNT prey, was obtained cloning the *rad9ΔNT* sequence, amplified from the yeast strain DLY2236, into pJG4-5.

Gene deletions were obtained by PCR-mediated gene replacement [70].

The YNOV15 (*rad9-F1140L*) and YNOV31 (*rad9-W1280L*) strains were obtained from YFL871. The *kanMX4* and *KlURA3* CORE cassettes, amplified from pCORE [71], were integrated in a K699 strain at position 1941 of the *RAD9* gene. Subsequently, the CORE cassette was replaced with the C-terminus of the *rad9-F1104L* or *rad9-W1280L* alleles, amplified respectively from pFL75.5 or pFL69.1, thus restoring the full-length *RAD9* open reading frame bearing the intended mutation. *RAD9* site-specific mutations on plasmids pFL75.5 and pFL69.1 were obtained by PCR with mutagenic oligonucleotides on the pFL36.1 plasmid [50]. Recombination events were selected on 5-fluoroorotic acid plates, and the strains were verified by sequencing.

The *rad9ΔBRCT::13MYC* and the *rad9ΔBRCT::GST* mutant alleles were obtained by introducing the 13-MYC or GST tags at the 984 aa, using the one-step PCR method [70], thus eliminating the whole Rad9 BRCT domain.

The *cdc28-as1* mutant allele was obtained by ClaI-directed integration of plasmid pVF6 [72] at the *CDC28* locus into the desired background. Plasmid pop-out events were selected on 5-fluoroorotic acid plates, and the presence of the *cdc28-as1* mutation was verified by assessing sensitivity to 1NMPP1 on plate.

Strains encoding the *rad9-S11A* mutant allele were obtained by MscI-directed integration of pRS306-NTRAD9^{cdk1} into the desired background. The transversion TCT-GCT causing the *rad9-S11A* mutation and the reversion GCT to ACT generating the *rad9-S11T* allele were produced by site- directed mutagenesis (Stratagene) of pGEMTeasyRAD9, containing a 2547 bp fragment from position -445 to position +2102 within the *RAD9* ORF. The 1.8 Kb BamHI-MscI fragment from the pGEMTeasyRAD9 vector was swapped with the equivalent fragment from an existing 6.3 Kb pRS306-NTRAD9 integrative vector, containing a BamHI-SpeI *RAD9* fragment from position −445 to position 1478 within the *RAD9* ORF and the presence of the mutation verified by sequencing. Plasmid pop-out events were selected on 5-fluoroorotic acid plates, and the *rad9-S11A* mutation was confirmed by PCR sequencing.

The *dpb11ΔCT* mutant allele was obtained by introducing a premature stop codon at the 583 aa and the *HPH* cassette after the codon with the one step PCR method previously described [73], thus mimicking the *dpb11-1* mutation [74].

Strain YFL921 was obtained by using the one-step PCR strategy described in Longtine 1998, using pFA6-*FKBP2x-13MYC-KanMX6*, as template. This plasmid was generated by cloning in PacI-linearized pFA6-*13MYC-KanMX6* the *FKBP2x* sequence amplified from pC4M-FV2E (ARGENT Regulated Homodimerization kit, ARIAD Pharmaceutical).

The yeast two hybrid was performed using the B42/lexA system with strain EGY42 (*MATa his3 ura3 trp1 6lexAOP-LEU2; lex- AOP-lacZ* reporter on plasmid pBH18-34) as the host strain [69].

Chromatin binding

To analyze chromatin binding of proteins, yeast extracts were prepared from G1- or M-arrested cells following published procedures [22].

Cell cycle blocks and DNA damage treatments

Cells were grown in YPD medium at 28°C (25°C in the experiments with strains harboring the *dpb11ΔCT* mutation) to a concentration of 6×10^{6} cells/ml and arrested in G1 or M with α-factor (20 µg/ml) or nocodazole (20 µg/ml), respectively. 50 ml of cultures were centrifuged, resuspended in 500 µl of fresh YPD and plated on a Petri dish (14 cm diameter). Plates were quickly

irradiated with a Stratalinker at 75 J/m^2 and cells resuspended in 50 ml of YPD plus α-factor or nocodazole. A 25 ml sample was taken 10 min after the treatment and processed for protein extraction with trichloroacetic acid (TCA) [75]. For analysis of the double-strand breaks (DSBs) checkpoint response, cells arrested at the proper cell cycle phase were treated with 150 µg/ml of zeocin. Samples were taken 45 min after treatment and processed for protein extraction.

FKBP dimerization

To analyze FKBP-driven (FK506 binding protein) dimerization, overnight cell cultures were diluted at a concentration of 1×10^6 cells/ml and treated for 6 h with 1 µM AP20187 (ARGENT Regulated Homodimerization kit, ARIAD Pharmaceutical). UV sensitivity assays or chromatin binding analysis were performed as described elsewhere in this section.

Inactivation of the Cdc28 kinase activity

Exponentially growing cells in a *cdc28-as1* background were harvested at a concentration of 4×10^6 cells/ml and blocked in M phase as described above. To selectively inhibit Cdc28 activity [56], the ATP analogue 1NMPP1 was then added to a concentration of 5 µM to half of the cultures; after 2 h of incubation at 28°C, cells were either mock- or UV-irradiated and protein extracts were prepared.

SDS-PAGE and western blotting

TCA protein extracts or chromatin binding samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% acrylamide gels. For the analysis of Rad9 phosphorylation, NuPAGE Tris-acetate 3% to 8% gels were used following the manufacturer's instructions. Western blotting was performed with anti-Rad9 (D. Stern), anti-Rad53 (C. Santocanale), with anti-phosphorylated Rad53 F9 Mab antibodies [76] anti-ORC2 (Abcam) and anti-tubulin (ML. Carbone), using standard techniques.

UV–sensitivity assay

To assess cell survival after UV irradiation, serials dilutions of overnight cultures were spotted onto YPD plates, which were either irradiated with different UV doses or mock-treated. For survival curves, yeast strains were cultured overnight to exponentially growing phase. Cells were diluted and approximately 500 cells/plate were plated, and then either irradiated with various UV doses or mock-treated. After 3 days, the total number of colonies formed on each plate was counted.

Yeast two-hybrid analysis

Protein interaction between Rad9 and Dpb11 in the G1 and M phase of the cell cycle was assessed by measuring β-galactosidase activity with ortho-Nitrophenyl-β-galactoside (ONPG) assay. Briefly, cells expressing Rad9 bait and/or Dpb11 prey were cultured overnight in yeast synthetic media (-Ura, -His, -Trp) with 2% (w/v) raffinose to a concentration of 5×10^6 cells/ml. Cultures were centrifuged and cells resuspended in YP plus raffinose and arrested in G1 or M phases, as described above. Galactose to a 2% w/v final concentration was added to the medium to induce prey expression. A 15 ml sample was taken after 1 h of galactose induction, centrifuged and resuspended in 250 µl of breaking buffer (100 mM Tris HCl at pH 8.0, Glycerol 10%; DTT 1 mM, 1 tablet of complete Roche antiproteolytic cocktail. Cells were lysed by using a FastPrep cell disruptor; the optical density (OD) of protein extract at 600 nm was determined using the Bio-Rad protein assay reagent. 1 ml of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol at pH 7.0) plus ONPG 4 mg/ml was aliquoted in a small glass tube for each sample. 20 µl of protein extract was added to each tube and incubated at 37°C until a yellow color developed. The reaction was stopped by adding 400 ml of 1 M $NaCO_3$ and the OD at 420 nm of each sample was measured. β-Galactosidase activity was calculated by using the formula units $=10^3$ $OD_{420}/(OD_{600}$ x reaction time in min).

Rad9-Dpb11-MYC immunoprecipitation

1.5 l cultures of strains YFP38 and YMAG281 expressing, respectively, the tagged Dpb11-MYC fusion protein under the control of the endogenous *DPB11* promoter in a wild-type or *rad9S11A* mutant background were grown in YPD medium at a cell density of 1×10^7 cells/ml. Cells were then arrested in M phase by addition of 10 µg/ml of nocodazole and were either mock treated or treated with 150 µg/ml of zeocin for 30 min. Cells were washed twice with pre-cooled ddH_2O and once in 2× lysis buffer (300 mM KCl, 100 mM Hepes (pH 7.5), 20% glycerol, 8 mM β-mercaptethanol, 2 mM EDTA, 0.1% Tween20, 0.01% NP-40). Resuspended cells were frozen as droplets in liquid nitrogen. Aliquots of frozen cells were manually ground in a mortar in liquid nitrogen. One volume of 2× lysis buffer, containing a protein inhibitor cocktail (2.8 µM leupeptin, 8 µM pepstatin A, 4 mM PMSF, 50 mM benzamidine, 25 µM antipain, 4 µM chymostatin in ethanol) and phosphatase inhibitors (2 mM sodium fluoride, 1.2 mM β-glycerophosphate, 0.04 mM sodium vanadate, 2 mM EGTA, 10 mM sodium pyrophosphate), was added. Cell extract was clarified by a low speed centrifugation followed by additional centrifugation for 1 h at 42.000 rpm in a Beckman Sw55Ti rotor. The clarified crude extract (Ext) was adjusted to 10 mg/ml in the various immunoprecipitation experiments. 1 ml of Ext was precleared by incubation with 40 µl of 50% (v/v beads/1× lysis buffer) Protein G slurry for 1 hour at 4°C on a rotating wheel. Precleared supernatants were incubated with either 20 µg of the anti-myc Mab 9E11 or 20 µg of unspecific mouse IgG. Samples were incubated for 2 h at 4°C on a rotating wheel and centrifuged at 14.000 rpm for 15 min at 4°C. 40 µl of 50% protein G slurry were added to the supernatants, incubated on a rotating wheel for 2 h at 4°C and recovered by centrifugation. Immunoprecipitated Dpb11-MYC samples were washed four times with 1 ml of lysis buffer containing protease and phosphatase inhibitors. Beads were finally resuspended in 40 µl of 3× Laemmli buffer (IP), boiled for 5 min and released proteins separated on 6.5% (80/1 acrylamide/bisacrylamide) SDS-PAGE gels. After blotting, Rad9 was visualized with the NLO5 Rad9 polyclonal antibody [13] or the 9E11 Mab (Abcam).

Supporting Information

Figure S1 (A) wt (K699) cells were arrested in G1 with α-factor and either mock or UV irradiated (75 J/m^2). 10 min after irradiation, samples were collected and analyzed in their total (T), soluble (S) and chromatin-enriched (Ch) fractions. Blots were probed with anti Rad9 polyclonal antibodies. After UV irradiation the hyper-phosphorylated Rad9 isoform migrates and it is detected on Western blots probed with anti-Rad9 antibodies near to an aspecific protein species (mostly present in the supernatant fraction) [50]. Such band was omitted in the Western blots shown in Figure 1, Figure 2, and Figure 7 for clarity. The positions of Rad9 and its hyper-phosphorylated isoform (pRad9) are indicated; * marks the background protein species unrelated to Rad9. (B) The Western blots in which the presence of Rad9 was analyzed in

the total (T), soluble (S) and chromatin-enriched (Ch) fractions were controlled for proper fractionation of control proteins, known to remain in the soluble fraction (Tubulin) or to bind to chromatin (Orc2). The blots in S1 Panel B show the results obtained with the same protein samples analyzed in Figure 1A. (C) Quantitative analysis of the percentage of hyper-phosphorylated and hypo-phosphorylated Rad9 isoforms in the total (T), soluble (S) and chromatin-enriched (Ch) fractions in α-factor and nocodazole arrested wild-type cells. Quantification was obtained with a Versadoc (Biorad) after incubation with fluorescent secondary antibodies, and error bars were obtained from 4 independent experiments. The percentages of hyper- and hypo- phosphorylated isoforms were calculated respectively to the total amount of Rad9. Found at: doi:10.1371/journal.pgen.1001047.s001 (1.16 MB TIF)

Figure S2 (A) The histograms show the M/G1 ratio increase in β-galactosidase activity, when the interaction between Dpb11/Rad9 or the positive controls p53 and SV40-TAg was measured by two-hybrid analysis in nocodazole (M) or α-factor (G1) arrested cells. Error bars were obtained from three independent two-hybrid experiments. (B) Amino acid sequence of the Rad9 ORF; the basic CDK1 (S/T-P) and PIKK (S/T-Q) consensus phosphorylation sites are shown in black or gray, respectively. (C) wt (K699) and *rad9-S11A* (YMAG162) strains were arrested in M with nocodazole and samples were collected to prepare protein extracts. Rad9 phosphorylation was analyzed by SDS-PAGE and Western blotting with anti-Rad9 antibodies.
Found at: doi:10.1371/journal.pgen.1001047.s002 (0.77 MB TIF)

Figure S3 wt (YMAG149/7B), *H2A-S129A* (YMAG168), *dpb11ΔCT* (YMAG145/20C), *H2A-S129A dpb11ΔCT* (YMAG155), *dot1Δ* (YMAG150/4A), *H2A-S129A dot1Δ* (YMAG170), *dpb11ΔCT dot1Δ* (YMAG148) and *H2A-S129A dpb11ΔCT dot1Δ* (YMAG157) strains were arrested in M with nocodazole and treated with zeocin (150 μg/ml). After 45 min, samples were collected and protein extracts were analyzed by SDS-PAGE and Western blotting with anti Rad53 antibodies to monitor checkpoint activation.
Found at: doi:10.1371/journal.pgen.1001047.s003 (0.76 MB TIF)

Table S1 Strains used in this study. All of the strains used in this work are derivatives of W303 [*MAT*a *ade2-1 trp1-1 can1-100 leu2-3,12 his3-11,15 ura3 rad5-535*]; only strains YFP91 and DLY2236 (provided by D. Lydall), are *RAD5*$^{+}$.
Found at: doi:10.1371/journal.pgen.1001047.s004 (0.06 MB DOC)

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Author Contributions

Conceived and designed the experiments: M Granata, F Lazzaro, R Kumar, M Grenon, NF Lowndes, P Plevani, M Muzi-Falconi. Performed the experiments: M Granata, F Lazzaro, D Novarina, D Panigada, F Puddu, CM Abreu. Analyzed the data: M Granata, F Lazzaro, D Novarina, D Panigada, F Puddu, M Grenon, NF Lowndes, P Plevani, M Muzi-Fal. Contributed reagents/materials/analysis tools: M Granata, F Lazzaro, D Novarina, D Panigada, F Puddu. Wrote the paper: NF Lowndes, P Plevani, M Muzi-Falconi.

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Supplementary Fig.1

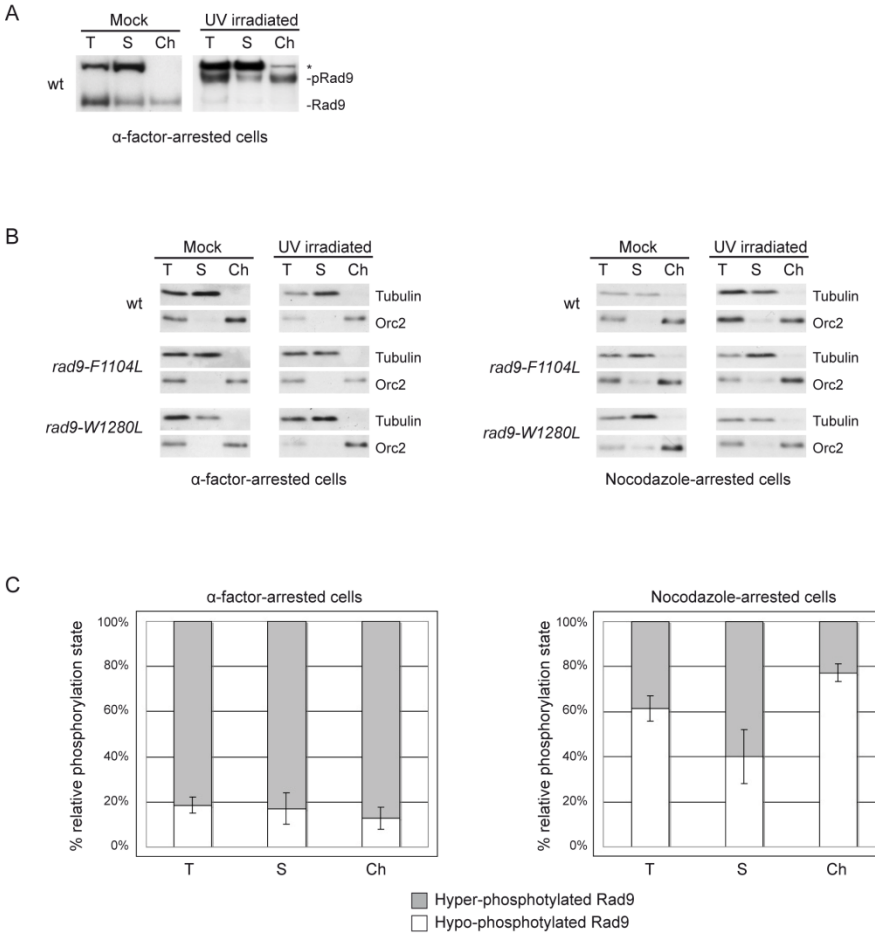

Figure S1.

(A) wt (K699) cells were arrested in G1 with α-factor and either mock or UV irradiated (75 J/m^2). 10 min after irradiation, samples were collected and analyzed in their total (T), soluble (S) and chromatin-enriched (Ch) fractions. Blots were probed with anti Rad9 polyclonal antibodies. After UV irradiation the hyper-phosphorylated Rad9 isoform migrates and it is detected on Western blots probed with anti-Rad9 antibodies near to an aspecific protein species (mostly present in the supernatant fraction) [50]. Such band was omitted in the Western blots shown in Figure 1, Figure 2, and Figure 7 for clarity. The positions of Rad9 and its hyper-phosphorylated isoform (pRad9) are indicated; * marks the background protein species unrelated to Rad9. **(B)** The Western blots in which the presence of Rad9 was analyzed in the total (T), soluble (S) and chromatin-enriched (Ch) fractions were controlled for proper fractionation of control proteins, known to remain in the soluble fraction (Tubulin) or to bind to chromatin (Orc2). The blots in S1 Panel B show the results obtained with the same protein samples analyzed in Figure 1A. **(C)** Quantitative analysis of the percentage of hyper-phosphorylated and hypo-phosphorylated Rad9 isoforms in the total (T), soluble (S) and chromatin-enriched (Ch) fractions in α-factor and nocodazole arrested wild-type cells. Quantification was obtained with a Versadoc (Biorad) after incubation with fluorescent secondary antibodies, and error bars were obtained from 4 independent experiments. The percentages of hyper- and hypo- phosphorylated isoforms were calculated respectively to the total amount of Rad9.

Supplementary Fig. 2

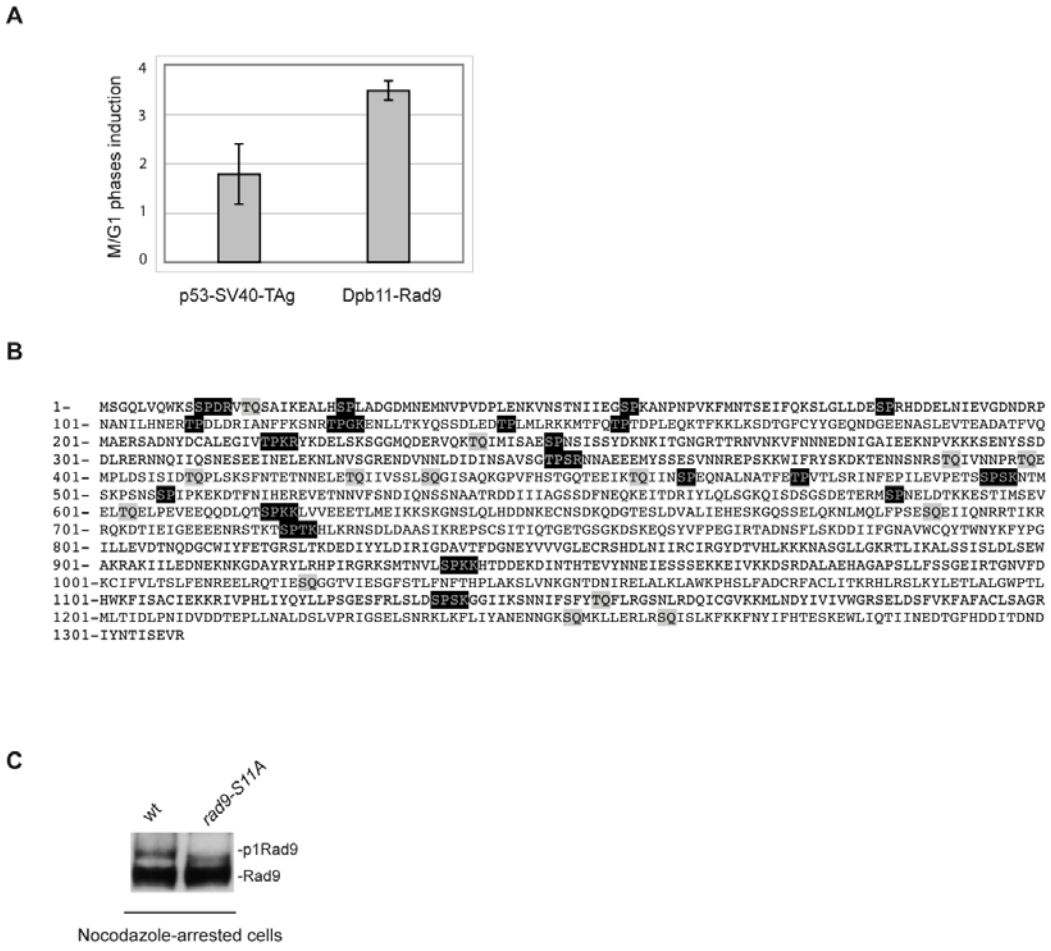

Figure S2.

(A) The histograms show the M/G1 ratio increase in β-galactosidase activity, when the interaction between Dpb11/Rad9 or the positive controls p53 and SV40-TAg was measured by two-hybrid analysis in nocodazole (M) or α-factor (G1) arrested cells. Error bars were obtained from three independent two-hybrid experiments. **(B)** Amino acid sequence of the Rad9 ORF; the basic CDK1 (S/T-P) and PIKK (S/T-Q) consensus phosphorylation sites are shown in black or gray, respectively. **(C)** wt (K699) and *rad9-S11A* (YMAG162) strains were arrested in M with nocodazole and samples were collected to prepare protein extracts. Rad9 phosphorylation was analyzed by SDS-PAGE and Western blotting with anti-Rad9 antibodies.

Supplementary Fig. 3

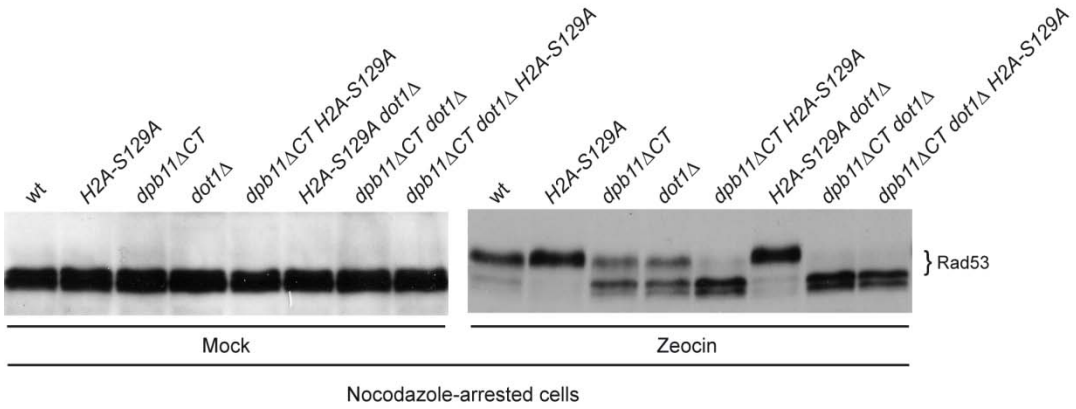

Figure S3.

wt (YMAG149/7B), *H2A-S129A* (YMAG168), *dpb11ΔCT* (YMAG145/20C), *H2A-S129A dpb11ΔCT* (YMAG155), *dot1Δ* (YMAG150/4A), *H2A-S129A dot1Δ* (YMAG170), *dpb11ΔCT dot1Δ* (YMAG148) and *H2A-S129A dpb11ΔCT dot1Δ* (YMAG157) strains were arrested in M with nocodazole and treated with zeocin (150 µg/ml). After 45 min, samples were collected and protein extracts were analyzed by SDS-PAGE and Western blotting with anti Rad53 antibodies to monitor checkpoint activation.

Table S1.

Strains used in this study. All of the strains used in this work are derivatives of W303 [*MAT*a *ade2-1 trp1-1 can1-100 leu2-3,12 his3-11,15 ura3 rad5-535*]; only strains YFP91 and DLY2236 (provided by D. Lydall), are *RAD5*$^{+}$.

Strain	Relevant genotype	Source
K699	*MAT*a *ade2-1 trp1-1 leu2-3,112 his3-11 ura3 can1-100 rad5-535*	K. Nasmyth
YFL871	K699 *rad9aa1-646:kanMX4:URA3*	This work
YNOV15	K699 *rad9-F1104L*	This work
YNOV31	K699 *rad9-W1280L*	This work
YMAG88	K699 *rad9::HIS3*	This work
YFL696/1b	K699 *rad9ΔBRCT::13MYC:TRP1*	This work
YMAG74	K699 *rad9ΔBRCT::GST:kanMX6*	This work
YFL773/2c	K699 *dot1::kanMX6 rad9ΔBRCT::GST:kanMX6*	This work
YFL921	K699 *rad9ΔBRCT::2xFKBP-13MYC:kanMX6*	This work
YMAG149/7B	K699 *hta1_htb1::LEU2 hta2_htb2::TRP1* (pSAB6)	(50)
YMAG145/20C	K699 *hta1_htb1::LEU2 hta2_htb2::TRP1 dpb11ΔCT::HPH* (pSAB6)	This work
YMAG150/4A	K699 *hta1_htb1::LEU2 hta2_htb2::TRP1 dot1::kanMX6* (pSAB6)	(50)
YMAG148	K699 *hta1_htb1::LEU2 hta2_htb2::TRP1 dpb11ΔCT::HPH dot1::kanMX6* (pSAB6)	This work
EGY42	*MAT*α *his3 ura3 trp1 leu2::6LexAop-LEU2*	R. Brent
JAU01	K699 *cdc28-as1*	(56)
YNOV4	K699 *cdc28-as1 dot1::kanMX6*	This work
YFL234	K699 *dot1::kanMX6*	(33)
DLY2236	K699 *rad9::LEU2 ura3::rad9-M232:URA3 RAD5+*	(58)
YFP91	K699 *rad9::LEU2 ura3::rad9-M232-URA3 dot1::kanMX6 RAD5+*	This work
YMAG162	K699 *rad9-S11A*	This work
YMAG164	K699 *rad9-S11A dot1::kanMX6*	This work
YMAG168	K699 *hta1_htb1::LEU2 hta2_htb2::TRP1* (pJD151)	(50)
YMAG170	K699 *hta1_htb1::LEU2 hta2_htb2::TRP1 dot1::kanMX6* (pJD151)	(50)
YMAG155	K699 *hta1_htb1::LEU2 hta2_htb2::TRP1 dpb11ΔCT::HPH* (pJD151)	This work
YMAG157	K699 *hta1_htb1::LEU2 hta2_htb2::TRP1 dpb11ΔCT::HPH dot1::kanMX6* (pJD151)	This work
YFL1177	K699 *rad9-S11AΔBRCT::GST:kanMX6*	This work

CHECKPOINT MECHANISMS AT THE INTERSECTION BETWEEN DNA DAMAGE AND REPAIR.

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Checkpoint mechanisms at the intersection between DNA damage and repair

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ABSTRACT

In response to genomic insults cells trigger a signal transduction pathway, known as DNA damage checkpoint, whose role is to help the cell to cope with the damage by coordinating cell cycle progression, DNA replication and DNA repair mechanisms. Accumulating evidence suggests that activation of the first checkpoint kinase in the cascade is not due to the lesion itself, but it requires recognition and initial processing of the lesion by a specific repair mechanism. Repair enzymes likely convert a variety of physically and chemically different lesions to a unique common structure, a ssDNA region, which is the checkpoint triggering signal. Checkpoint kinases can modify the activity of repair mechanisms, allowing for efficient repair, on one side, and modulating the generation of the ssDNA signal, on the other. This strategy may be important to allow the most effective repair and a prompt recovery from the damage condition. Interestingly, at least in some cases, if the damage level is low enough the cell can deal with the lesions and it does not need to activate the checkpoint response. On the other hand if damage level is high or if the lesions are not rapidly repairable, checkpoint mechanisms become important for cell survival and preservation of genome integrity.

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1. DNA damage checkpoints

Maintenance of genomic stability is of fundamental importance for dividing and non-dividing cells. Any alteration of genome structure can lead to loss of proliferative controls and/or cell death. Genome integrity is continuously challenged by endogenous and exogenous agents, which generate an ample spectrum of physically distinct lesions. To ensure the accurate transmission of genetic information throughout generations and through the DNA to protein flow, cells have evolved an intricate set of surveillance and DNA repair mechanisms, which prevent damaged DNA from being converted into heritable mutations. These surveillance systems, called DNA damage checkpoints, are signal transduction cascades triggered by DNA damage, and result in inhibition of cell cycle progression and DNA replication, and, in some cases, stimulation of the apoptotic pathway. Moreover, checkpoint activation frequently brings about a change in the transcriptional program of the cell [1–3] and modifications of DNA repair factors, resulting in a more efficient removal of the lesions and an increased resistance to further damage [4–6].

DNA repair requires the recognition of the presence of even the least intruding lesion and the activation of the correct repair machinery; if the triggering lesions are not rapidly cleared, a temporary arrest of cell cycle progression allows more time for removal. There are indeed lesions which are so rapidly repaired that cell cycle arrest is not required [7]; others, like thymidine dimers, are removed slowly and are obstacles for DNA replication and transcription; frequently leading to cell cycle arrest [8–10]. The general belief is that this arrest will last until DNA repair has finished processing the lesions. There are indeed suggestions that more lesions will lead to a longer arrest [11]. On the other hand, in some cases it may be more sensible for the cell to keep cycling and reach a cell cycle phase where the specific damage is less toxic or more easily dealt with; there is evidence that sometimes lesions are left in place until the next cell cycle round [12]. It is thus obvious that checkpoint pathways must be able to tune repair mechanisms integrating the specificity of the lesions with the cell cycle position.

In this review we will describe how physically different damages trigger the same checkpoint signal transduction cascade and we will discuss the relevance of DNA repair mechanisms in recognizing and processing the primary lesions to generate a common structure that will recruit the first checkpoint proteins. Moreover, we will summarize recent findings on how the checkpoint phosphorylation cascade feeds back and regulates DNA repair.

One important question is how checkpoint mechanisms can recognize the presence of DNA lesions, which are dispersed within large amounts of normal DNA that is packed in chromatin and is constantly involved in DNA metabolic processes, undergoing dynamic structural changes. Moreover, the chemical nature of the

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possible lesions is so diverse that there have to be specialized factors which are specific for different DNA damages [13]. At the top of the checkpoint cascades are two protein kinases of the PIKK family (ATM and ATR). ATM responds mostly to double strand breaks, while ATR responds to a variety of DNA lesions and to replication stress [14]. In recent years several papers contributed evidence that primary lesions are not directly recognized by checkpoint factors, but generally it is the processing of such lesions by repair mechanisms which is responsible for activating the apical checkpoint kinases. How this is achieved depends on the kind of primary lesion and thus on the repair mechanism invoked.

2. Double strand breaks

Although, in principle, a double strand break (DSB) can be visualized as a cut in a chromosome, a cell can encounter different types of DSBs which may differ from each other for some features and thus undergo specific processing by repair enzymes. Also the chromatin architecture around a DSB affects how the lesion will be repaired, and chromatin remodeling complexes, such as RSC, INO80 or others belonging to the SWI/SNF2 family, play important roles during recombination [15,16]. The physical ends of DSBs may have chemical structures which are not easily handled by some DNA repair proteins. The nature of these structures may depend on how DSBs are generated: it has been proposed that the action of certain nucleases leave "clean" and well defined ends at the DSB, whereas ionizing radiation (IR), the most common DSB-inducing agent, may generate "ragged" ends at the breaks that require further processing [17]. The occurrence of different types of DSBs may thus influence the choice of the recombination pathway used by the cell to repair the lesions. Cells can adopt two different alternative strategies to repair DSBs. The ends of the break can be directly rejoined by ligation through a pathway that does not require homology between the two halves of the broken molecule (non-homologous-end-to-end-joining, NHEJ). Alternatively DSBs can be repaired through homologous-directed recombination (HDR), which requires pairing of the regions surrounding the broken DNA ends with a homologous sequence (called "donor") [18] (Fig. 1). There is clear evidence that the balance between the two pathways is regulated during the cell cycle, NHEJ being the favorite in G1 [18]. DSB lesions in G1 cannot be engaged in HDR because initial processing of the break to generate the ssDNA filaments requires CDK/cyclin B activity and because the preferred donor sequence utilized in the process, usually located on the sister chromatid, is missing.

Many of the enzymes and events occurring during the recombination processes are well characterized. Using different molecular approaches, including chromatin immunoprecipitation (ChIP) and in vivo protein co-localization studies, several checkpoint and repair factors have been shown to be loaded onto DSBs. Many of these proteins, among which are the KU and the Mre11–Rad50–Nbs1 (MRN) complexes, compete for the same structure and are directly involved in the DSB repair mechanisms [19]. It can be assumed that binding of a given factor drives the DSB into a certain recombination mechanism, excluding access to alter-

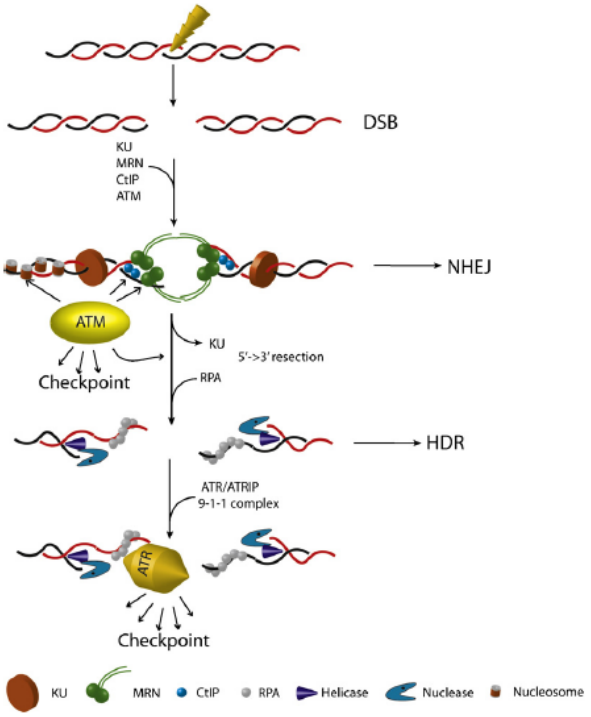

Fig. 1. Double strand break processing and checkpoint activation. As soon as a DSB is generated, the DNA ends are captured by the KU heterodimer, which is required to channel the cut molecule into the NHEJ repair pathway. CtIP and the MRN complex also bind the broken chromosome and hold the ends together, allowing activation of the checkpoint, mediated by the recruitment of ATM. If the broken ends are not rapidly rejoined, they are resected by nucleolytic activities, generating long 3′-ended ssDNA filaments, which are covered by RPA. It is this structure that, recruiting ATR/ATRIP and the 9-1-1 complex, activates the checkpoint response.

native factors and the use of a different recombination pathway. Regulating the kinetics and/or the order of recruitment of these factors onto a DSB may be crucial to promote the most effective way to repair a specific lesion.

2.1. DSBs signaling to ATM

Different processing of the DSBs will generate different structures that will be treated differently by the DNA damage checkpoint signaling machines. The main PIKK apical checkpoint kinases, ATM and ATR, are loaded onto DNA after DSBs induction and recognize different types of signals generated in the process of DSB repair. ATM activation requires the presence of the MRN complex, which binds DSBs, has a DNA molecules tethering capacity and possesses both endo- and exo-nucleolytic activities [20] (Fig. 1). Accordingly, patients affected by Nijmegen breakage syndrome (NBS) or by AT-like disorder (ATLD), who carry mutations in the MRN complex, exhibit phenotypes that are similar to those of AT patients who are mutated in ATM [21–23]. These findings are supported by molecular evidence showing that loss of MRN complex prevents phosphorylation of ATM substrates [24,25] and loading of ATM onto damaged chromatin [26]. These observations suggest that MRN and ATM act in the same biological process. A new twist to this question came from some very elegant recent work showing that mouse cells expressing only a nuclease-defective Mre11 protein are indistinguishable from cells lacking Mre11; they are sensitive to DNA damaging agents and exhibit chromosomal instability, similarly to ATLD cells. Surprisingly, while cells lacking Mre11 do not trigger ATM kinase, nuclease-defective Mre11 mutant cells can activate the ATM pathway just fine [27,28]. All these observations seem to suggest that nuclease activity of Mre11 is not critical for ATM activation and the presence of a physically assembled MRN complex is necessary and sufficient to satisfy the MRN requirement for ATM activation; mutations in MRN subunits defective in ATM activation may do so by destabilizing the complex. A major question now is what is the essential role of Mre11 nuclease activity: the most likely possibility is cleaning up the termini of the broken DNA ends to make them repairable.

ATM normally exists as an inactive dimer, which is converted to an active monomer upon damage; such activation has been reported to be induced also by alterations of chromatin structure that do not cause DSBs, suggesting that short unwound regions exposing ssDNA may be sufficient to trigger the kinase [29]. Supporting evidence has shown that in vitro, dimeric ATM can be activated by DNA and MRN, which unwinds the double helix and also increases the affinity of ATM for its substrates [30,31]. In the S/G2 phase of the cell cycle, ATM and the MRN complex, together with the CtIP/Sae2 protein, initiate processing of DSB ends [32–36], promoting the formation of the 3′-ssDNA intermediate, which is the structure that will recruit ATR [37] (Fig. 1). Moreover, the short oligonucleotides generated by the nucleolytic reaction sustain the activity of ATM likely acting as allosteric cofactors [38], suggesting that initial activation due to ATM–MRN loading at the DSB is maintained by nucleolytic processing of the broken molecule and is important for the formation of a 3′-ssDNA filament that is a prerequisite for homologous recombination and for ATR activation.

The situation is somewhat different in budding yeast, where Tel1, the orthologue of ATM, participates only marginally in the DSB-induced checkpoint response and its role becomes evident only in the presence of multiple DSBs and/or when the initiation of DSB ends processing is defective [39]. In this organism, a single irrepairable DSB triggers the Mec1 (ATR) pathway of the checkpoint response, activated through extensive resection of the double stranded DNA end [40]. In fact, genetic and biochemical analysis using yeast systems where one DSB can be induced at a specific locus in the genome, established that, if the break is not immediately repaired, nucleolytic activities produce long ssDNA regions that, via the ssDNA binding protein RPA, recruit Mec1 kinase activating the checkpoint [41,42]. The same DNA intermediate is also the substrate for homologous recombination and, indeed, RPA, Rad51 and Mec1 co-localize with DSB lesions in cells treated with IR (or other agents causing DSB formation), providing a useful tool to study the in vivo activation and choreography of the DNA damage response [43].

2.2. DSBs signaling to ATR

The biochemical mechanism responsible for ATR kinase activation has been recently reviewed [44,45], but is still not completely understood. Briefly, full ATR activation requires a long stretch of ssDNA, a functional ATRIP (mediating binding to RPA–ssDNA), the 9-1-1 checkpoint clamp and TopBP1, which are also recruited at the site of damage. This functional model for ATR activation is very well conserved in budding yeast, were Mec1 activation is influenced by the Rad17–Mec3–Ddc1 clamp and by Dpb11 (the TopBP1 orthologue) [45–47]. Although the ATM requirement for ATR activation could be explained in term of ssDNA generation as a pre-requisite for ATR recruitment onto DNA, it will be interesting to investigate whether ATM directly activates ATR, during the early events of the DSBs response. In this view, it has been shown that TopBP1 is converted to a more efficient ATR activator through its phosphorylation by ATM [48]. Indeed, the interdependency between the ATM and ATR kinases is one of the main questions that needs to be addressed to better understand the mechanism of checkpoint activation. Much work in the last few years has been devoted to understanding how ssDNA is generated from a DSB. In yeast, several groups have shown that the broken DNA ends are resected by nuclease activities that convert the DSB to ssDNA filaments. It was established that MRX played a role in this nucleolytic processing, indeed *mre11*Δ cells are clearly affected in DSB resection. On the other hand, Mre11–nuclease-defective yeast mutants fail to process ends blocked by covalent adducts, but still resect DNA ends generated by HO [49,50]. Sae2 cooperates with the MRX complex in processing DSB ends. Exo1 is also involved and together with Dna2 and the Sgs1 helicase has been suggested to be responsible for taking over the ends primed by MRX/Sae2 and generate the long ssDNA stretch which is required for HDR and full checkpoint activation [36,35].

An interesting twist has been added by recent data obtained using a genetic system in yeast and human cells, where large amounts of the apical kinase complex Mec1–Ddc2 and ATR–ATRIP, respectively, were targeted at an undamaged genomic locus [51,52]. In this context, the checkpoint response was activated in the apparent absence of any DSB or ssDNA intermediate, suggesting that DSBs and ssDNA structures may not be directly required for checkpoint activation, but rather may serve as scaffold to co-localize and concentrate checkpoint proteins, which is sufficient to trigger the checkpoint cascade. It will be interesting to further exploit this system to investigate more precisely an interesting issue in the current model of DSB-induced checkpoint activation, namely the existence of a threshold level of damaged DNA that can trigger the signaling cascade. Correlating the activity of checkpoint kinases to the number of checkpoint molecules bound at a specific site will help in defining the mechanism of checkpoint activation. The potency of the checkpoint response has been reported to correlate to the amount of damage in the genome [53]; moreover, it is known that if recombination/repair is fast enough and the length of the ssDNA regions generated through processing of the DSB is less than 10 kb, the ATR-dependent checkpoint is not activated in yeast aploid cells [54,55]. All this may indicate that a minimal amount of ssDNA has to be present in order to activate the checkpoint kinase. The existence of a ssDNA-dependent threshold to activate the ATR-

mediated checkpoint is an interesting hypothesis requiring more investigation.

2.3. Activation of DNA damage checkpoints affects local chromatin structure

From this discussion it is evident that various DSB repair enzymes share the responsibility to generate the signal triggering the checkpoint cascade; but the reverse is also true. In fact, there is clear evidence that one of effects of activating checkpoint kinases is to regulate repair efficiency. This can involve a general effect, such as modifications of chromosome packaging or cohesion, or may be due to a more direct action on the actual repair machineries.

In response to DSBs, KAP-1, a co-repressor of transcription, is phosphorylated by ATM specifically at damage sites and spreads out through chromatin, promoting chromatin relaxation. Mutations blocking KAP-1 phosphorylation increase the cellular sensitivity to DNA damaging agents, suggesting that ATM-induced chromatin relaxation may facilitate the repair of broken DNA, possibly by making the donor sequence more accessible [56,57]. Histone proteins are also modified upon DSB-induction, as was originally shown by Bonner [58]. In yeast, a subset of H2A localized close to the DSB is phosphorylated near its C-terminus, and a similar modification has been identified in human H2AX variant histone [59]. Phosphorylation of histone H2AX is an early event in the cellular response to DSBs that contributes to the recruitment of other checkpoint and repair factors to "nuclear foci" and promotes efficient repair of the lesions [59–62]. Similarly, Rnf8 and Ubc13 ubiquitylate histones at DSBs in vertebrates; this modification is dependent upon the apical checkpoint kinases and is thought to be particularly relevant for the assembly of checkpoint factors and repair machines [63–66]. Generally, repair of DSBs via the homologous recombination pathway utilizes the donor sequence from the sister chromatid rather than the homologous chromosome. This can explain why mutations affecting sister chromatid cohesion have a negative effect on DSB repair [67]. This aspect is also tuned by the checkpoint response; indeed, it has been shown that phosphorylation of yeast H2A by the apical PIKK kinases is responsible for deposition of cohesins onto a DSB, which may contribute to hold together the arms of the cut chromosome, thus facilitating the recombination process with the sister chromatid [68,69] and possibly preventing the highly reactive ends from undergoing aberrant reactions that would lead to translocations or other gross chromosomal rearrangements [70].

2.4. Checkpoint factors modulate DSB repair

The interconnections between DSB repair and the checkpoint response are further supported by the finding that various repair factors undergo post-translational modification (mainly by phosphorylation) in a checkpoint-dependent manner. These observations rise the stimulating question whether the checkpoint pathway directly regulates the efficiency of the DSB repair. While in many cases the physiological significance of such DNA damage-induced post-translational modifications is not clear, there are cases where phosphorylation of repair factors actually leads to more effective repair. For example, the checkpoint response finely regulates DSB ends processing, which is a crucial stage in the recombination process; unbalancing this control step can have the most severe effects on recombination, preventing HDR and allowing NHEJ to take over. For instance, inactivation of CDK1 leads to an increase of NHEJ events in the G2 phase of the cell cycle [40]. Further, checkpoint-dependent phosphorylation of recombination factor BRCA1 and Nej1 seems to affect whether HDR or NHEJ pathways are used to repair DSBs [71–73]. The nucleases involved in the conversion of a DSB to ssDNA filaments, namely MRN/MRX, CtIP/Sae2 and Exo1, are targets of CDK and of the checkpoint kinases [33,74–78]. While in most cases we still do not know the relevance of these phosphorylation events, it is tempting to speculate that this may be a way to control resection. In the case of Sae2, who is involved in tethering the DSB ends and in converting them to ssDNA, mutations mimicking constitutive phosphorylation of one CDK target site allow resection to occur also in G1, tilting the balance between NHEJ and HDR. Moreover, mutations preventing its phosphorylation by Tel1 kinase cause break-induced chromosome translocations due to uncontrolled NHEJ events [33,70]. One interpretation of these results is that Tel1 activation may be relevant to discern which ends to join and to suppress end joining between different chromosomes. It is known that the length of the 3′-ssDNA nucleoprotein filament affects the recombination partner choice through a mechanism dependent on the length of the interacting homologous donor sequences [79]. In *Saccharomyces cerevisiae* it has been shown that a mutation in Rad24, a subunit of the RFC-like complex required to load the 9-1-1 checkpoint clamp onto DSB, affects activation of the Mec1-dependent pathway, and slows down the kinetics of DSB resection promoting ectopic recombination with short homologous donor sequences [79]. In fission yeast, Crb2, the putative Rad9 ortholog, is phosphorylated by CDK1 and this modification is important to mediate later steps of HDR-mediated DSB repair implicating the RecQ helicase Rqh1 and the Top3 topoisomerase [80].

Studies in budding yeast established that checkpoint kinases phosphorylate and regulate the recombination factors Srs2, Rad55 and Slx4 [6,81,82], suggesting that the checkpoint pathway may play additional roles in regulating DSB repair at later steps after ends resection. Srs2 is a DNA helicase/translocase that by removing Rad51 from DNA influences various steps of the recombination process. In fact, Srs2 warrants the formation of adequate amount of the Rad51 nucleoprotein filament. This Srs2-dependent regulation is crucial for the outcome of DSB repair as a gene conversion or crossing over event [55,83–85]. Whether checkpoint-dependent phosphorylation of Srs2 influences its role in recombination is still unknown; however, it was proposed that Srs2 might be involved in removing checkpoint factors from DSBs after repair [55], suggesting that checkpoint-dependent Srs2 phosphorylation might be required to resume cell cycle progression (the recovery step). In yeast cells, the recombination protein Rad55, a Rad51 paralog, is targeted by Mec1 kinase upon DNA damage; data obtained studying point mutations removing the phosphorylation sites suggest a role for such modification in activating recombinational repair [6,86]. Tel1 and Mec1, through damage-dependent phosphorylation of Slx4, also control the single-strand annealing (SSA) sub-pathway of DSB repair [81].

Finally, checkpoint kinases feed back onto the factors that were responsible for their activation. It has been shown that in budding yeast DSB resection, is regulated by the checkpoint factor Rad9; its binding to chromatin, mediated by methylated histone H3-K79 (H3-K79me), is important for checkpoint activation but also for restraining nucleolytic processing of the DSBs. Loss of Rad9 binding to H3-K79me leads to increased resection activity and partially bypasses the requirement for CDK activation of DSB processing [87]. These data suggest that removal of Rad9 from methylated histone may be part of the mechanism through which the checkpoint response regulates processing of damaged DNA.

3. UV-induced lesions

As discussed in the previous section, the response to DSBs requires the formation of DNA intermediates containing ssDNA, which seem to be the major signal activating the DNA damage checkpoint. On the other hand, for different types of DNA lesions, the nature of the structures acting as the signal activating checkpoint is still poorly defined.

Ultraviolet light induces the formation of photoproducts, mainly cyclobutane pyrimidine dimers and 6-4 pyrimidine–pyrimidone photoproducts. The presence of these lesions in DNA represents physical obstacles for replication and transcription, greatly affecting DNA metabolic processes. Such lesions are removed by direct reversal, through the activity of photolyases, and by nucleotide excision repair (NER), a versatile pathway and in mammals the only mechanism that repairs DNA damage due to UV- and chemical-induced bulky lesions. NER requires the function of over 30 factors and results in the excision of the DNA region containing the lesion, generating a short ssDNA gap (approximately 30 nt), which is then refilled by DNA polymerases.

3.1. The cell cycle modulates activation of the UV checkpoint response

Recent findings in yeast and in human cells revealed that activation of G1 and G2 DNA damage checkpoints following UV irradiation of non cycling cells requires NER-dependent processing of the lesions. On the other hand, if cells are cycling and thus replicating their genome, then NER activity is not required to trigger the checkpoint response. This may be due to replication fork stalling when it encounters a UV lesion, leading to exposure of ssDNA regions [88–90]. If NER is not sufficient, due to mutations affecting recognition of the lesions or incision of the damaged strand, activation of the checkpoint kinase Rad53 in G1 and G2 is incomplete and noticeably delayed. Moreover, this residual Rad53 phosphorylation is not functional to prevent the G1-S transition and it is likely due to DNA fragmentation occurring when NER is not functional [88,91,92]. Studying cells derived from Xeroderma pigmentosum and Cockayne syndrome patients, it has been shown that NER processing is required for G1 and G2 checkpoint activation also in human primary fibroblasts [89,93–95]. Interestingly, while in yeast cells either the global genome repair (GG-NER) or the transcription-coupled repair (TC-NER) sub-branches of NER is sufficient to generate the checkpoint signal [88], in human cells activation of the checkpoint requires a functional GG-NER [89]. This difference in the requirement of the GGR-NER and TC-NER sub-branches between yeast and human cells may be ascribed to the different fractions of genome that is transcribed in these organisms.

One important question is whether the short gaps produced by NER enzymes are sufficient to activate the checkpoint kinases, or they are converted into other structures (i.e. long ssDNA regions) leading to activation of the DNA damage response. It has been suggested that if the UV dosage is low (5 J/m^2) the UV-induced checkpoint is not immediately activated, but the DNA damage response is triggered only after DNA replication has occurred [96]. This effect could be ascribed to DNA lesions that persist through G1 so that cells enter S-phase in the presence of such alterations, which are then detected by the replication apparatus. Indeed, passage of the replication forks on UV-damaged DNA leads to the creation of long ssDNA gaps [97] that likely lead to checkpoint activation following DNA replication. Such question has been recently discussed in an intriguing study of the effect of chronic low UV doses exposure (CLUV) on yeast cells. This paper shows that in CLUV conditions cell survival is guaranteed by post-replication repair, and that NER and checkpoint factors play only a marginal role [98]. While interesting, it is difficult to reconcile these results with the fact that Xeroderma pigmentosum patients, defective in NER, exhibit an extreme sunlight sensitive phenotype, raising the question of how the CLUV results obtained in yeast may be applicable to other systems.

3.2. Processing of UV lesions signals to checkpoint kinases

On the other hand, exposure of eukaryotic cells to a higher UVC dose leads to a rapid replication-independent checkpoint activation in G1, G2 or M-phase depending on the model organism used and on the cell cycle phase at which UV irradiation is delivered [88,89,99]. At high UV doses NER may generate DNA structures that are detected by the checkpoint sensors. This could happen, for example, if the DNA repair capacity of the cell becomes limiting at some sites and other enzymatic activities (such as exonucleases, endonucleases or helicases) may gain access to the damaged region producing larger ssDNA stretches, capable to activate the checkpoint (Fig. 2). In budding yeast, Exo1 has a role in the activation of the UV-induced G2 checkpoint [100]; (Giannattasio et al., in prepa-

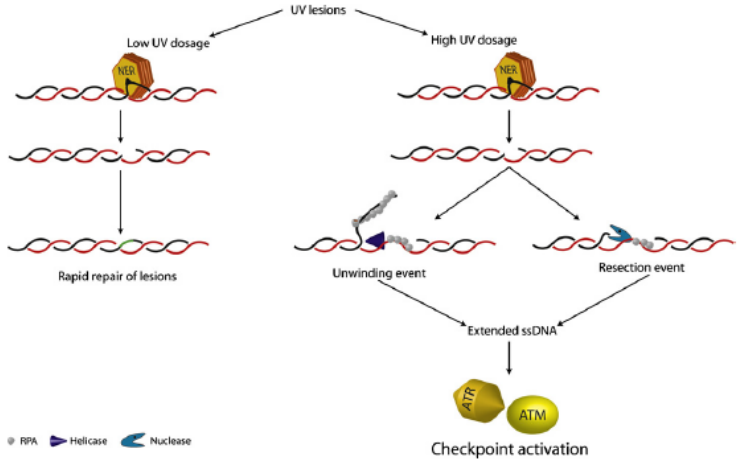

Fig. 2. In non-cycling cells, UV lesions lead to checkpoint activation only after being processed by NER and nucleolytic activities. At low doses of UV light, non-cycling cells do not activate the checkpoint response. The lesions are rapidly removed by NER. At elevated UV doses, the level of lesions is too high and somehow the repair system may become limiting; this allows further processing of damaged DNA by nuclease/helicase enzymes. The resulting ssDNA region could be the actual signal triggering the checkpoint cascade. If cells are cycling, replication forks will stall in front of any lesion that is still present during S-phase. The uncoupling between the stalled replicative polymerases and the helicase leads to the formation of a long ssDNA region, which activates the checkpoint response.

ration), and in human cells the Nbs1 subunit of the MRN complex is also required for the G2 arrest after UV irradiation [95]. While the idea that nucleases may contribute to checkpoint activation after UV damage especially in non-cycling conditions is intriguing, the substrates or structures recognized by such nucleases and their physical and functional crosstalk with the NER machinery are unknown. The hypothesis that DNA repair stalling at some UV-damaged sites may correlate with checkpoint activation is in agreement with recent findings. Matsumoto et al. have observed hyper-activation of ATR (measured as hyper-phosphorylation of histone H2AX) in non-cycling human fibroblasts where, after UV irradiation, NER-dependent DNA repair synthesis is blocked with the cytosine analog AraC [101]. The authors of this work proposed a model in which perturbed gap-filling synthesis during NER leads to the exposition of ssDNA gaps that could contribute to hyper-activation of the ATR kinase. The alternative explanation for the rapid G1 and G2/M checkpoint activation observed at high UV doses assumes the possible production of DSBs in these conditions (reviewed in [99]). Generation of DSBs in UV-irradiated cycling cells has been known for a long time, and is dependent upon DNA replication [102]. On the other hand, clear data indicate that, in non-cycling conditions and in the presence of a functional NER, exposition of human fibroblasts to UVC light does not lead to DSBs formation [94].

In the future, it will be important to verify the existence of extended ssDNA gaps generated after UV irradiation in non-cycling cells. During S-phase, the UV-induced ssDNA regions exist, as shown by electron microscopy observations [97], and could originate from the lesion-induced uncoupling of leading and lagging strand DNA synthesis. These ssDNA gaps are also likely responsible for the observed UV-induced sister chromatid recombination [103].

3.3. Checkpoint pathways influence repair of UV lesions

Besides a role for DNA repair in activating the response to UV irradiation, several observations suggest an effect of checkpoint pathways in regulating DNA repair of UV-induced lesions. In budding yeast, the checkpoint genes *RAD9* and *RAD24* have been reported to be required for a proper DNA damage-dependent induction of NER [104]. A more direct functional connection between checkpoints and regulation of NER comes from the observation that XPA, a fundamental NER factor, co-localizes with ATR, requires ATR for nuclear translocation and is phosphorylated on serine 196 in an ATR-dependent manner after UV treatment. The significance of these events is not yet clear, since XPA becomes phosphorylated at late time-points after irradiation. Nonetheless, substitution of this serine residue with alanine causes UV sensitivity, suggesting that such ATR-dependent modification may have a relevant role in the control of the NER reaction, in the removal of persisting lesions or in a feedback regulative loop on the repair process [105,106].

Similarly to what has been shown in response to DSBs, recent reports demonstrate that after UV treatment histones undergo post-translational modifications in a manner that is DNA repair- and checkpoint-dependent. In particular, in the regions containing the damage, histone H2A is mono-ubiquitylated after UV irradiation, and this modification requires NER, ATR and the Ring2 ubiquitin ligase, while it is independent upon phosphorylation of the H2AX variant [107]. A different ubiquitin ligase, DDB1–CULLIN4–DDB2, was also reported to target H2A after UV irradiation, and this is supposed to facilitate initiation of NER [108,109]. The observation that different ubiquitin ligase complexes can target histone H2A is intriguing; while DDB2 participates to NER, in fact XP patients belonging to XP-E complementation group have mutations in DDB2 [108], the ubiquitylation mediated by Ring2 seems to occur after incision of the UV-damaged strand [107]. The analysis of histone mutants lacking these ubiquitylations will help to shed light on their functions and regulation; unfortunately, the particular H2A residues ubiquitylated in response to UV treatment are still not known. Damage-induced H2A ubiquitylation may remodel the chromatin structure in the proximity of the lesions, thus facilitating recognition of the UV photoproducts by the NER machinery or may more directly influence the NER reaction. Histones H3 and H4 also undergo mono-ubiquitylation in response to UV light; this modification requires CULLIN4–DDB1–ROC1 [110], but the relationships among all these ubiquitylation events and their influence on repair of UV-induced lesions is not yet clear. In response to DSBs both histone H2A and the H2AX variant are mono-ubiquitylated by RNF8, which is recruited by phospho-MDC1 [65]. Such nucleosome modification around the lesion would induce the chromatin flanking the DSB to concentrate essential DNA repair factors and facilitate fixing of the lesion. A similar model may also explain the relevance of the ATR-dependent H2A mono-ubiquitylation in response to UV irradiation, described above. The scenario that is emerging is that after UV irradiation different nucleosome components are targeted by various ubiquitin ligases and these modifications may play a role in the crosstalk between NER and the checkpoint response and/or may be more directly connected to the first events of the NER reaction.

In conclusion, two major questions need to be answered to better understand the connections between checkpoint activation and repair of UV lesions. The first issue is related to the signal required to activate the checkpoint: at least in non-cycling conditions and at high UV doses, NER-dependent lesion processing seems to be required both in yeast and mammalian cells. However, the level of ssDNA generated during the NER reaction is limited and most of the ssDNA stretches generated in the course of the repair reaction may never be effectively exposed. It is possible that, over a certain threshold of UV dose or if DNA repair synthesis stalls at some damaged sites, some nucleases may act in conjunction with NER generating larger ssDNA regions and activating the checkpoint. Further investigations are also required to understand whether the DNA damage checkpoint regulates removal of UV lesions in a specific chromatin contest. In this direction a better understanding of the role of the checkpoint in influencing histone dynamics and modifications would be very informative.

4. Base alterations

Base excision repair (BER) is involved in the removal of a variety of endogenous and exogenous DNA lesions, and it requires the activity of more than 20 proteins to repair mostly alkylated, deaminated and oxidized bases. Indeed, mutations in BER genes are associated with aging, cancer susceptibility and neurodegeneration. In the BER pathway, the damaged base is initially removed by a specific DNA glycosylase, which leaves behind an abasic site. A successive recruitment of an AP endonuclease will incise the damaged strand leaving a 3-OH and a 5′-deoxyribose phosphate group (5′-dRP). In order to refill and close the gap, the 5′-dRP moiety needs to be removed by the dRP lyase activity of DNA polymerase β, which also synthesizes DNA through the gap from the 3′-OH end. Finally DNA ligase will seal the nick, completing the process.

Largely because of the broad spectrum of lesions repaired by BER and the partial overlap with other DNA repair system, it is not yet clear what are the functional connections between BER and DNA damage checkpoint activation. A recent paper, addressing this problem, analyzes the cellular response to MMS in cycling and in nocodazole arrested mammalian cells and suggests that initial processing of lesions by a glycosylase encoded by *MPG* may be required for the activation of the ATM–CHK2 pathway by MMS [111]. On the other hand, many studies reported physical interactions between the 9-1-1 checkpoint clamp and BER factors. These include proteins involved in the initial steps of BER, such as the DNA glycosylase MutY in fission yeast, and thymine DNA glycosylase and

AP endonuclease I in human cells [112–114]. These interactions may lead to a stimulation of lesion processing activities, but may also be seen as a way to recruit checkpoint factors at the damage sites [115]. Moreover, the 9-1-1 complex also interacts and stimulates the activity of a number of other BER proteins, such as: human DNA polymerase β, the major polymerase involved in BER [116]; human FEN1 endonuclease, a key nuclease required for long-patch BER and for the removal of RNA primers in DNA replication [117]; DNA ligase I, who seals the final product of the long patch BER reaction [115,118,119]. This last interaction is promoted by DNA damage and influences ligase activity both by increasing its affinity for the nicked DNA substrate, and by facilitating the sequential action of FEN1 and DNA ligase I [119]. It is clear from these data that the checkpoint clamp interacts with proteins acting in almost every step of the long patch-BER pathway, positively regulating each passage. It is quite intriguing that many of these BER factors interact also with PCNA. One possible interpretation is that PCNA is very efficient during S phase, stimulating both the replication and repair activities of FEN1 and DNA ligase I. Once cells enter the G2-M phase, PCNA may be replaced by the 9-1-1 clamp, which is active for the repair functions of these enzymes [119]. This model is supported by the finding that caseine kinase II suppresses ligase I activity in G2-M by phosphorylation; this form of the enzyme cannot be enhanced by PCNA, but is still activated by the 9-1-1 complex [120]. Moreover, as a result of the cellular response to DNA damage, the PCNA inhibiting factor p21 is induced; this may cause inhibition of DNA replication, but efficient repair processes may be supported by the stimulatory effect of the checkpoint clamp [121].

Recent evidence suggested yet another involvement of the checkpoint pathway in BER. The CHK2 kinase was shown to interact in vitro and in vivo with XRCC1, a scaffold protein that interacts with each BER component and is required for efficient BER [122,123]. In fact, activation of the ATM–CHK2 pathway by base alkylation and oxidative damage was shown to result in CHK2-dependent phosphorylation of XRCC1 on threonine 284. Interestingly a phospho-mimic mutant displays an increased affinity for glycosylases, and cells expressing a T284A XRCC1 mutant were significantly less effective in BER, compared to wild type cells [111]. These observations suggest that the checkpoint response may modulate the repair capacity of the cell through regulated interactions with BER factors and by their post-translational modification.

5. Mispairings

Mismatch repair (MMR) is a specialized mechanism that targets base substitution mismatches and insertion–deletion mismatches resulting from errors occurred during normal DNA replication and escaped from the proofreading activity of DNA polymerases, an event happening with a frequency of about 1 in 10^9–10^{10} base pairs per cell division [124]. Nucleotide mispairing can also arise as a consequence of exposure to exogenous agents or endogenous reactive chemical species produced by the cellular metabolism that may cause base modifications [125]. Loss of MMR leads to a mutator phenotype, which causes cancer predisposition and also affects DNA damage signaling, recombination, and several other DNA metabolic processes [126]. The functional relevance of MMR is emphasized by the consideration that in cells with a non-functional MMR, the spontaneous mutation rate is increased, particularly in repeated sequence elements, with characteristic microsatellite instability [127–129]. Finally, mutations in genes coding for MMR factors are associated with hereditary non-polyposis colon cancer (HNPCC) that represent about 1–5% of all cases of colon cancer [130].

The MMR system has been highly conserved throughout evolution and this has allowed the use of simple organisms, such as bacteria and yeasts, to define the molecular details of this process. In *Escherichia coli*, where the process is best understood, MutS binds the mismatched region of DNA and recruits MutL; the formation of this complex, which requires the expense of ATP, activates a latent endonucleolytic activity of MutH. This enzyme is bound to hemimethylated GATC sites and cleaves, in the area containing the mismatch, the newly synthesized DNA strand which is still unmethylated. UvrD helicase is loaded at the nick and together with RPA generates a ssDNA filament containing the mismatched base, which is then digested by nuclease activities. Finally, DNA polymerase III refills the gap faithfully and DNA ligase III seals the last nick [131]. Eukaryotic MMR, although similar, is more complicated due to the presence of distinct partially redundant MutS homologues (MSH), which recognize different types of mismatches, and different MutL homologues (MLH). Moreover, there is no known MutH protein in eukaryotic cells; this leaves the problem to find an entry point for the strand excision activities. The solution seems to rest on using nicks or gaps left behind by the progressing replication forks, which may explain the relevant role of PCNA in MMR [126,131].

Several studies have shown that genes coding for MMR factors are involved in cell cycle arrest after treatment with DNA alkylating agents [132–136]. hMSH2 has been reported to physically interact with ATR and CHK2, while hMLH1 has been shown to interact with ATM [137–139]. Moreover, after treating cells with alkylating agents, proper phosphorylation of p53 on Ser15 and Ser392, and phosphorylation of CHK1 and CHK2 require the function of hMutSα and hMutLα [137,133,136,140]. These observations indicate that this repair system is required for checkpoint activation in response to DNA alkylation damage.

The simplest model assumes that repair proteins are required to localize checkpoint factors at the lesions, generating a high local concentration of apical kinases and downstream checkpoint factors [140–142]. In a second model the simple recognition of mispairs is not sufficient to activate the DNA damage response, but processing of the lesions is required [143,136,144]. In agreement with this hypothesis is the observation that, after treatment with S_N1 DNA methylating agents, cell cycle arrest at the G2/M transition is observed only in the second cell cycle [136]; this observation remained unexplained for several years. It was initially shown that when MMR attempts to repair an O6-methyl guanine (MeG)-T mispair, if the incision affects the DNA strand opposite to the lesion (containing the T), the MeG is not removed, thus the mismatch is not repaired and it is subject to re-excision [144]. This observation found strong support from the work of Mojas et al. who elegantly showed that after treating cells with S_N1 DNA methylating agents in G1 or early S-phase, MMR is unable to take care of the mispairs because the modified nucleotides are in the template strand and MMR only targets the newly synthesized DNA filament. This futile attempt to repair causes the formation of short ssDNA gaps, which are insufficient to activate the DNA damage response. Only after a second transit through S-phase these gaps lead to replication fork collapse, triggering the checkpoint response [143] (Fig. 3).

Moreover, the mechanism of checkpoint activation in response to S_N1 methylators is also influenced by the dosage of the drug. In fact, high concentrations of methylating agents lead to an MMR-dependent cell cycle arrest immediately after the first S-phase [145,146]. In this case, the large number of MMR-processed regions during first cell cycle may be sufficient to activate the DNA damage checkpoint or may be further processed by the action of nucleases as proposed in a previous section.

The complexity of the role of MMR in checkpoint activation is underlined by the existence of specific mutants of MSH2 and MSH6, which cause defects in MMR but are proficient in DNA damage signaling [142,141]. These separation of function alleles strongly indicate that MMR proteins participate to checkpoint activation also through another pathway, which is distinct from the processing-mediated activation.

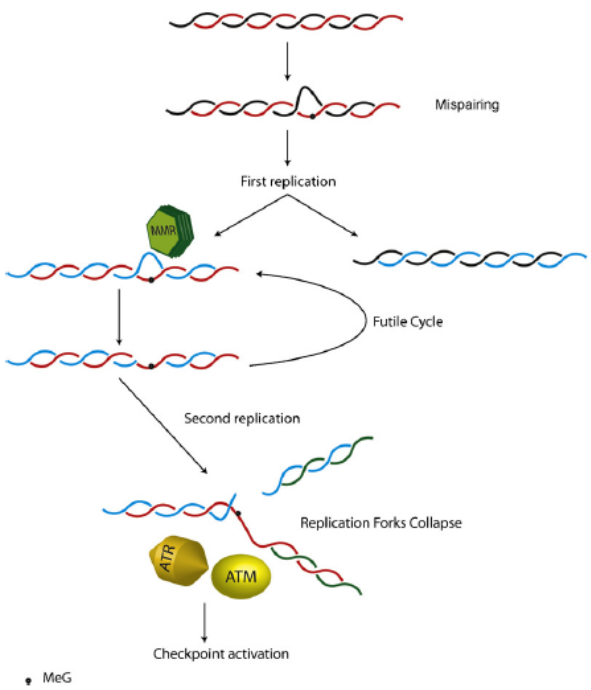

Fig. 3. MMR attempts to remove a mispair where the mutated base is in the template strand, results in checkpoint activation. Treatment with SN1 methylating agents modify bases, generating mispairs that upon replication are recognized by MMR. If the lesion is on the parental strand (red), MMR will attempt to remove the mispair by correcting the newly synthesized strand (blue), which does not contain the MeG, and the mispair will be maintained. This will cause a series of futile MMR cycles to start, leading to the production of a gapped molecule that will cause replication fork collapse and checkpoint activation, during the second round of DNA replication.

It will be important to better understand the connections between MMR and the DNA damage response because this process is likely relevant for cancer development and for cancer treatment, since MMR deficiencies are responsible for increased resistance to S_N1 methylating agents that are commonly used in therapy.

6. Crosstalk between translesion synthesis and DNA damage checkpoint

When the mechanisms discussed above do not efficiently repair a lesion in DNA, cycling cells enter S-phase with a damaged genome. This frequently causes stalling of the replication fork in the area of the lesion, which cannot be used as template by replicative DNA polymerases. In this case, the lesion threatens the capacity of the cell to complete the cell cycle, and cells adopt a set of non-repair strategies known as DNA-damage tolerance pathways or post-replication repair (PRR) that allow completion of replication, leaving to the repair mechanisms the possibility to remove the offending lesion at some other cell cycle stage. PRR comes in two flavors: an error-free pathway, which is based on a recombination-like mechanism and a temporary switching of the replication machinery to the undamaged sister chromatid, and an error-prone pathway, which uses translesion DNA polymerases to copy the filament containing the damage. Translesion DNA synthesis (TLS) can replicate faithfully or be mutagenic, depending on the nature of the lesion and on which TLS polymerase is used [147]. The choice between these pathways seems to depend on the modification state of the replicative clamp PCNA, which can be mono-ubiquitylated, poly-ubiquitylated and sumoylated [148]. Mono-ubiquitylation of PCNA, mediated by Rad6 and Rad18, leads to TLS, while its poly-ubiquitylation and sumoylation stimulate the error-free pathway [148–150].

Replication fork blockage is thought to result in the accumulation of ssDNA between the stalled polymerase and the uncoupled MCM helicase activity [151] and this molecular intermediate is likely responsible for the activation of the DNA damage checkpoint [42] (Fig. 4).

On the other hand, recent findings implicate the TLS machinery in modulating the signal that activates the checkpoint, but the molecular mechanisms underlying this action are not yet clear. It was recently found in budding yeast that the Rad6–Rad18 complex, which has a well established role in TLS by mono-ubiquitylating PCNA, is also involved in the ubiquitylation of the Rad17 subunit of the 9-1-1 checkpoint clamp and this modification is required for the induction of the transcriptional response to MMS treatment and it is also partially required for the activation of the Chk2 homolog Rad53 [152]. A more direct connection between TLS and checkpoints derive from the recent observation that XP-V cells, which are mutated in POLH, the gene coding for DNA polymerase η (Polη), display an enhanced ATR signaling after UV irradiation [90], possibly because a decreased capacity in UV-lesion bypass may lead to increased fork stalling and accumulation of checkpoint signal (Fig. 4). On the other hand, the situation is complicated by data suggesting that down regulation of POLH causes a defective phosphorylation of Chk2 and p53 by ATM, when human fibroblasts are exposed to IR or camptothecin; however no effect was detected on ATM autophosphorylation or histone H2AX modification, suggesting that Polη may also play a role in allowing proper signal

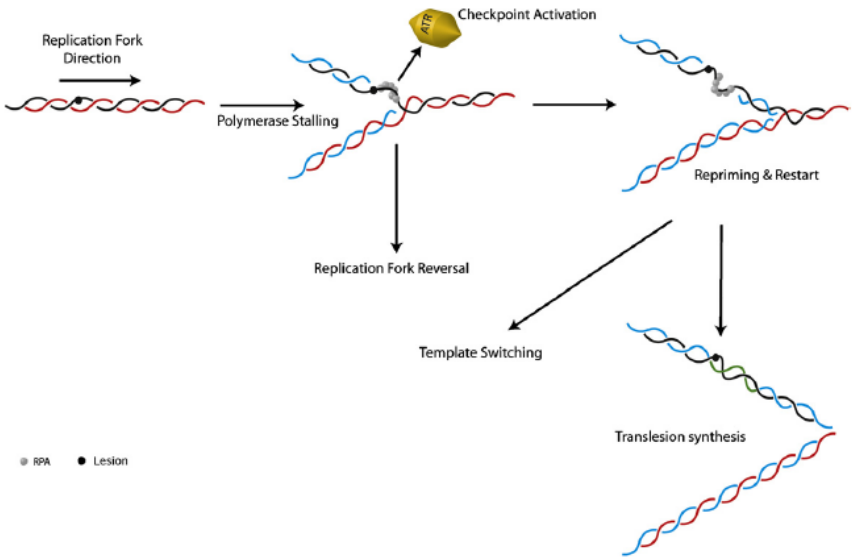

Fig. 4. Replication of a damaged template. If lesions are allowed to persist into S-phase, they can act as obstacles for the progressing replication fork, which will stall at the lesion. Uncoupling between the polymerase and the helicase leads to formation of long RPA-covered ssDNA regions, which trigger checkpoint kinase activation. DNA replication can be completed thanks to a damage avoidance pathway (PRR) which can go through a fork reversal, a template switching or a translesion (TLS) mechanism. Inhibition of TLS leads to increased checkpoint activation, likely due to the accumulation of ssDNA regions.

transduction downstream of ATM, without affecting ATM activation [153].

A large amount of data support a role for DNA damage checkpoint mechanisms in regulating translesion synthesis. The first demonstration that a PI-3 kinase pathway was involved in the modulation of TLS in human cells came from the study of DNA replication in XP-V fibroblasts: in the absence of the relatively accurate Polη, the production of high molecular weight DNA in S-phase after UV [159] or cisplatin [160] treatments is abolished by the PI-3 kinase inhibitor caffeine. This observation can be explained invoking a direct effect of checkpoint kinases on the translesion process performed by other polymerases, such as Polζ, or it may be due to the loss of stabilization of stalled replication forks resulting form checkpoint inhibition [161]. Another suggestion along these lines is the fact that, in mammalian cells, the accumulation of hRad18 at replication forks after MMS is abrogated by wortmannin, another PI-3 kinase inhibitor [162].

Why are PI-3 kinases so important for the TLS pathway? One possibility is that when the replication machinery encounters an irreparable damage, PI-3 kinases stimulate the activation of translesion synthesis, allowing the cell to cope with the damage. In this direction, it has been recently demonstrated that the kinase activity of Mec1, the budding yeast ATR homolog, is required for the association of Polζ–Rev1 complex with sites near DSBs [163]. Since Rev1 has been shown to be phosphorylated in response to DNA damage by Mec1 [164], a possible explanation is that a Mec1-directed phosphorylation of Rev1 may be necessary for the loading of Polζ onto damaged chromatin. In fission yeast, a clear role for the upstream kinase Rad3 (equivalent to ATR) in PRR has been established: Rad3 phosphorylates the Rad9 subunit of the 9-1-1 checkpoint clamp at Thr225 and this event channels the DNA repair mode into the error-free branch of the Rad6 repair pathway [165]. For this activity is crucial the damage-dependent physical interaction of Rad9 phospho-T225 with the Mms2 protein which, in complex with Ubc13 and Rad5, participates in the polyubiquitylation of PCNA, in the error-free pathway [166,148]. Moreover, the involvement of checkpoint genes in replication of irreparable DNA damage is supported also by the finding that, in NER defective yeast cells, the accumulation of UV-induced, Polζ-mediated, mutations is almost completely dependent on the presence of an intact checkpoint cascade [154,155]. Further studies in fission yeast revealed that a mutation affecting the binding of the RFC-like complex to chromatin (*rad17-K118E*) [156] reduces the 9-1-1 clamp loading efficiency and abrogates the MMS-induced recruitment of Polκ to chromatin, leading to a drastic reduction in Polκ-dependent TLS activity [157]. A prominent role for the checkpoint clamp in regulating translesion synthesis is supported by the finding of physical and functional interactions between this complex and translesion DNA polymerases. In budding yeast it has been shown that, in a NER defective background, the checkpoint clamp regulates spontaneous Polζ-dependent mutagenesis through a physical interaction between the polymerase and the Mec3 and Ddc1 subunits of the 9-1-1 clamp [158]. Therefore, beside its checkpoint signaling role, the 9-1-1 clamp is an important player in the post replication repair pathway.

A clear evidence of the role of the downstream checkpoint kinase Chk1, in the activation of the TLS repair pathway has been recently reported in higher eukaryotes. Cells expressing a kinase-inactive, dominant negative, form of Chk1 display a reduction in PCNA mono-ubiquitylation and in the amount of Polκ associated PCNA in damaged cells, suggesting that the ATR/Chk1 signaling is directly involved in the recruitment of Polκ and in promoting TLS in response to DNA damage [167]. But things are often not as easy as they appear and the underlying mechanisms are still elusive: recent findings seem to indicate that checkpoint activation may also negatively regulate translesion synthesis. Strains lacking the histone metyltransferase Dot1 are partially defective in the intra-S checkpoint induced by MMS [168] but are unexpectedly more

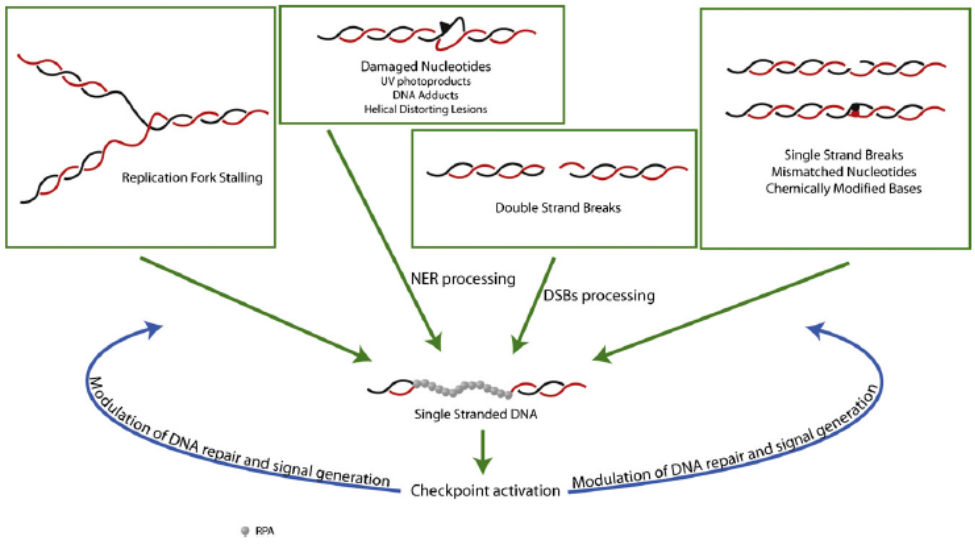

Fig. 5. The DNA damage checkpoint response is activated by DNA repair intermediates. Cells respond to an ample variety of genomic lesions by triggering a common signal transduction cascade. This is achieved by exploiting the repair mechanisms that are specific for the different lesions. Recognition and initial processing of the damaged DNA brings about the formation of a common intermediate, namely ssDNA regions, which are covered by RPA and represent the signal that activates the checkpoint response. Checkpoint factors functionally and physically interact with repair machineries to regulate repair of the DNA lesions and modulate the level of the ssDNA structure, keeping a balanced integration between damage, repair and cell cycle progression.

MMS resistant than wild-type cells and display an increased level of MMS-induced mutagenesis. Both these phenotypes are dependent upon Polζ and are not due to the lack of silencing caused by *DOT1* deletion, but can reasonably be ascribed to a reduced DNA damage checkpoint activity [169].

All these evidences support a close interdependency between checkpoint and TLS. A better understanding of this crosstalk is auspicable, since it seems to overturn the classical view of the relationship between DNA damage checkpoint and mutagenesis. It has been shown, that in *S. cerevisiae* DNA damage causes an increase in dNTPs pool and in mutation rates [170]. Loss of checkpoint functionality prevents the induction of ribonuclease reductase activity and leads to a reduction in mutation rates [170,155]. Since TLS polymerases require more than 10 times higher concentration of dNTPs compared with a replicative polymerase [171], it is reasonable to speculate that this mutator phenotype is due to the high dNTPs concentration, which may stimulate the TLS pathway, suggesting that checkpoint activation contributes to the generation of mutations, rather than to its prevention.

7. Concluding remarks

DNA damage checkpoints are mechanisms that allow cells to respond to critical situations such as an acute exposure to elevated doses of genotoxic agents or to cope with DNA lesions that cannot be immediately repaired. This task is executed by activating a variety of strategies: preventing cell cycle progression, cells avoid replication/segregation of damaged chromosomes; controlling replication efficiency cells ensure the completion of duplication of the entire genome and prevent the generation of secondary dangerous lesions; modifying the transcriptional program and acting on repair/chromatin proteins, checkpoint kinases potentiate repair reactions and channel the lesions into the most appropriate repair process, according to the cell cycle stage of the cell. This response is common to a whole variety of chemically different lesions, and its efficiency is at least partially due to the hijacking of specialized modules (namely the repair system specific for that kind of lesion) to generate a common intermediate structure (most likely ssDNA), which will be the actual signal for triggering the checkpoint cascade (Fig. 5). Once active, the checkpoint mechanism may modulate both the repair systems and further generation of the ssDNA signal, integrating damage formation with repair reactions, to allow an efficient removal of the lesions and an effective resumption of proliferation.

Conflict of interest

The authors declare that there are no conflicts of interest.

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PART III -

GENERAL

DISCUSSION

DPB11: A NEW PLAYER IN THE DNA DAMAGE CHECKPOINT

DNA damage checkpoints represent an important component of the cellular response to DNA damage, since they guarantee a constant surveillance of the state of the genome and, in case a lesion is present, they activate and regulate the appropriate biological response, including DNA repair, a transient cell cycle arrest and a change in the transcriptional programme of the cell (Harrison and Haber, 2006). Defects in these mechanisms lead to increased genomic instability, cancer susceptibility, ageing and several human pathologies (Lazzaro et al., 2009).

DNA damage checkpoints are organized as signal transduction cascades, whose players have been conserved throughout the evolution (Harrison and Haber, 2006). These pathways are orchestrated by the activity of phosphatidylinositol-like kinases (PIKKs), namely Mec1 and Tel1 in budding yeast, and ATM and ATR in higher eukaryotes. Once activated, PIKKs phosphorylate different targets, allowing transmission of the signal from the "sensor" proteins, to the "effector " checkpoint kinases Rad53 and Chk1in budding yeast and Chk2 and Chk1 in mammalian cells, which are able to activate the cellular responses to DNA damage.(Harrison and Haber, 2006). The exact order of function of the players in the signal transduction cascade has been defined by monitoring their phosphorylation status.

Budding yeast *RAD9* was the first checkpoint gene to be identified in a pioneering study performed by Hartwell and colleagues (Weinert and Hartwell, 1988). It is classified as an "adaptor" checkpoint protein, being responsible of the transmission of the signal from the apical PIKKs to the kinases Rad53 and Chk1 (Weinert and Hartwell, 1988; Gilbert et al., 2001; Blankley and Lydall, 2004; Sweeney et al., 2005). In particular, Rad9 recruits and catalyzes the activation of Rad53, functioning as a scaffold protein bringing Rad53 molecules in close proximity, thus facilitating the Rad53 autophosphorylation reaction, essential for the checkpoint activation (Vialard et al., 1998). Notwithstanding its importance in the checkpoint cascade, the mechanism responsible for Rad9 and Mec1 recruitment to the proximity of the lesion remained unknown for a long time.

Evidence collected in recent years suggested that histone modifications are important actors in the Rad9 and its orthologues loading onto DNA. Indeed, it has been demonstrated that Rad9 can bind histone H3 lysine 79 methylated by Dot1, accommodating the methylated residue in the binding pocket of its tandem Tudor domain (Giannattasio et al., 2005; Toh et al., 2006; Grenon et al., 2007; Hammet et al., 2007). Consistently, in the absence of H3-K79 methylation or if the Rad9 Tudor domain is mutated, yeast cells in G1 do not exhibit Rad9 loading onto DNA and are

deficient in transmitting the checkpoint signal from Mec1 to Rad53 (Wysocki et al., 2005; , Giannattasio et al., 2005; Hammet et al., 2007). A similar pathway has been described also in fission yeast and in higher eukaryotes, highlighting the importance of this "histone-depenent" branch in the recruitment of adaptor proteins (Sanders et al., 2004; Huyen et al., 2004; Botuyan et al., 2006; Du et al., 2006).

Surprisingly, histone H3 methylation is only partially required for an effective checkpoint activation in the G2/M phase of the cell cycle. G2/M-arrested *dot1Δ* cells normally delay the nuclear division after UV irradiation and display a significant Rad53 phosphorylation after treatment with UV or with the double-strand break inducing agent zeocin (Giannattasio et al., 2005). This observation suggested, therefore, that in this specific phase of the cell cycle, a different mechanism of Rad9 recruitment to damaged chromatin and to Mec1 kinase must exist and can compensate for the loss of the histone H3 methylation. To define the nature of this second pathway we looked for deletion mutants that, when combined with the *DOT1* deletion, completely turn off the G2/M checkpoint signal, that is the Rad53 phosphorylation after UV treatment.

With this analysis, we concluded that the residual phosphorylation of Rad9 and Rad53 observed in *dot1Δ* nocodazole-arrested cells was not due to an unscheduled activation Tel1-dependent or Chk1-dependent pathway, whereas it was still dependent upon Mec1. In fact, deletion of *TEL1* or *CHK1* did not affect the residual Rad53 phosphorylation observed in the absence of histone H3 methylation, which was instead abolished in a *mec1-1* background. (**Puddu et al., 2008 – Fig. S1A**). Then we tested whether other histone residues could be used as Rad9 docking sites in the absence of H3K79 methylation. However, nor H4K59, which is in close proximity to H3K9, neither H4K20, that represent the recruitment site for the fission yeast-Rad9 orthologue Crb2, seemed to be redundant with lysine 79 methylation. When mutations in these histone aminoacid were combined with *dot1Δ*, we could not detect any synthetic effects on G2/M checkpoint activation (**Puddu et al., 2008 - Fig S1C**). A similar negative result was achieved when we delete, in the *dot1Δ* background, the *SET1* or *SET2* histone methyltransferase gene, responsible for the methylation of H3K4 and H3K36, respectively (**Puddu et al., 2008 – Fig. S1B**).

The function of Rad9 in the G1 DNA damage checkpoint is dependent upon the presence of both H3K79me and phosphorylation of H2AS129. It has been suggested that these two histone modifications constitutes two different, but interdependent pathway for Rad9 recruitment to damaged chromatin (Javaheri et al., 2006; Hammet et al., 2007). We therefore decided to test also the contribution of histone H2A phosphorylation in G2/M, combining an H2A mutant in which S129 cannot be phosphorylated because mutated to alanine, with the *DOT1* deletion. A S129A mutation not only does not further reduce the Rad9 or Rad53 phosphorylation observed in the absence of Dot1, but, surprisingly, it seems also to rescue the mild Rad53 phosphorylation defect

exhibited by the *dot1Δ* strain (**Puddu et al., 2008 – Fig. S1D**). A similar results was observed also after the treatment of zeocin (**Granata et al., 2010 – Fig. 7** (discussed below).

Fission yeast Crb2 forms damage foci after IR treatment and at a single persistent DSB. The focal accumulation of this protein, necessary for the activation of the checkpoint response, was shown to require the physical interaction of Crb2 with the H4-K20 methylated, phosphorylation of H2A C-terminus and a functional Cut5/Rad4 protein (Furuya et al., 2004; Du et al., 2006). We analyzed whether Dpb11, the budding yeast orthologue of Cut5/Rad4, might be analogously involved in recruiting Rad9 to the proximity of Mec1, allowing its phosphorylation and, therefore, Rad53 activation in G2/M. We combined a *DOT1* deletion with the temperature sensitive *dpb11-1* mutation – which encodes for a truncated protein, lacking the last 182 aminoacids - and analyzed the G2/M checkpoint response and cellular survival both after UV irradiation and zeocin treatment. In both situations, the *dpb11-1* mutation on its own had no significant effects on cellular survival (**Puddu et al., 2008 – Fig. 1A and 2A**). On the other hand, in combination with *dot1Δ* it exhibited synergistic effects on sensitivity to UV or DSBs inducing agents (**Puddu et al., 2008 – Fig. 1A and 2A**). Moreover, the double mutant completely lost the checkpoint-dependent delay of nuclear division after UV irradiation, suggesting that the checkpoint response was completely abrogated in this background. Consistently, the two mutations completely abolished the DNA damage-dependent phosphorylation of Rad9 and Rad53, while Mec1 activity did not seem to be significantly reduced, as indicated by the phosphorylation state of Ddc2 (**Puddu et al., 2008 – Fig. 1B/C and 2B and data not shown**). These data indicated that, in the G2/M phase of the cell cycle, *DPB11* and *DOT1* work in two parallel pathways leading to Rad9 recruitment and Rad53 phosphorylation.

Dpb11 has been widely characterized in its replication function; however, its precise role in the DNA damage response remained unknown. Dpb11 has been reported to physically and genetically interact with the Ddc1component of the 9-1-1 checkpoint clamp: this interaction seems to involve the last BRCT of Dpb11, which is a phospho-protein binding motif (Wang and Elledge, 2002). Since Ddc1 is subjected 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. Ddc1 sequence analysis revealed the presence of eight putative target sites for Mec1-dependent phosphorylation ([S/T]Q) and three consensus sites for Cdk1-dependent phosphorylation ([S/T]P) (**Puddu et al., 2008 – Fig. 3A**). 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, where all the putative phosphorylation sites have been mutated. By western blot analysis we observed that both *ddc1-M8* and *ddc1-M11 cells* lose the DNA-damage dependent phosphorylation of Ddc1after UV treatment (**Puddu et al., 2008 – Fig. 3B**), but they are not defective in the checkpoint response,

since they can still phosphorylate Rad9 and Rad53 after UV irradiation in nocodazole-arrested coltures (**Puddu et al., 2008 – Fig.4A/B and data not shown**). On the other hand, when combined with *DOT1* deletion, both *ddc1-M8* and *ddc1-M11* produce a synthetic phenotype: in fact, bot *dot1Δ ddc1-M8* and *dot1Δ ddc1-M11* mutant strains lose the ability to hyperphosphorylate Rad9 and Rad53 and display a synthetic lethality after UV irradiation (**Puddu et al., 2008 – Fig. 4A/B**). These phenotypes are recapitulated by the single *ddc1T602A* mutation and strongly resembles the *dpb11-1* phenotype previously described (**Puddu et al., 2008 – Fig. 5A/B, 6B**). Moreover, *ddc1T602A* and *dpb11-1* appear to be in the same epistasis group both for what concerns Rad53 phosphorylation and also for sensitivity to UV irradiation (**Puddu et al., 2008 – Fig. 5A/B, 6B**). Such observations suggest that a pathway requiring Dpb11 and Mec1-dependent phosphorylation of Ddc1 on T602 collaborates with methylated H3K79 in G2/M checkpoint activation and is required to phosphorylate Rad9 in the absence of the histone-mediated pathway.

Phospho-Ddc1 may be involved in recruiting Dpb11 to the lesion, bringing it close to checkpoint kinases. To test this hypothesis, we investigated whether Dpb1 is be phosphorylated after DNA damage and whether this may be dependent upon phospho-Ddc1. After UV irradiation of nocodazole-arrested cells, we detected a modification of Dpb11 which is DNA damage and Mec1-dependent (**Puddu et al., 2008 – Fig. 7A/B**). Further analysis demonstrated that this modification is greatly reduced in *ddc1-T602A* cells (**Puddu et al., 2008 – Fig. 7A/B/C**). This defect in Dpb11 phosphorylation could be explained if phospho-Ddc1 is required to recruit Dpb11 to the lesion and, therefore, close to Mec1. Our hypothesis was confirmed by two-hybrid data, which showed that the physical interaction between Dpb11 and Ddc1 is lost in a *mec1-1* mutant background and when we use the *ddc1-M8* bait (**Puddu et al., 2008 – Fig. 8A/B**).

LINKING STRUCTURE AND FUNCTION: RAD9 PHOSPHORYLATION AND ITS CHECKPOINT FUNCTION

The finding that Dpb11 participates to the DNA damage checkpoint facilitating the recruitment of Rad9 to the lesion, shed light on an important step of the signal transduction cascade, but this opened many questions.

First, it remain unknown why this "histone independent" branch of checkpoint activation is active only in the G2/M phase of the cell cycle. This evidence strongly suggested that the pathway might be somehow regulated by cell cycle-dependent control mechanism (e.g. high CDK1 activity in the G2 and M phases of the cell cycle). We therefore decided to analyze the contribution of Cdc28 to the G2/M checkpoint response in the absence of the histone methyltransferase Dot1. To

establish if the residual Rad53 activation we observed in *dot1Δ* cells was attributable to CDK1 activity, we examined Rad53 phosphorylation in cells expressing *cdc28-as1,* a Cdc28 mutant which allows conditional turn off of CDK1 kinase activity through the use of 1NMPP1-ATP analogue (Bishop et al., 2000). We found that the inhibition of CDK1 has a weak effect on Rad53 activation *per se*, whereas it has synergistic effect when combined with *DOT1* deletion, behaving as the *dpb11-1* mutation (**Granata et al., 2010 – Fig. 4A/C**). This evidence confirmed that CDK1 activity is required for the function of the histone-independent branch becessary for Rad53 activation in nocodazole-arrested cells.

Having shown that CDK1 activity was important for the Dpb11-dependent branch of checkpoint activation in G2/M cells, we next wanted to identify the CDK1 target responsible for this requirement. Rad9 contains 9 full ([S/T]-P-x-[K/R]) and 11 partial ([S/T]-P) CDK1 consensus sites and shows a cell cycle-dependent mobility shift indicative of phosphorylation (**Granata et al., 2010 – Fig. S2B/C**). We thus hypothesized that Rad9 could be a relevant Cdc28 target in the histone-independent branch. Indeed, yeast cells carrying a truncated Rad9 version lacking 9 putative CDK1 target sites in the N-terminus displayed a defect in checkpoint activation in the absence of Dot1, resembling the *dpb11-1* mutant strain (**Granata et al., 2010 – Fig. 5A**). In order to identify the residue/s critical for this phenotype, all the 9 putative CDK1 target sites in the Rad9 N-terminus were then mutagenized by site-specific mutagenesis and different mutant combinations were tested. Of all the mutant analyzed, *rad9S11A* was the only one that displayed a detectable defect in cell cycle-regulated Rad9 phosphorylation (**Granata et al., 2010 – Fig. S2C**). Moreover, after genotoxic treatment, it recapitulated the phenotypes observed in *rad9ΔNT* cells when combined with *DOT1* deletion, namely, severe impairment of Rad53 phosphorylation in nocodazole-arrested cells after UV irradiation, and UV hypersensitivity (**Granata et al., 2010 – Fig. 5B/C**). To prove that this synthetic effect was indeed due to a loss of Rad9 S11 phosphorylation, we produced a mutant strain that restores a different phosphorylatable residue, *rad9S11T*. Unlike the *dot1Δrad9S11A* mutant, which is defective in Rad53 activation after UV treatment, the *dot1Δrad9S11T* double mutant and *dot1Δ* single mutant displayed a similar level of Rad53 phosphorylation, which means that the *rad9S11T* mutation can almost completely rescue the defect observed in the *rad9S11A* mutant (**Granata et al., 2010 – Fig. 5D**).

To further demonstrate that Dpb11 could play a role in recruiting Rad9 to the proximity of the lesion, we tested whether these two factor physically interact. We analyzed this interaction by two-hybrid performed at different cell cycle stages. We observed that Rad9 and Dpb11 physically interact and that the interaction is more evident in G2/M- rather than in G1-arrested cells (**Granata et al., 2010 – Fig. 4A and data not shown**). Further experiments showed that this interaction was reduced in the presence of Rad9NT isoform, lacking the 9 potential CDK1 phosphorylation sites, or when Cdc28 activity was inhibited by the conditional switch off of the

Cdc28 activity in *cdc28-as1* mutant background (**Granata et al., 2010 – Fig. 6A**). Co-immunoprecipitation experiments confirmed that Rad9 and Dpb11 physically interact also *in vivo* (**Granata et al., 2010 – Fig. 6B**) and that the interaction requires the phosphorylation of Rad9 on S11, since it is abrogated in the *rad9S11A* mutant (**Granata et al., 2010 – Fig. 6C**).

We were then interested in understanding the spatio-temporal dynamics of Rad9 interaction with chromatin during the DNA damage response. For this reason, we monitored Rad9 chromatin binding and Rad53 phosphorylation in strains harbouring defects in all the different branches known to regulate Rad9 checkpoint function in G2/M phase (**Granata et al., 2010 – Fig. 7**). As previously demonstrated in other studies, Rad9 binding to damaged chromatin requires two histones post-translational modifications: the H3K79 methylation and H2AS129 phosphorylation. Surprisingly, impairment of the Dpb11-dependent pathway, obtained in the *dpb11ΔCT* mutant, which mimics the *dpb11-1* mutant previously used, did not affect Rad9 recruitment to chromatin. Rad53 phosphorylation was significantly defective in double mutant combinations carrying the *dpb11ΔCT* mutation. On the other hand, as already observed (**Puddu et al., 2008 – Fig. S1D**), abrogation of Rad9 binding to chromatin (as in the single *dot1Δ* and *H2AS129A* or in the double *dot1Δ H2AS129A* mutant strains) does not affect Rad53 phosphorylation; in these conditions Dpb11 becomes necessary and sufficient to guarantee checkpoint activation, which is in fact impaired in the triple mutant *dot1Δ H2AS129A dpb11ΔCT* (**Granata et al., 2010 – Fig. 7**).

The second aspect which we investigated was the the relationship between the functional role of the "histone-dependent" (that is H3K79 and H2AS129-dependent) and "Dpb11-dependent" branches of Rad9 recruitment and the molecular structure this protein assumes when it is bound to these different docking sites.

Rad9 contains many domains, each one implicated in a precise function. Productive Rad9-chromatin interactions are guaranteed, already in unperturbed conditions, trough interaction of its Tudor domain with methylated histone H3 (Hammet et al., 2007). It is commonly thought that constitutive Rad9 recruitment might facilitate the efficiency and speed of the Rad9-dependent response to genotoxins. After DNA damage, Rad9 binding to chromatin is strengthened by a physical interaction between its C-terminal BRCT domains and phosphorylated histone H2A (Lancelot et al., 2007). Finally, Rad9 has a Ser/Thr-Gln phosphorylation site cluster domain (SCD) that is a PIKK substrate. Colocalization of Mec1 with Rad9 enables Mec1 to phosphorylate the Rad9 SCD and this permits docking of Rad53 through FHA domains that recognize Rad9 phospho-SCD (Emili, 1998; Vialard et al., 1998; Durocher et al., 1999; Schwartz et al., 2002) This event is followed by Rad53 autophosphorylation, which is required for full activation of the kinase and of the checkpoint response (Pellicioli et al., 1999; Usui and Petrini, 2007).

It has been recently shown that mutations in a conserved region of the first BRCT motif affect binding of Rad9 to γ-H2A, thus altering the G1 checkpoint signaling in response to DSBs (Javaheri

et al., 2006; Hammet et al., 2007) and the G2/M response to uncapped telomeres (Nnakwe et al., 2009). Moreover, previous studies indicated that the BRCT domains modulate Rad9-Rad9 interactions after DNA damage, promoting oligomeric assembly of phosphorylated Rad9. This provides a platform where Rad53 can bind at high concentration, triggering the autophosphorylation step (Soulier and Lowndes, 1999; Gilbert et al., 2001). To further explore the functional role of the Rad9 BRCT domains and the ability of the protein to bind chromatin, we analyzed whether mutation of two highly conserved aromatic residues of the BRCTs, affecting the whole folding of these domains (Soulier and Lowndes, 1999), might alter the Rad9 binding to chromatin. Indeed, we observed that both *rad9F1104L* and *rad9W1289L* are completely unable to be loaded onto chromatin, both in G1- and in G2/M-arrested cells, in UV-treated and in unperturbed conditions (**Granata et al., 2010 – Fig. 1**). These evidences suggested that the intactness of the BRCT domains is necessary not only for the Rad9 binding to chromatin via γH2A, but also for the recruitment of this protein in unperturbed conditions.

To evaluate whether the Rad9-Rad9 interactions were necessary for its chromatin binding, we generated a set of yeast strains in which the C-terminal BRCT domains were substituted with either a 13-MYC epitope or a GST-tag, which has been shown to act as a heterologous constitutive dimerization domain (Walker et al., 1993; Du et al., 2004). We then analyzed both Rad9 chromatin recruitment and checkpoint functions in cells expressing all these constructs. The substitution of the BRCT domains with the heterologous dimerization motif restore the Rad9 binding to chromatin in the G1 phase of the cell cycle, both before and after genotixic treatment. This recruitment was still dependent upon histone H3 methylation, suggesting that the BRCT-mediated dimerization of Rad9 is a pre-requisite for the loading of the protein on K79 by its Tudor domain (**Granata et al., 2010 – Fig. 2A**). GST-forced Rad9 dimerization significantly recovered also the Rad9 hyperphosphorylation after UV irradiation and full checkpoint activation (**Granata et al., 2010 – Fig. 2A and data not shown**).

Contrary to what observed in G1-arrested cells, the forced Rad9 dmerization by the GST tag did not restore the Rad9 binding to chromatin in nocodazole-arested cells (**Granata et al., 2010 – Fig. 2D**). Surprisingly, despite undetectable recruitment on the chromatin, rad9ΔBRCT::GST rescues the Rad9 and Rad53 phosphorylation after UV irradiation and also the checkpoint function (**Granata et al., 2010 – Fig. 2D and Fig. 3**). Since we had already observed that in nocodazole-arrested cells checkpoint activation in the absence of the histone-dependent branch of Rad9 recruitment was completely dependent upon Dpb11, we wanted to test whether Rad53 phosporylation supported by the heterologous dimerization motif in the *rad9ΔBRCT::GST* mutant strain was dependent upon Dpb11. To address this question, we introduced the *rad9S11A* mutation in *rad9ΔBRCT::GST* mutant strain and we analyzed checkpoint activation and cellular survival after UV treatment. Whilst either single mutant strain was only partially defective in Rad53 phosphorylation and was slightly sensitive to UV, in the double mutant checkpoint activation was

severly impaired and the UV sensitivity was significantly higher, confirming again that the residual DNA damage response observed in *rad9ΔBRCT::GST* was due to Dpb11-branch acting through the S11 residue at the N-terminus of Rad9 (**Granata et al., 2010 – Fig. 2D and Fig. 8**).

In our experimental conditions, the *dpb11-1* mutant allele did not exhibit a significant effect on Ddc2 phosphorylation. However, in these condtions, the Dpb11 protein, albeit missing its C-terminal part, is still present in the cells and is likely to be partially functional. Since accumulating evidences in higher eukaryotes suggested a role of the Dpb11 orthologue, TopBP1 in the activation of ATR kinase (Kumagai and Dunphy, 2006), we exploited a temperature-sensitive degron version of Dpb11 (*dpb11td*), which can be conditionally eliminated from the cells to evaluate the possible role of Dpb11 in the control of Mec1 kinase activity. We therefore monitor the phosphorylation state of Ddc2, commonly used as a marker for Mec1 activation, in nocodazole-arrested cells after UV irradiation. We were able to detect a significant defect in the DNA-damage Ddc2 phosphorylation, and thus in Mec1 activation, after cells had been depleted of Dpb11, suggesting that, as in higher eukaryotes Dpb11 participate in the robust activation of Mec1, maybe in strengthening its kinase activity (**Puddu et al., 2008 – Fig. 9**).

FINAL CONSIDERATIONS

The data collected during these years shed light on an important step of the checkpoint signal transduction cascade, regarding the dynamics of recruitment of the adaptor Rad9 to the apical kinase Mec1 and the proximity of the lesion and in the G2/M phase of the cell cycle.

The working model we propose (**Granata et al., 2010 – Fig. 9**) suggests that, during an unperturbed cell cycle, Rad9 is already present on the chromatin, thank to its physical interaction with the H3K79 methylated, bound by its Tudor domains. Constitutive Rad9 chromatin binding might be necessary to facilitate and speed the Rad9 functions in the DNA Damage Response. Its BRCT-mediated dimerization seems to be a pre-requisite for a functional recruitment on the chromatin. Indeed, given the symmetrical structure of the histone octamer within the nucleosome core, dimerization might facilitate the correct orientation and positioning of two Rad9 molecules on the nucleosome, allowing productive interactions with unmodified histones.

In the presence of DNA damage, activated Rad9 may change its conformations, interacting also with γ-H2A throught its BRCT domain. Histone H2A phosphorylation and histone H3 methylation represent the two docking sites for Rad9 recruitment in the close proximity to the chromatin. However, in M-phase, an alternative mean of Rad9 recruitment exists and involves its interaction with Dpb11. Dpb11 is brought near the Mec1-Ddc2 complex through its interaction with the Ddc1 subunit of the 9-1-1 checkpoint clamp, phosphorylated by Mec1 on T602. Once loaded close to

the lesion, it binds Rad9 phosphorylated at S11 by CDK1. This Dpb11-dependent localization of Rad9 to sites of DNA damage can compensate for the loss of the histone-dependent branch of checkpoint activation, allowing rapid Rad9 hyper-phosphorylation and thus checkpoint activation.

PART IV -

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