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**Regulation of the DNA damage response  
by cyclin-dependent kinase in  
*Saccharomyces cerevisiae***

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## ***SUMMARY***



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***RIASSUNTO***





Il ciclo di divisione cellulare eucariotico comprende una serie di eventi il cui ordine e la cui corretta successione dipende dall'attività oscillante delle chinasi ciclina-dipendenti (*cyclin-dependent kinases* o Cdk), che salvaguarda la tempestiva duplicazione e segregazione del genoma. Dato che l'integrità del genoma è costantemente minacciata da fonti endogene ed esogene di danni al DNA, il ciclo di divisione cellulare è intimamente connesso con un complessa risposta cellulare al danno al DNA (*DNA damage response* o DDR) evolutivamente conservata. Tale risposta garantisce la trasmissione fedele dell'informazione genetica dalla cellula madre alla cellula figlia e assicura la sopravvivenza cellulare. Infatti, la DDR comprende diversi sistemi di riparazione delle lesioni così come meccanismi di sorveglianza, chiamati checkpoint da danni al DNA, che inibiscono la progressione del ciclo cellulare fintanto che la lesione non viene riparata. Il checkpoint da danno al DNA è attivato in presenza di danni al DNA o di stress replicativo ed è basato su cascate di trasduzione del segnale di protein chinasi, che riconoscono il DNA danneggiato, trasmettono e amplificano il segnale di danno, e regolano diverse proteine effettrici per prevenire la progressione del ciclo cellulare e coordinarlo con la capacità di riparare la lesione. Oltre a controllare la progressione del ciclo cellulare, il checkpoint promuove l'attivazione dei sistemi di riparazione della lesione e il corretto completamento della replicazione del DNA, attiva specifici programmi trascrizionali e, in alcuni casi, indirizza la cellula alla morte per apoptosi.

Crescenti evidenze suggeriscono che le Cdk non siano solo i bersagli attraverso cui la DDR modula la progressione del ciclo cellulare, ma che esse partecipino

anche alla regolazione della DDR, sia promuovendo la piena attivazione del checkpoint che stimolando la riparazione del DNA per ricombinazione omologa (*homologous recombination* o HR). I danni al DNA più pericolosi sono le rotture a doppio filamento del DNA (*double-strand breaks* o DSB), che possono essere riparate per *non-homologous end joining* (NHEJ) o per HR. Mentre il NHEJ ricongiunge direttamente le estremità della lesione, la HR utilizza una sequenza di DNA omologa come template per la riparazione per dare origine a prodotti di ricombinazione che si distinguono in crossover o noncrossover in riferimento alle sequenze parentali fiancheggianti il sito di danno. In aggiunta, un DSB posizionato tra due sequenze ripetute dirette può essere riparato tramite un particolare meccanismo di ricombinazione omologa chiamato *single-strand annealing* o SSA, che risulta nella riparazione della lesione con concomitante perdita di una delle due ripetizioni e della regione compresa tra esse. Tutti i meccanismi di HR iniziano con un esteso processamento in direzione 5'-3' (noto come resection 5'-3') delle estremità della lesione per generare code di DNA a singolo filamento in 3' (*single-stranded DNA* o ssDNA), che sono legate dalla proteina di replicazione A (*Replication Protein A* o RPA). Successivamente, RPA viene spiazzata da Rad51 a formare filamenti nucleoproteici che possono catalizzare l'appaiamento e l'invasione di una doppia elica di DNA omologa.

La scelta tra NHEJ e HR è finemente regolata durante il ciclo cellulare e la HR è generalmente limitata alle fasi S/G2 del ciclo cellulare, quando il DNA è stato replicato e il cromatidio fratello è disponibile come stampo per la riparazione. Questa specificità legata al ciclo cellulare dipende dall'attività di Cdk (Cdk1 in *Saccharomyces cerevisiae*), che dà inizio alla HR stimolando la degradazione nucleolitica in direzione 5'-3' delle estremità del DSB. Non era noto se Cdk1

regolasse anche altri passaggi richiesti per la HR. Per rispondere a questa domanda abbiamo indagato quale fosse la richiesta di Cdk1 nell'esecuzione di diversi processi di HR in *S. cerevisiae*. Per aggirare la richiesta di Cdk1 per la resection, abbiamo utilizzato cellule prive dell'eterodimero Yku e/o della proteina di checkpoint Rad9, che sono entrambi noti come regolatori negativi della resection al DSB. Abbiamo dimostrato che cellule *yku70Δ*, che accumulano ssDNA alle estremità di un DSB indipendentemente dall'attività di Cdk1, sono in grado di riparare un DSB per SSA in fase G1, quando l'attività di Cdk1 è bassa. Questa capacità di riparare per SSA dipende dal processamento delle estremità del DSB, poiché tanto l'efficienza di resection quanto quella di SSA aumentano in cellule *yku70Δ* in G1 prive anche di Rad9. Abbiamo inoltre osservato che in cellule *yku70Δ* e *yku70Δ rad9Δ* in G1 si generano prodotti noncrossover come risultato della ricombinazione intercromosomica, indicando che la generazione di ssDNA al DSB è sufficiente per compensare la richiesta di Cdk1 per portare a termine anche questo tipo di ricombinazione. Al contrario, cellule *yku70Δ* e *yku70Δ rad9Δ* sono specificamente difettive nella formazione di prodotti crossover come risultato della ricombinazione intercromosomica quando l'attività di Cdk1 è bassa. Pertanto, Cdk1 promuove la riparazione di DSB per SSA e ricombinazione noncrossover agendo principalmente a livello della resection, mentre ulteriori passaggi richiedono l'attività di Cdk1 per generare prodotti crossover. Dato che durante il ciclo cellulare mitotico i crossover possono potenzialmente portare a pericolosi riarrangiamenti cromosomici quando il cromatide fratello non viene usato come stampo per la riparazione, questa richiesta addizionale di Cdk1 nel promuovere la formazione

di prodotti crossover può fornire un ulteriore meccanismo di sicurezza per garantire la stabilità genomica.

Durante la replicazione del DNA le cellule sono particolarmente sensibili ai danni al DNA. Le cellule eucariotiche rispondono allo stress replicativo attraverso una complessa via di trasduzione del segnale, chiamata checkpoint di fase S, le cui componenti chiave in *S. cerevisiae* sono le chinasi Mec1 e Rad53. Sia Mec1 che Rad53 sono essenziali per la vitalità cellulare e mutanti di checkpoint *mec1* e *rad53* sono estremamente sensibili agli agenti che causano stress replicativo, come l'idrossiurea (HU) e il metil metan sulfonato (MMS). La protein chinasi sensore Mec1 è reclutata a livello delle forche replicative bloccate, dove attiva la chinasi effettrice Rad53. L'attivazione di queste protein chinasi mantiene l'integrità/attività delle forche replicative, stimola la produzione di desossiribonucleotidi, inibisce l'attivazione delle origini di replicazione tardive e previene l'accumulo di strutture aberranti del DNA. Una questione fondamentale da risolvere era determinare quale/i dei suddetti processi regolati dal checkpoint di fase S sia/siano cruciale/i per il mantenimento della vitalità cellulare. Abbiamo investigato questo aspetto cercando mutazioni extrageniche capaci di sopprimere la forte sensibilità alla HU di mutanti *mec1*. Tramite la caratterizzazione di una delle mutazioni individuate, abbiamo potuto constatare che una diminuita attività di Cdk1 allevia gli effetti letali di mutazioni *mec1* e *rad53* tanto in assenza quanto in presenza di stress replicativo, suggerendo che l'esecuzione di alcuni eventi cellulari mediati da Cdk1 è deleteria in assenza di Mec1 e Rad53. Questa letalità è ricollegabile a funzioni di Cdk1 sia in G1 che in mitosi. Infatti, è possibile preservare la vitalità di cellule *mec1* e *rad53* in presenza e in assenza di HU sia

ritardando la transizione G1/S che rallentando l'allungamento del fuso mitotico. I dati raccolti evidenziano come l'inappropriato ingresso in fase S o la segregazione di cromosomi non completamente replicati porti a morte cellulare quando il checkpoint di fase S non è funzionale. In aggiunta, quanto osservato suggerisce che la funzione essenziale di Mec1 e Rad53 non sia necessariamente distinta dalla funzione che queste chinasi svolgono nel supportare la sintesi del DNA in condizioni di stress.

In conclusione, i risultati ottenuti indicano che Cdk1 influenza la DDR attraverso molteplici meccanismi. Cdk1 è infatti richiesta per la completa attivazione del checkpoint in seguito a DSB, per la riparazione del DSB per ricombinazione omologa e per la formazione di crossover durante il ciclo cellulare mitotico. D'altra parte l'attività di Cdk1 deve essere strettamente regolata, poiché un'eccessiva attività di Cdk1 può minacciare la stabilità genomica, quantomeno quando il checkpoint non è funzionale.



# ***ABSTRACT***





The eukaryotic cell division cycle comprises a series of events, whose ordering and correct progression depends on the oscillating activity of cyclin-dependent kinases (Cdks), which safeguard timely duplication and segregation of the genome. Since genome integrity is constantly threatened by both endogenous and exogenous sources of DNA damage, cell division cycle is intimately connected to an evolutionarily conserved DNA damage response (DDR) in order to guarantee the faithful transmission of genetic information from one cell to its daughter and to ensure cell survival. In fact, the DDR involves DNA repair pathways that reverse DNA lesions, as well as DNA damage checkpoint pathways that inhibit cell-cycle progression while repair occurs. The DNA damage checkpoint is activated in the presence of DNA damage or replicative stress and is based on signal transduction cascades of protein kinases that recognize damaged DNA, transduce and amplify the damage signal, and target several effector proteins to prevent cell cycle progression and to couple it with the DNA repair capacity. In addition to driving cell-cycle arrest, these pathways control the activation of DNA repair pathways, the proper completion of DNA replication, the activation of transcriptional programs and, in some cases, the commitment to cell death by apoptosis.

There is increasing evidence that Cdks are not only downstream targets of the DDR, but also participate in the DDR regulation, both by leading to a strong checkpoint activation and by promoting DNA repair by homologous recombination (HR). The most dangerous DNA lesions are the DNA double-strand breaks (DSBs) that can be repaired either by non-homologous end

joining (NHEJ) or by HR. While NHEJ directly relegates the broken DNA ends, HR uses homologous DNA sequences as a template to form recombinants that are either crossover or noncrossover with regard to flanking parental sequences. Furthermore, a DSB flanked by direct DNA repeats can be repaired by a particular HR pathway called single-strand annealing (SSA), which results in DSB repair with concomitant deletion of one repeat and of the intervening sequence. All HR processes initiate with extensive 5' to 3' end-processing (a process referred to as 5'-3' resection) of the broken ends to yield 3'-ended single-stranded DNA (ssDNA) tails, which are bound by Replication Protein A (RPA). RPA is then displaced by Rad51 to form nucleoprotein filaments that can catalyse homologous pairing and strand invasion.

The choice between NHEJ and HR pathways is tightly regulated during the cell cycle and HR is generally restricted to S/G2 cell cycle phases, when DNA has been replicated and a sister chromatid is available as a repair template. This cell cycle specificity depends on Cdk (Cdk1 in *Saccharomyces cerevisiae*) activity, which initiates HR by promoting 5'-3' nucleolytic degradation of the DSB ends. Whether Cdk1 regulates other HR steps was unknown. To address this question, we explored the Cdk1 requirement in the execution of different HR processes in *S. cerevisiae*. In order to bypass the Cdk1 requirement for resection we analyzed cells lacking Yku heterodimer and/or the checkpoint protein Rad9, which are known as negative regulators of DSB resection. We showed that *yku70Δ* cells, which accumulate ssDNA at the DSB ends independently of Cdk1 activity, are able to repair a DSB by SSA in the G1 cell cycle phase, when Cdk1 activity is low. This ability to perform SSA depends on DSB resection, because both resection and SSA are enhanced by the lack of

Rad9 in *yku70Δ* G1 cells. Furthermore, we found that interchromosomal noncrossover recombinants are generated in *yku70Δ* and *yku70Δ rad9Δ* G1 cells, indicating that DSB resection bypasses Cdk1 requirement also for carrying out these recombination events. By contrast, *yku70Δ* and *yku70Δ rad9Δ* cells are specifically defective in interchromosomal crossover recombination when Cdk1 activity is low. Thus, Cdk1 promotes DSB repair by SSA and noncrossover recombination by acting mostly at the resection level, whereas additional events require Cdk1-dependent regulation in order to generate crossover outcomes. As crossovers during mitotic cell growth have the potential for deleterious genome rearrangements when the sister chromatid is not used as repair template, this additional function of Cdk1 in promoting crossovers can provide another safety mechanism to ensure genome stability.

During DNA replication cells are particularly sensitive to DNA damage. Eukaryotic cells respond to replication interference through a complex signal-transduction pathway, known as the S-phase checkpoint, whose key players in *S. cerevisiae* are the Mec1 and Rad53 kinases. Both Mec1 and Rad53 are essential for budding yeast cell viability and *mec1* and *rad53* checkpoint mutants are extremely sensitive to agents that cause replicative stress, such as hydroxyurea (HU) and methyl methanesulfonate (MMS). The sensor kinase Mec1 is recruited to stalled replication forks, where it activates the effector kinase Rad53. The activation of both these kinases maintains the integrity/activity of the replication forks, stimulates deoxyribonucleotides (dNTPs) production, inhibits the firing of late replication origins and prevents accumulation of aberrant DNA structures. A fundamental question to be addressed was which of the above checkpoint-regulated process(es) is/are

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critical for the maintenance of cell viability. We investigated this question searching for extragenic mutations suppressing the hypersensitivity to HU of *mec1* mutant. By characterizing one of the identified suppressor mutations, we provided evidence that decreased activity of Cdk1 alleviates the lethal effects of *mec1* and *rad53* mutations both in the absence and in the presence of replication stress, indicating that the execution of certain Cdk1-mediated event(s) is detrimental in the absence of Mec1 and Rad53. This lethality involves Cdk1 functions in both G1 and mitosis. In fact, delaying either the G1/S transition or spindle elongation in *mec1* and *rad53* mutants allows their survival both after exposure to HU and under unperturbed conditions. Altogether, our studies indicate that inappropriate entry into S phase and segregation of incompletely replicated chromosomes contribute to cell death when the S-phase checkpoint is not functional. Moreover, these findings suggest that the essential function of Mec1 and Rad53 is not necessarily separated from the function of these kinases in supporting DNA synthesis under stress conditions. In conclusion, our results suggest that Cdk1 influences the DDR through multiple mechanisms. Indeed, Cdk1 is required for DSB-induced checkpoint activation, DSB repair by homologous recombination, and crossover formation. On the other hand, Cdk1 activity must be carefully regulated, because too much Cdk1 activity can affect genome integrity, at least when the checkpoint is not functional.

# ***INTRODUCTION***



## **MAINTAINING GENOME INTEGRITY: THE DNA DAMAGE RESPONSE**

The faithful transmission of genetic information from one cell to its daughters is fundamental for the survival of organisms. To achieve such faithful transmission, cells must ensure an accurate DNA replication and subsequent precise chromosome segregation. Errors in these essential processes could mean death for unicellular organisms and may lead to cancer development in multicellular organisms (Hartwell and Kastan, 1994).

Genomes are constantly threatened by both endogenous and exogenous genotoxic agents. Spontaneous DNA alterations can be due to deoxyribonucleotides (dNTPs) misincorporation during DNA replication and loss or modification of DNA bases by alkylation and deamination. Furthermore, reactive oxygen species produced by normal cellular metabolism can oxidize DNA bases and generate DNA breaks. DNA damage can be also produced environmentally by chemicals or by physical sources like ionizing radiation (IR) and ultraviolet light (UV). Thus, cells need to recognize and repair DNA alterations in order to survive and transmit a complete and undamaged genome to the offspring. To achieve this accuracy, cells have evolved a complex cellular response, named DNA damage response (DDR), orchestrated by specialized surveillance mechanisms, known as DNA damage checkpoints, that monitor the structure of chromosomes and coordinate DNA repair and cell-cycle progression (Zhou and Elledge, 2000). Hence, these checkpoints ensure the correct completion of DNA replication during S-phase and supervise the

integrity of DNA throughout the entire cell cycle to minimize the number of heritable mutations. Indeed, DNA damage checkpoints are able to recognize and respond to DNA lesions by arresting the cell cycle progression and by promoting repair of the damage. Only when DNA repair is correctly accomplished, the arrest is relieved to allow the cell to move to the next cell cycle phase (Nyberg *et al.*, 2002).

In humans, the importance of the DDR has been unveiled by the identification of severe genetic disorders characterized by mutations in many checkpoint and repair genes (Shiloh, 2003). Indeed, genetic defects that perturb these mechanisms almost invariably cause severe syndromes associated with catastrophic phenotypes including degeneration of specific tissues, growth and developmental retardation, premature signs of ageing, chromosomal instability, sensitivity to DNA-damaging agents and predisposition to cancer. The existence of such genomic instability disorders, that are associated with a moderate to severe increase in the incidence of cancers, underscores the functional link between genomic instability and cancer development (Vessey *et al.*, 1999). Furthermore, recent work has also shown that the checkpoint is activated in early cancerous lesions and may function more generally to prevent human tumorigenesis (Bartkova *et al.*, 2005; Gorgoulis *et al.*, 2005). Given the importance of DDR in maintaining genome stability and the correlation of loss of this function with human pathology and cancer, scientists have taken advantage of the extreme conservation of these mechanisms to use model organisms to identify the genes and uncover the molecular events at the basis of the DNA damage response in eukaryotes. In the past years, studies in different organisms such as the budding yeast *Saccharomyces cerevisiae* and



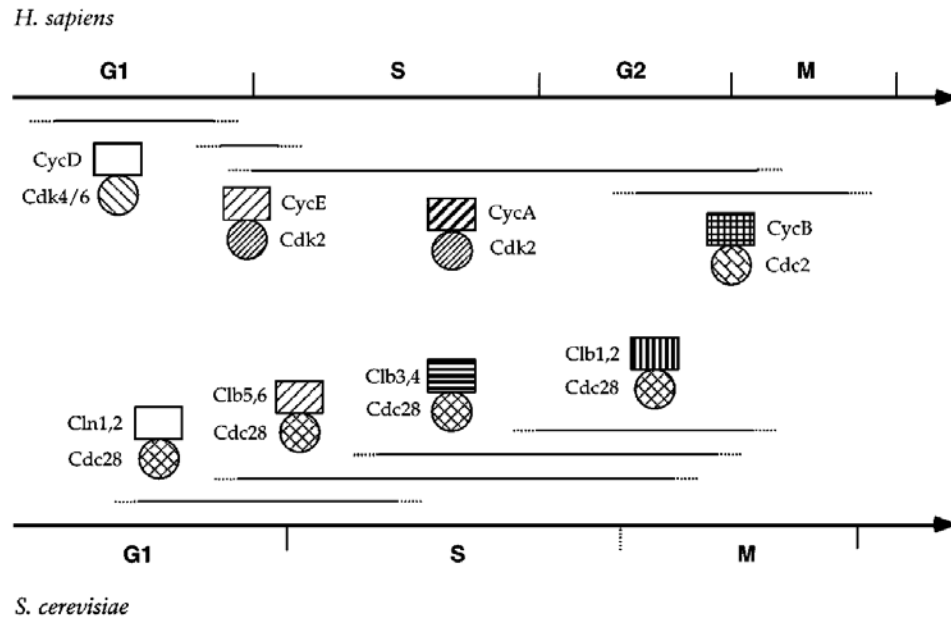
the fission yeast *Schizosaccharomyces pombe*, the fruit fly *Drosophila melanogaster*, the frog *Xenopus laevis*, mouse and mammalian systems have combined to increase our understanding of the molecular basis of the DDR. Although researchers have partially unveiled the genetic, biochemical and molecular mechanisms underlying the DNA damage response, many aspects of these surveillance mechanisms are still obscure and further studies are required to shed light on them. The tight correlation between failures in the DDR and human cancer development implies that improving our knowledge in this field would help in providing new therapies for cancer treatment.

During the DDR, there is a strong correlation between cell cycle, DNA damage checkpoint and DNA repair mechanisms. In fact, different types of DNA damage are recognized and repaired by specialized mechanisms depending on the type of lesion and on the cell cycle phase in which they occur, indicating that the cell cycle plays a role in the choice of the proper DNA repair mechanism. Furthermore, the checkpoint is strongly activated only in specific cell cycle phases. On the other hand, the checkpoint pathways are able to stop/slow down the progression of the cell cycle and activate the DNA repair systems. Hence, this complex interconnection between cell cycle, DNA repair and checkpoint ensures a functional and well-organized DNA damage response that guarantees the maintenance of genome integrity and is therefore highly conserved during evolution.

## CYCLIN-DEPENDENT PROTEIN KINASES CONTROL CELL CYCLE PROGRESSION

The eukaryotic cell cycle is considered to be composed of four phases: the gap before DNA replication (G1), the DNA synthetic phase (S), the gap after DNA replication (G2), and the mitotic phase, which culminates in cell division (M). During cell cycle cells duplicate their genome and then segregate it equally between two daughter cells. These processes must be accurately controlled to ensure that eukaryotic cells never duplicate twice their chromosomes during the same cell cycle, never undergo mitosis before DNA replication is completed, nor segregate their sister chromatids until all pairs are aligned on the mitotic spindle. This cell cycle ordering is achieved by the oscillating activity of cyclin-dependent kinases (Cdks), which act as master regulators of cell cycle progression.

Cdks are Ser/Thr protein kinases that drive and coordinate the events of the eukaryotic cell cycle. Cdk catalytic subunits do not act alone, they are allosterically activated by binding to regulatory subunits, named cyclins. Cyclin levels are strictly controlled by timed expression, degradation and localization, so that their oscillating concentrations underlie the stage-specific timing of Cdk activity. In yeast, a single Cdk (Cdk1 or Cdc28 in the budding yeast *Saccharomyces cerevisiae* and Cdc2 in the fission yeast *Schizosaccharomyces pombe*) is able to regulate diverse cell cycle transitions by associating with multiple stage-specific cyclins (**Figure 1**).



**Figure 1. Major Cdk-cyclin complexes involved in cell cycle control in humans and budding yeast.**

Lines indicate approximate timing of activation and function of the indicated complexes. Note that S and M phases overlap in *S. cerevisiae* (adapted from (Morgan, 1997)).

In *S. cerevisiae*, the G1 function of Cdk1 requires a set of three G1 cyclins, Cln1–3, with overlapping functions, while a partially redundant family of six cyclins, Clb1–6, governs entry into S phase (primarily Clb5, 6) and mitosis (Clb1–4) (Nasmyth, 1996).

The situation appears less complex in *S. pombe*, where a single cyclin, Cdc13, is required for the mitotic function of Cdc2, whereas initiation of DNA synthesis involves the cyclin Cig2 and, to a lesser extent, Cig1 (Stern and Nurse, 1996). In vertebrates, Cdc2 (Cdk1) and Cdk2 are functionally homologous to yeast Cdc2/Cdk1 and are the main Cdks involved in central cell cycle functions. Cdk2

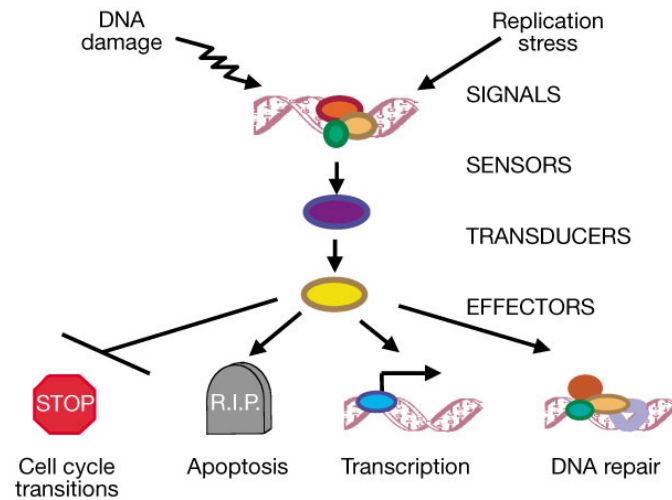
interacts with cyclin E at the beginning of the S phase to induce the initiation of DNA synthesis, and then binds cyclin A throughout the S phase. Mitosis is then initiated by the Cdc2-cyclin B complex, also known as M-phase promoting factor (MPF). Complexes between Cdc2 and cyclin A may also contribute to the preparation for mitosis (Nigg, 1995; Edgar and Lehner, 1996). Cell proliferation in vertebrates often requires the presence of external growth factors, which usually influence the cell's commitment to division in G1. A key response to growth factors in many cell types is the activation of Cdk4 or its close relative Cdk6 by members of the cyclin D family (Sherr, 1996). These complexes are required for progression through the G1 phase, apparently by virtue of their ability to suppress the antiproliferative effects of the pRb protein. In cells lacking pRb, G1 progression occurs in the absence of Cdk4/6-cyclin D activity (Bartek *et al.*, 1996; Sherr, 1996). Thus these complexes are not central components of the machine that drives the cell through division, but serve to couple this machine with extracellular signals.

Although cyclin binding is the primary determinant of Cdk function, layers of additional regulatory subunits and protein kinases also modulate Cdk activity, substrate recognition, and subcellular localization. In fact, Cdk must be phosphorylated within the activation loop of the kinase domain by a Cdk-activating kinase (CAK) to attain full activity. Conversely, Cdk activity is inhibited by phosphorylation near the N-terminus of the protein or by binding to Cdk-inhibitory subunits (CKIs) (Morgan, 1997). This finely tuned regulatory network ensures the precise timing and coordination of the mechanical events that duplicate and divide the cell.

## THE DNA DAMAGE CHECKPOINTS: PROTEINS AND PATHWAYS

DNA damage checkpoints are defined as stress response pathways, based on signal transduction cascades, that specifically delay cell cycle transitions in response to DNA damage, allowing time for repair. The checkpoint pathways involve three major groups of proteins that act in concert to transduce the signal of damage in order to promote cell cycle arrest and DNA repair. These groups include: (a) sensor proteins that recognize damaged DNA directly or indirectly and signal the presence of alterations in DNA structure, initiating the transduction cascade; (b) transducer proteins, typically protein kinases, that relay and amplify the damage signal from the sensors by phosphorylating other kinases or downstream target proteins; and (c) effector proteins, which include the most downstream targets of the transducer protein kinases, and are thus regulated, usually by phosphorylation, to prevent cell cycle progression (Nyberg *et al.*, 2002). In addition to driving cell-cycle arrest, these pathways have been shown to control the activation of DNA repair pathways, the proper completion of DNA replication, the telomere homeostasis, the activation of transcriptional programs and, in some cases, the commitment to cell death by apoptosis. However, thinking of checkpoints as unidirectional pathways seems to be an oversimplification. For example, DNA repair proteins or some components of the DNA replication complexes can act as both checkpoint sensors and effectors. This complexity suggests that the checkpoint responses likely involve

complex regulatory networks that incorporate both feedback loops and threshold responses (Putnam *et al.*, 2009). Thus, it is clear that the checkpoint pathways are the central part of a larger and multifaceted DNA damage response (Figure 2).



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**Figure 2. The DNA damage response.**

Arrowheads represent activating events and perpendicular ends represent inhibitory events. Cell-cycle arrest is depicted with a stop sign, apoptosis with a tombstone. The DNA helix with an arrow represents damage-induced transcription, while the DNA helix with several oval-shaped subunits represents damage-induced repair. For the purpose of simplicity, the network of interacting pathways are depicted as a linear pathway consisting of signals, sensors, transducers and effectors (adapted from (Zhou and Elledge, 2000)).

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Moreover, several checkpoint genes are essential for cell and organism survival, implying that these pathways are not only surveyors of occasional damage, but are firmly integrated components of cellular physiology (Zhou and Elledge, 2000).

**Table 1.** Homologs of the components of the central kinase cascade.

Class	<i>S. cerevisiae</i>	<i>S. pombe</i>	<i>H. sapiens</i>	
<b>Sensors</b>	<i>MEC1</i>	<i>RAD3</i>	<i>ATR</i>	
	<i>DDC2</i>	<i>RAD26</i>	<i>ATRIP</i>	
	<i>RAD24</i>	<i>RAD17</i>	<i>RAD17</i>	
	<i>DDC1</i>	<i>RAD9</i>	<i>RAD9</i>	
	<i>MEC3</i>	<i>HUS1</i>	<i>HUS1</i>	
	<i>RAD17</i>	<i>RAD1</i>	<i>RAD1</i>	
	<i>DPB11</i>	<i>CUT5/RAD4</i>	<i>TOPBP1</i>	
	<i>TEL1</i>	<i>TEL1</i>	<i>ATM</i>	
	<i>MRE11</i>	<i>MRE11</i>	<i>MRE11</i>	
	<i>RAD50</i>	<i>RAD50</i>	<i>RAD50</i>	
	<i>XRS2</i>	<i>NBS1</i>	<i>NBS1</i>	
	-	-	<i>DNA-PKcs</i>	
	<b>Adaptors</b>	<i>RAD9</i>	<i>CRB2</i>	<i>53BP1</i>
		<i>MRC1</i>	<i>MRC1</i>	<i>CLSPN</i>
<b>Effector kinases</b>	<i>CHK1</i>	<i>CHK1</i>	<i>CHK1</i>	
	<i>RAD53</i>	<i>CDS1</i>	<i>CHK2</i>	

adapted from (Putnam *et al.*, 2009)

In *S. cerevisiae*, DNA damage checkpoints delay the G1/S transition and block the G2/M transition of the cell cycle (Weinert and Hartwell, 1988; Siede *et al.*, 1993). In addition, the S-phase checkpoint has been defined, which arrests cell cycle progression and inhibits firing of late replication origins in response to replication stress and DNA damage that blocks the progression of the replication machinery (Santocanale and Diffley, 1998; Paulovich *et al.*, 1997). The different DNA damage checkpoints share many components that are evolutionarily well conserved (**Table 1**). The highly conservation of DNA damage response pathways throughout evolution emphasizes the importance of preserving genome integrity for all organisms. This allows the combined

study of these surveillance mechanisms in a variety of organisms ranging from yeast to humans.

**THE PI3K-RELATED FAMILY OF KINASES.** The key components of DNA damage checkpoint pathways are protein kinases belonging to phosphatidylinositol 3' kinase-like kinases (PIKKs) family. The members of this family are protein kinases structurally related to phosphatidylinositol 3' kinase and play an essential role in the DDR. *S. cerevisiae* has two PIKK proteins, Mec1, the homolog of human ataxia-telangiectasia and Rad3-related (ATR) and *S. pombe* Rad3, and Tel1, the homolog of human ataxia telangiectasia mutated (ATM) and *S. pombe* Tel1, that function as both damage sensors and signal transducers, but lacks a homolog of the human DNA-dependent protein kinase, DNA-PKcs (Putnam *et al.*, 2009). Both Mec1 and Tel1 kinases preferentially phosphorylate serine and threonine residues followed by a glutamine residue (SQ/TQ consensus motif) on numerous target proteins in response to DNA damage. Although several Mec1/Tel1 targets were already characterized, the use of improved proteomics approaches has led to the identification of many new putative Mec1/Tel1 targets, thus expanding our knowledge of checkpoint-mediated response.

Mec1 and Tel1 are loaded onto damaged DNA through the interaction with protein complexes that recognize specific DNA structures or with DNA intermediates generated by DNA repair processes. Indeed, Mec1 binds to Ddc2, the homolog of the human ATR interacting protein (ATRIP), which recognizes single-stranded DNA (ssDNA) bound by replication protein A (RPA) (Zou and



Elledge, 2003), while Tel1 binds to the DNA end-binding Mre11-Rad50-Xrs2 (MRX) complex (Nakada *et al.*, 2003).

Since the *mec1Δ tel1Δ* double mutant shows a synergistically increased sensitivity to DNA damaging agents (Morrow *et al.*, 1995) and has an higher rate of spontaneous genome rearrangement, compared to single mutants (Myung and Kolodner, 2002), it is thought that Mec1 and Tel1 have partially redundant functions. However, several lines of evidence indicate a functional distinction between ATM- and ATR-dependent pathways in both yeasts and mammals. In fact, human ATR responds to UV-induced DNA damage, double-strand breaks (DSBs), and stalled replication forks, while human ATM seems to respond primarily to DSBs (Shiloh, 2003). Similarly, *S. cerevisiae* Mec1 and *S. pombe* Rad3, more closely related to human ATR, are the principal transducers of the DNA damage and replication stress signals, while yeast Tel1, homolog of human ATM, is likely involved only in signaling DSBs (Nyberg *et al.*, 2002).

**Mec1/ATR ACTIVATION.** It has been shown that *S. cerevisiae* Mec1, as well as other ATR-related proteins, works in stably association with a partner that likely functions as a regulatory subunit for the kinase. In fact, Mec1 physically interacts with the checkpoint protein Ddc2 (also called Lcd1 or Pie1) (Paciotti *et al.*, 2000; Rouse and Jackson, 2000; Wakayama *et al.*, 2001), functionally related to Rad26 and ATRIP, which binds Rad3 and ATR in *S. pombe* and human cells, respectively (Edwards *et al.*, 1999; Cortez *et al.*, 2001). Following DNA damage, Mec1 and Ddc2 are recruited onto the lesion independently of other checkpoint proteins (Kondo *et al.*, 2001; Melo *et al.*, 2001; Rouse and Jackson, 2002) and Mec1-dependent Ddc2 phosphorylation does not require other

known checkpoint factors *in vivo* (Paciotti *et al.*, 2000). This suggests a crucial role for Mec1-Ddc2 complex in recognizing DNA alterations. Similarly, ATR colocalizes with ATRIP in nuclear foci after DNA damage, indicating that the ATR-ATRIP complex is recruited onto damaged DNA as well as Mec1-Ddc2 (Zou *et al.*, 2002). Furthermore, the ssDNA binding complex RPA has been shown to promote the binding of ATRIP to ssDNA in human cells *in vitro*, indicating that ATRIP binding to RPA-coated ssDNA enables the ATR-ATRIP complex to associate with damaged DNA and stimulates checkpoint activation (Zou and Elledge, 2003). Similarly, Ddc2 is recruited to DSBs in an RPA-dependent manner.

Even if Mec1-Ddc2 complex seems to respond directly to DNA insults, a full Mec1-dependent activation of downstream targets requires other damage sensors, like Rad24 and the PCNA-like Ddc1-Rad17-Mec3 complex (the *S. cerevisiae* homologs of the human Rad9-Hus1-Rad1 or 9-1-1 complex). The PCNA-like Ddc1-Rad17-Mec3 complex is loaded onto partial duplex DNA via the Rad24-Rfc2-5 alternative replication factor C (RFC) complex independently of Mec1-Ddc2 (Kondo *et al.*, 2001; Melo *et al.*, 2001). Colocalization of Mec1-Ddc2-RPA and the 9-1-1 complex in the context of partial duplex DNA lesion sites results in Mec1 full activation (Majka *et al.*, 2006), indicating that DNA plays a passive scaffolding role in checkpoint activation. Activation of Mec1 is also mediated by Dpb11, the *S. cerevisiae* homolog of TopBP1, and Dpb11 is synergistic with the 9-1-1 complex (Mordes *et al.*, 2008; Navadgi-Patil and Burgers, 2008). The combination of the 9-1-1 complex and Dpb11 in activation of Mec1 is highly conserved, and differs from fission yeast, *Xenopus* and human systems only in that the 9-1-1 complex in those organisms cannot activate the

Mec1 homolog in the absence of the Dpb11 homolog (Furuya *et al.*, 2004; Delacroix *et al.*, 2007; Lee *et al.*, 2007).

**Tel1/ATM ACTIVATION.** In budding yeast, Mec1 is central in responding to different types of DNA lesions and replication stress, whereas Tel1 has only a minor role in the DNA damage response. Indeed, Tel1 seems to respond to unprocessed DSBs by controlling a checkpoint that becomes apparent only in the absence of Mec1 (Usui *et al.*, 2001). The ATM/Tel1-dependent checkpoint involves additional proteins. In fact, a highly conserved trimeric complex, known as Mre11–Rad50–Nbs1 (MRN) in mammals and Mre11–Rad50–Xrs2 (MRX) in *S. cerevisiae*, is substrate for the ATM/Tel1 kinase (Gatei *et al.*, 2000; Lim *et al.*, 2000; D’Amours and Jackson, 2001, 2002; Grenon *et al.*, 2001). In response to DNA damage, mammalian Mre11 and Nbs1 are phosphorylated in an ATM-dependent manner (Gatei *et al.*, 2000; Lim *et al.*, 2000; Wu *et al.*, 2000; Zhao *et al.*, 2000; D’Amours and Jackson, 2002; Falck *et al.*, 2002), as well as DNA damage stimulates Tel1-dependent phosphorylation of *S. cerevisiae* Mre11 and Xrs2 (D’Amours and Jackson, 2001; Grenon *et al.*, 2001). The MRN/MRX complex is required for checkpoint response in both mammals (D’Amours and Jackson, 2002) and yeast, as in *S. cerevisiae* *mre11*, *rad50* and *xrs2* mutant cells are defective in the activation of the S-phase checkpoint in response to DSB-inducing agents and hydroxyurea (HU), a drug that causes replicative stress by inhibiting the synthesis of deoxyribonucleotides (dNTPs) (D’Amours and Jackson, 2001; Grenon *et al.*, 2001). Moreover, it has been shown that the MRN complex stimulates ATM kinase activity *in vitro* by facilitating stable substrate binding (Lee and Paull, 2004).

Aside from checkpoint activation, the MRN/MRX has been implicated in multiple eukaryotic functions, all fundamental for the maintenance of genome integrity and mainly related to the response to DSBs. These functions include: DSB repair, telomere maintenance, checkpoint signaling, meiotic recombination and, during DNA replication, response to stalled replication forks and resolution of DNA hairpins (Slijepcevic, 2006; Borde, 2007; Williams *et al.*, 2007). MRN/MRX has a wide spectrum of biochemical abilities: DNA binding by multiple subunits and tethering of DNA molecules through interactions between MRN/MRX complexes, incision of the DNA phosphodiester backbone through its single-stranded endonuclease activities and ATP hydrolysis. Thanks to its numerous abilities, the MRN/MRX complex is a key component of the immediate early response to DNA damage, involved in a cross-talk between the repair and the checkpoint machinery (Rupnik *et al.*, 2009).

Although Mec1 and Tel1 do not respond equally to different types of DNA damage, they play some overlapping functions. In fact, *TEL1* deletion increases the sensitivity of *mec1* mutants to DNA damaging agents (Ritchie *et al.*, 1999). Moreover, high levels of Tel1 can suppress both cell lethality and hypersensitivity to DNA damaging agents of *mec1Δ* strains, indicating that an excess of Tel1 can bypass both the essential and the DNA damage response function of Mec1 (Sanchez *et al.*, 1996). In contrast to Mec1, Tel1 function does not require Ddc2. In fact, overexpression of *TEL1*, but not that of *MEC1*, can suppress the hypersensitivity to genotoxic agents of *ddc2Δ* cells, implying that high levels of Tel1 can bypass the requirement for Ddc2 (Clerici *et al.*, 2001).

**Rad53/Chk2 AND Chk1 EFFECTOR KINASES AND THEIR MEDIATORS.** Once DNA lesions are recognized, checkpoint signals are propagated through the evolutionary conserved effector kinases Chk1 and Rad53/Chk2, which also undergo phosphorylation in response to DNA damage in a Mec1/ATR- and Tel1/ATM-dependent manner (Sanchez *et al.*, 1996, 1999; Usui *et al.*, 2001). In response to DNA damage and replication stress, it seems that the requirement for effector kinases in checkpoint signal propagation has evolved differently in different eukaryotes. In *S. cerevisiae*, while Chk1 is required only for the DNA damage G2/M checkpoint, Rad53 is essential for the proper response to DNA damage in all cell cycle phases and to replication blocks (Gardner *et al.*, 1999; Sanchez *et al.*, 1999). Phosphorylation of the Rad53 protein kinase by Mec1 and Tel1 leads to its activation and subsequent autophosphorylation; the resulting hyperphosphorylated Rad53 is frequently used as an experimental marker for monitoring activation of the DNA damage response. In contrast, the fission yeast orthologue of Rad53, Cds1, is a specific effector of the checkpoint responding to DNA damage during S-phase and replication blocks, while Chk1 is specifically involved in checkpoint signaling in G2 (Lindsay *et al.*, 1998; Brondello *et al.*, 1999). Conversely, in human cells and *Xenopus*, Chk1 appears to be the principal effector for the DNA replication checkpoint, while the Rad53 homologue, Chk2, is mainly involved in the response to DSBs (Guo *et al.*, 2000; Nyberg *et al.*, 2002). The divergent specialization of these proteins in different eukaryotic systems may depend on the presence of DNA damage-specific or S-phase-specific mediators that link the DNA damage-sensing functions with the downstream effectors. The principal *S. cerevisiae* mediator is Rad9 that acts as a scaffold protein upon which Rad53 autophosphorylates and self-activates. It

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has been proposed that, upon Mec1-dependent phosphorylation (Emili, 1998; Sun *et al.*, 1998; Vialard *et al.*, 1998), Rad9 acts first as an adaptor to promote Mec1-Rad53 interaction and Mec1-mediated Rad53 phosphorylation/activation (Schwartz *et al.*, 2002; Sweeney *et al.*, 2005). Then, phosphorylated Rad9 protein completes the Rad53 activation process by promoting in trans Rad53 autophosphorylation, perhaps by increasing the Rad53 local concentration on the Rad9 surface, thus indicating that Mec1 can regulate both sensing and transducing checkpoint signals (Gilbert *et al.*, 2001). Rad9 also contributes to Chk1 activation with a mechanism involving its N-terminal portion, which is not required for Rad53 activation (Blankley and Lydall, 2004). However, Rad9 has no role in responding to DNA replication stress (Navas *et al.*, 1996; Neecke *et al.*, 1999). Rad9 counterpart in response to replication stress is Mrc1, which also actively participates in DNA replication (Alcasabas *et al.*, 2001; Tanaka and Russell, 2001; Katou *et al.*, 2003; Osborn and Elledge, 2003). Similarly, different mediators function during S and G2 phases in *S. pombe*, where Mrc1 promotes Cds1-mediated checkpoint signaling in S-phase, while Crb2 mediates full Chk1 activation in G2 (Furuya and Carr, 2003).

#### **THE CHECKPOINT-MEDIATED CONTROL OF THE CELL CYCLE PROGRESSION.**

Once activated, the checkpoint effector kinases phosphorylate several downstream targets, thus regulating a variety of cellular processes. One of the primary events governed by the checkpoint response is the cell cycle arrest, which is induced by the phosphorylation of different substrates depending on the cell cycle phase in which the DNA damage is detected.

When the damage occurs during the G1 phase, most eukaryotic cells show a pronounced delay of the G1/S transition that allows vital time to repair the lesion and avoids replication of a damaged template. Otherwise, uncontrolled DNA replication in the presence of a lesion may, for example, convert a gap chromosome in two sister chromosomes, one of which containing a DSB. However, the role of the G1 checkpoint remains controversial since it has different relevance in vertebrates compared to yeasts. For instance, in budding yeast the G1 checkpoint exists but is very weak and it usually leaves the damage unrepaired (Siede *et al.*, 1994; Gerald *et al.*, 2002). This delay in the G1/S transition is due to Rad53-dependent phosphorylation of the Swi4/6 transcription factors, thus inhibiting the transcription of G1 cyclins, thereby slowing entry into S-phase (Sidorova and Breeden, 1997). In contrast, DNA damage induces a very robust G1 arrest in higher eukaryotes, that appears to have two wave of action (Bartek and Lukas, 2001). The first one is immediate and p53-independent, it provides time for repair but it lasts only few hours. This first delay is due to ATM-dependent activation of Chk2 that targets Cdc25A to degradation (Mailand *et al.*, 2000), thus resulting in inhibition of Cdk2-cyclin E association, an essential step for S-phase entry. Then, a slower and sometimes irreversible second G1 delay involves ATM/ATR- and Chk1/Chk2-dependent activation and stabilization of p53, causing transcriptional induction of p21, which then inhibits the Cdk2-cyclin E complex (Sherr and Roberts, 1999; Ekholm and Reed, 2000). Both these mechanisms function to remove damaged cells from cycling, and so they may both play prominent roles in cancer evasion.

In budding yeast, the G2/M checkpoint arrests cells at the metaphase to anaphase transition. At the onset of anaphase, a caspase-related protease (separase) destroys the link between sister chromatids by cleaving the cohesin subunit Scc1 (Uhlmann, 2003). During most of the cell cycle, separase is kept inactive by binding to an inhibitory protein called Pds1/securin, which is degraded by ubiquitin-mediated proteolysis shortly before the metaphase to anaphase transition (Cohen-Fix *et al.*, 1996). Hence, one of the mechanisms by which the DNA damage checkpoint prevents this cell cycle transition is the inhibition of Pds1 ubiquitination by the ubiquitin ligase anaphase-promoting complex (APC) and its regulatory subunit Cdc20, thus stabilizing the securin and preventing anaphase to occur. The stabilization of Pds1 in response to DNA damage is achieved by at least two independent mechanisms involving both Chk1 and Rad53 checkpoint effector kinases. On the one hand, phosphorylation of Pds1 by Chk1 blocks its APC-dependent degradation (Sanchez *et al.*, 1999), on the other hand, Pds1 is further stabilized through Rad53-dependent inhibition of the Pds1–Cdc20 interaction, which recruits the APC ubiquitin ligase (Agarwal *et al.*, 2003). Beside the DNA damage checkpoint, even the spindle assembly checkpoint (SAC) increases Pds1 stability to inhibit anaphase onset when the mitotic apparatus is not properly assembled. Indeed, the SAC protein Bub3, Mad2 and Mad3 physically interact with the APC regulatory subunit Cdc20, required for Pds1 ubiquitination, likely leading to Cdc20 inhibition (Musacchio and Hardwick, 2002). There seems to be an interaction between the DNA damage checkpoint and the spindle assembly checkpoint, as Rad9 and Rad53 are phosphorylated after nocodazole arrest, which both activates the spindle assembly checkpoint and prevents the degradation of Pds1. This



phosphorylation is independent of Mec1 and Tel1, but is abolished by deletion of the spindle assembly checkpoint genes *MAD2* or *BUB1* (Clémenson and Marsolier-Kergoat, 2006). These types of checkpoints crosstalk may provide a mechanism for generating a more robust cell cycle arrest phenotype.

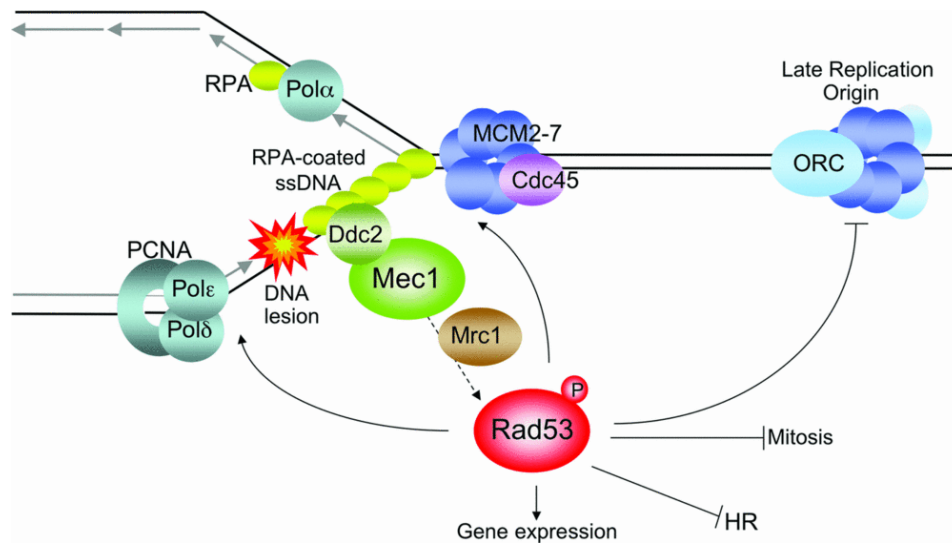
In addition, both Rad53 and Chk1 appear to suppress also the later stage of mitotic exit by preventing the release of the Cdc14 phosphatase from a complex with Net1 in the nucleolus, thus inhibiting the mitotic exit network (MEN) and Cdc-fourteen early anaphase release (FEAR) pathways (Liang and Wang, 2007). Release of Cdc14 marks the end of mitosis through dephosphorylation of Cdk1 targets, leading to the degradation of mitotic cyclins, induction of transcription of the *SIC1* gene encoding a Cdk1 inhibitor, and stabilization of the expressed Sic1 protein (Visintin *et al.*, 1998).

The scenario is quite different in other organisms. For example, both fission yeast and higher eukaryotes target the Cdk Cdc2 to maintain its inhibitory phosphorylations as a principal mean to block the G2/M transition. This is achieved by acting on various parallel pathways, including the phosphatases that promote mitosis, the kinases that block Cdc2 function, and other proteins that act on these regulators, which all converge in modulating Cdc2 activity. In mammals, ATM, ATR, Chk1, Chk2, the polo kinase Plk1, PCNA, p21, the 14-3-3 proteins and many other factors are involved in these different mechanisms that act in concert to delay G2/M transition in the presence of DNA damaged molecules (Nyberg *et al.*, 2002).

**THE S-PHASE CHECKPOINT.** The replication of DNA is a very complex process, that needs to occur accurately, rapidly, and only once per cell cycle in order to

prevent genome abnormalities and deleterious loss of genetic information. Hence, problems arising during chromosome replication are inherent to the complexity of the process and a major source of genomic instability. They are aggravated and frequently caused by exogenous environmental agents and reactive metabolic products that constantly damage the DNA, thus generating potential obstacles to the progression of replication forks. In addition, particular regions in the genome constitute a challenge to replication-fork movement and are associated to a high incidence of chromosomal rearrangements. In all these cases, replication forks must maintain their integrity in order to be able to finish chromosome replication accurately when conditions that halt them are eliminated (Paulsen and Cimprich, 2007; Tourrière and Pasero, 2007; Friedel *et al.*, 2009). To cope with such situations of replicative stress, eukaryotic cells activate the so-called S-phase checkpoint, which detects the replication problems and coordinates a global response to maintain genome integrity (Zhou and Elledge, 2000; Harrison and Haber, 2006). In budding yeast, the central players of the S-phase checkpoint are Mec1 and Rad53 kinases, which get activated under conditions that threaten DNA replication, such as DNA damage or nucleotide depletion (Branzei and Foiani, 2007; Paulsen and Cimprich, 2007; Tourrière and Pasero, 2007). The S-phase checkpoint activation requires the establishment of DNA replication forks (Lupardus *et al.*, 2002; Stokes *et al.*, 2002; Tercero *et al.*, 2003) and the generation of ssDNA. The accumulation of ssDNA regions at stalled forks occurs probably because the MCM (*minichromosome maintenance complex*) helicase continues DNA unwinding, although uncoupled from DNA synthesis (Sogo *et al.*, 2002; Byun *et al.*, 2005; Nedelcheva *et al.*, 2005). RPA binds the ssDNA and

triggers the recruitment of Mec1/ATR at stalled forks by its regulatory subunit, Ddc2/ATRIP (Zou *et al.*, 2003). Mec1 then phosphorylates Mrc1 (the homologue of human Claspin), a component of the replication machinery and a checkpoint mediator that transduces the signal from Mec1 to the effector kinase Rad53 (Alcasabas *et al.*, 2001), which becomes phosphorylated and activated. The checkpoint response during S-phase is depicted schematically in **Figure 3**.



**Figure 3. A schematic illustration of the S-phase checkpoint response.**

When replication forks hit DNA lesions or stall because of dNTP deprivation, the helicase and the polymerases may uncouple, exposing regions of ssDNA that cause the activation of the checkpoint response. RPA binds to ssDNA and triggers the recruitment of *S. cerevisiae* Mec1 to the stalled fork by its regulatory subunit Ddc2. Mec1 phosphorylates the mediator Mrc1 and the signal is transduced to the downstream effector kinase Rad53, which is phosphorylated and activated. Rad53 maintains stable, functional DNA replication forks, inhibits firing of late origins, activates gene expression and prevents entry into mitosis and unscheduled recombination. Abbreviations: HR, homologous recombination; PCNA, proliferating cell nuclear antigen (adapted from (Segurado and Tercero, 2009)).

The S-phase checkpoint response coordinates DNA replication, DNA repair and cell-cycle progression and regulates processes such as firing of replication origins (Santocanale and Diffley, 1998; Shirahige *et al.*, 1998; Santocanale *et al.*, 1999), stabilization of DNA replication forks in response to DNA damage or replicative stress (Lopes *et al.*, 2001; Tercero and Diffley, 2001), resumption of stalled DNA replication forks (Desany *et al.*, 1998; Szyjka *et al.*, 2008), transcriptional induction of DNA damage response genes (Allen *et al.*, 1994), choice of the repair pathway (Kai *et al.*, 2007) and inhibition of mitosis until replication is completed (Allen *et al.*, 1994).

Many studies have been performed with the aim to identify the targets of the checkpoint at DNA replication forks, but to date the whole view seems to be yet incomplete. Obvious checkpoint target candidates were the components of the replication machinery, and several studies suggest that the checkpoint stabilizes the association of the replisome with the replication fork. However, it is not completely clear whether those results reflect that the replisome is targeted directly by the Mec1/Rad53 kinases or are a consequence of an indirect effect (Segurado and Tercero, 2009). For example, checkpoint-dependent phosphorylation of the Pol $\alpha$  polymerase seems to be important to stabilize the replisome (Pelliccioli *et al.*, 1999; Lemoine *et al.*, 2005). Another putative target of the S-phase checkpoint is RPA (Zou and Elledge, 2003; Binz *et al.*, 2004), which is phosphorylated in a Mec1-dependent manner (Brush *et al.*, 1996), although the relevance of this modification for fork stabilization is currently unknown. Also the Mrc1 transducer is phosphorylated in a checkpoint-dependent manner (Alcasabas *et al.*, 2001). This protein is usually associated with the replication fork, but under replicative stress conditions

Mrc1 forms a pausing complex with Tof1 that is essential to avoid the uncoupling of the replication machinery components from the place of DNA replication (Katou *et al.*, 2003). Even the MCM-helicase complex seems to be an interesting checkpoint target, since disassembly of Mcm2-Mcm7 from stalled replication forks causes loss of viability in the presence of replicative stress (Labib *et al.*, 2000) and it has been shown that MCMs undergo ATR/ATM-dependent phosphorylation in metazoans (Ishimi *et al.*, 2003; Cortez *et al.*, 2004; Yoo *et al.*, 2004; Shi *et al.*, 2007; Trenz *et al.*, 2008). The stabilization of stalled replication forks by the S-phase checkpoint is also thought to prevent unscheduled recombination (Meister *et al.*, 2005; Lambert *et al.*, 2007). The mechanisms by which the S-phase checkpoint restrains recombination at the replication forks are not well understood, but several proteins required for this process appear to undergo checkpoint-dependent phosphorylation (Lambert *et al.*, 2007). For instance, in *S. pombe*, the recombination proteins Mus81 and Rad60 are regulated negatively by the checkpoint, with the former being dissociated from chromatin by Cds1-mediated phosphorylation (Kai *et al.*, 2005), and the last being delocalized from the nucleus upon checkpoint activation (Boddy *et al.*, 2003). Increasing evidence indicates that the checkpoint targets also chromatin remodelers, such as the Ies4 subunit of the Ino80 complex (Morrison *et al.*, 2007), and histone regulating enzymes, such as the Hst3 deacetylase (Thaminy *et al.*, 2007), both these classes of enzyme are required for maintenance of functional DNA replication forks.

Aside the stabilization of stalled replication forks, another key feature of the S-phase checkpoint response is the regulation of dNTPs production. In fact, cells need to tightly control ribonucleotide reductase (RNR) activity in order to

synthesize just enough dNTPs during S phase for efficient chromosome replication, as too much or too little dNTPs can be mutagenic (Chabes *et al.*, 2003). In fact, reduced dNTP levels slow fork progression and stimulate chromosome instability (Zhao *et al.*, 2001), thus making an important contribution in the early stages of cancer development, following oncogene activation (Bester *et al.*, 2011). Indeed, from yeasts to humans, the *RNR* genes are part of a large set of genes that are normally induced during S phase and then repressed afterwards (Elledge *et al.*, 1992; de Bruin and Wittenberg, 2009). In response to replication defects, the S-phase checkpoint kinases preserve the S-phase transcriptional program, maintaining the expression of *RNR* genes as well as many other genes that encode replication factors (De Bruin and Wittenberg, 2009). In fact, the *RNR* genes are regulated by a repressor known as Crt1 (constitutive *RNR3* transcription), which is inhibited by Dun1 kinase in response to checkpoint activation, leading to greatly increased expression of *RNR* (Zhou and Elledge, 1993; Huang *et al.*, 1998). Furthermore, *RNR* is also post-translationally regulated by the S-phase checkpoint pathway. In fact, the budding yeast *MEC1* and *RAD53* genes are essential for cell viability even in the absence of exogenous sources of replication stress or DNA damage. Mutation of a gene called *SML1* (suppressor of *Mec1* lethality) was found to suppress the lethality of *mec1Δ* or *rad53Δ*, as did increased expression of *RNR* genes (Desany *et al.*, 1998; Zhao *et al.*, 2001). The Sml1 protein represents a direct inhibitor of *RNR* that must be degraded in each round of the cell cycle when budding yeast cells enter S phase, and that is also degraded in response to DNA damage and replication defects (Zhao *et al.*, 2001). Moreover, it has been recently unveiled a new checkpoint-dependent regulation of the *RNR*

activity. The small subunit of RNR is normally hidden in the nucleus away from the mainly cytoplasmic large subunit, until S-phase or checkpoint activation. The Dif1 protein (*damage regulated import facilitator*) takes the small RNR subunit to the nucleus where it is tethered by another factor, and the Mec1–Rad53–Dun1 pathway phosphorylates Dif1 upon checkpoint activation and promotes its degradation (Lee *et al.*, 2008; Wu and Huang, 2008). This fine-tuned regulation of RNR activity in response to replication stress seems conserved even in mammalian cells, although metazoan orthologues of Sml1/Dif1 are still to be identified.

Finally, another key feature of the S-phase checkpoint response, which is conserved from yeasts to mammals, is the regulation of replication origins firing. As defective replication forks are a major potential source of chromosome instability, delaying the firing of new origins in response to replication defects will allow the cell to avoid the accumulation of more defective forks. Moreover, it seems likely that some of the factors that are needed at forks are present at limiting levels (dNTPs is one example), and this provides another reason for a mechanism that prevents too many forks from being formed at the same time (Labib and De Piccoli, 2011). Very recent work with budding yeast has identified the main features of the mechanism by which the Rad53 kinase blocks the initiation step of replication (Lopez-Mosqueda *et al.*, 2010; Zegerman and Diffley, 2010; Duch *et al.*, 2011). Rad53 phosphorylates two factors that play a key role during the initiation of chromosome replication at each origin: the Dbf4 subunit of the Cdc7 kinase, and the Sld3 protein. Inhibiting these factors prevents the activation of the replicative DNA helicase at origins and so blocks the establishment of DNA replication forks. Once the

source of the damage or replication defect has been dealt with and the checkpoint has been inactivated, the previously silenced origins can give rise to new forks that will aid the completion of chromosome replication.

As the S-phase checkpoint coordinates multiple cellular processes, such as DNA replication and repair and cell cycle progression, genome integrity is profoundly affected when the S-phase checkpoint is defective. Thus, yeast cells mutated for checkpoint components show significantly increased chromosomal rearrangement (Myung *et al.*, 2001; Kolodner *et al.*, 2002; Myung and Kolodner, 2002). Furthermore, in *S. cerevisiae*, *MEC1* and *RAD53* are essential genes, but the lethality can be avoided by increasing the levels of dNTP (Desany *et al.*, 1998; Zhao *et al.*, 1998). However, the checkpoint functions cannot be rescued by simply regulating dNTP levels (Desany *et al.*, 1998). In fact, *mec1* and *rad53* checkpoint mutants are extremely sensitive to agents that cause replicative stress, such as HU (hydroxyurea), that depletes the dNTP pool by inhibiting the ribonucleotide reductase. These mutants are also highly sensitive to different forms of DNA damage, such as DNA alkylation originated by MMS (methyl methanesulfonate), ionizing radiation or UV light (Segurado and Tercero, 2009).

The Mec1/Rad53 checkpoint regulates at least two aspects of DNA replication: forks stabilization and late origin firing. Using a hypomorphic *mec1* mutant, *mec1-100* (Paciotti *et al.*, 2001), which has a delayed and reduced level of Rad53 activation in response to HU or MMS, it was shown that forks stabilization and late origins firing are genetically separable functions (Tercero *et al.*, 2003). Even though *mec1-100* mutant cells cannot block the firing of late replication origins in HU or MMS, as *mec1Δ* mutant, they are not hypersensitive



to HU or MMS, thus indicating that the stabilization of DNA replication forks is the most critical checkpoint function to ensure cell survival in the presence of replicative stress.

In *S. cerevisiae*, this checkpoint function was proposed by parallel studies on the integrity of DNA replication forks in response to DNA damage by MMS or HU blocks (Lopes *et al.*, 2001; Tercero and Diffley, 2001). Monitoring the progression of replication forks in the presence of MMS, it was found that MMS diminishes drastically the replication fork rate in both wild-type and mutant cells, which is probably the consequence of a physical impediment of replication-fork progression either by methylated DNA bases or some intermediates formed during the processing of damaged DNA (Vázquez *et al.*, 2008). Unlike wild-type cells, whose replication forks move slowly through alkylated DNA, but continue to progress and finish replication, a high proportion of DNA remains unreplicated in checkpoint mutants under the same conditions. These experiments showed that DNA replication forks terminate irreversibly at a high rate in *mec1* and *rad53* mutants, very likely accounting for the elevated lethality of checkpoint mutant cells after exposure to MMS. Similarly, it has been demonstrated that the S-phase checkpoint was required to stabilize DNA replication forks under replicative stress originated by the decrease in dNTP levels caused by HU (Lopes *et al.*, 2001). The failure in completing DNA replication in *rad53* mutant cells after the HU block is irreversible, as unusual DNA structures, resulting from the collapse of stalled replication forks, persist if the HU is removed, and adding new Rad53 does not restore the replication defects (Lopes *et al.*, 2001; Tercero *et al.*, 2003; Rouse, 2004). Therefore the function of Mec1/Rad53 at replication forks is not only to

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resume DNA synthesis after fork stalling. Instead, these checkpoint proteins are required to avoid catastrophic irreversible events that cause the collapse of DNA replication forks and correlate with cell death.

Interestingly, the requirement of the S-phase checkpoint for the integrity of DNA replication forks seems to be conserved in both fission yeast and higher eukaryotes (Segurado and Tercero, 2009). However, despite extensive studies and considerable progress, the precise mechanisms by which the replication checkpoint prevents fork collapse remain elusive. The emerging picture from recent works suggests that it is a much more complex task than first thought.

**DNA DAMAGE CHECKPOINT AND REPAIR.** The arrest of the cell cycle progression and the control of DNA replication are key features of the checkpoint response. However, the checkpoint kinases, once activated, can phosphorylate many other cellular targets in order to regulate different cellular processes. For instance, the arrest of the cell cycle is likely required to allow DNA repair. Hence, numerous proteins directly involved in DNA repair pathways have been identified as targets of the checkpoint kinases (**Table 2**). In some cases, such as Rad55, Rtt107, and Nej1, the phosphorylation appears to play important functional roles (Rouse, 2004; Herzberg *et al.*, 2006; Roberts *et al.*, 2006; Ahnesorg and Jackson, 2007). In others, such as Rfa1, Rfa2, and Xrs2, the role of the phosphorylation during mitotic DNA repair is less clear (Brush and Kelly, 2000; D'Amours and Jackson, 2001; Mallory *et al.*, 2003; Bartrand *et al.*, 2006). Although the data accumulated to date are still insufficient to define the specific pathways targeted by checkpoint activation, they indicate that the activities of at least some DNA repair proteins in *S. cerevisiae* are modified after

DNA damage, suggesting a post-translational regulatory mechanism for DNA repair proteins.

**Table 2.** Known DNA repair substrates of checkpoint activation.

Substrate	Kinase
Rfa1	Mec1, Tel1
Rfa2	Mec1, Tel1
Xrs2	Tel1
Mre11	Tel1
Rad55	Rad53; possibly Mec1, Tel1
Slx4	Mec1, Tel1
Rtt107 (Esc4)	Mec1; requires Slx4
Nej1	Dun1
Exo1	Kinase pathway; possibly Rad53
Srs2	Kinase pathway+Cdk1
Sae2	Mec1, Tel1
Cdc13	Mec1, Tel1
Mdc1 (Scc1)	Chk1

adapted from (Putnam *et al.*, 2009)

The DNA damage checkpoint targets also chromatin factors. In fact, considering that DNA packaging into chromatin might restrict the access to the sites of damage by the repair machinery, it is not surprising that chromatin structure and composition can be manipulated to facilitate DNA repair (reviewed in (Wurtele and Verreault, 2006)). One of the best-characterized chromatin modification events in DNA damage response is the phosphorylation of the Ser129 on histone H2A by the Mec1 and Tel1 kinases (Morrow *et al.*, 1995), which is equivalent to the ATR/ATM-dependent phosphorylation of the alternative metazoan H2A subunit H2AX on Ser139 (also called  $\gamma$ -H2AX) and has been largely used as an experimental marker for checkpoint activation. In

response to an induced DSB, Mec1 and Tel1 generate a unique  $\gamma$ -H2AX-containing chromatin domain that can extend at least 10 kb from the lesion (Downs *et al.*, 2004). In yeast,  $\gamma$ -H2AX domains promote effective DSB repair by favouring the accumulation of checkpoint and repair proteins to DSBs and by recruiting cohesin complex, the NuA4 histone acetyltransferase and ATP-dependent chromatin remodelers, such as Ino80 and Swr1 complexes (Downs *et al.*, 2004; Morrison *et al.*, 2004; van Attikum *et al.*, 2004). In *S. cerevisiae*, the NuA4 catalytic subunit Esa1 acetylates the N-terminal tail of histone H4. Both lysine substitution in the N-terminal H4 tail and inactivation of the NuA4 subunits Esa1 or Yng2 cause hypersensitivity to DSB-inducing agents (Bird *et al.*, 2002; Downs *et al.*, 2004), suggesting a function for NuA4 and histone H4 acetylation in DSB repair. The Ino80 and Swr1 complexes belong to the SWI/SNF chromatin remodelers superfamily, and are implicated in both transcription and DSB repair. Based on lessons learned from transcription, NuA4, Ino80 and Swr1 could promote the formation of a nucleosome-free region around the damage sites in order to facilitate the access to DNA or its processing by repair proteins (Tsukuda *et al.*, 2005).

## **DNA REPAIR DURING THE CELL CYCLE**

DNA lesions are very heterogeneous and include dNTP misincorporation, interconversion between DNA bases caused by deamination and loss and modification of DNA bases following DNA depurination and alkylation respectively. Moreover, oxidized DNA bases and DNA breaks can be generated in response to both endogenous and exogenous sources of DNA damage. To counteract all these DNA lesions, cells have evolved different repair mechanisms specific for different types of DNA damage. For instance, mispaired DNA bases are replaced with correct bases by mismatch repair (MMR), whereas small chemical alterations of DNA bases are repaired by base excision repair (BER) through excision of the damaged base. More complex lesions, such as pyrimidine dimers and intrastrand crosslinks, are corrected by nucleotide excision repair (NER) through the removal of an oligonucleotide of approximately 30 bp containing the damaged bases (Ciccia and Elledge, 2010). DNA repair is carried out by a plethora of enzymatic activities that chemically modify the DNA to reverse the lesion. However, these repair tools must be precisely regulated, otherwise they can be dangerous for the integrity of DNA if misused or allowed to access DNA at the inappropriate time or place. Thus, eukaryotic cells have developed strategies to recruit and activate the right factors in the right place at the right time.

**DNA DOUBLE-STRAND BREAK REPAIR.** DNA double-strand breaks (DSBs) are among the most deleterious types of DNA damage, because failures to repair

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them can lead to loss of genetic information and chromosome rearrangements. DSBs can occur spontaneously during normal metabolism or after exposure to environmental factors, such as ionizing radiation (IR) and radiomimetic chemicals. Efficient DSB repair is crucial in safeguarding genome integrity, hence cells have evolved two major pathways to repair them: non-homologous end joining (NHEJ) (**Figure 4A**) and homologous recombination (HR) (**Figure 4B and C**). Whereas NHEJ is able to ligate the two chromosomal ends with no, or minimal, base pairing at the junction (Moore and Haber, 1996), HR uses the genetic information stored in the sister chromatid or in the homologous chromosome to repair the DSB with high fidelity. On the contrary, even if NHEJ is a very effective mechanism for DSB repair, its ability to ligate essentially any pair of DNA ends makes it a potentially mutagenic repair mechanism.

Key NHEJ proteins are conserved from yeast to mammals, and include the DNA end-binding Ku70-Ku80 heterodimer, DNA ligase IV (Dnl4/Lig4 in *S. cerevisiae*) and its accessory factors Lif1/Xrcc4 and Nej1/Xlf (Daley *et al.*, 2005). The Ku heterodimer is thought to form a ring-like structure that binds to DNA ends and initiates the NHEJ process by recruiting DNA ligase IV. The latter will in turn catalyze ligation once the DNA ends are aligned and base pairing of the overhangs can take place. Moreover, the *S. cerevisiae* MRX (Mre11-Rad50-Xrs2) complex, but not its vertebrate and *S. pombe* counterparts MRN (Mre11-Rad50-Nbs1) (Manolis *et al.*, 2001; Di Virgilio and Gautier, 2005), works together with Ku in ensuring efficient NHEJ. It is thought that MRX structure is involved in bridging two broken DNA ends together and this MRX-dependent intrachromosomal association requires the zinc-hook motifs located in the coiled-coil region of Rad50 (Kaye *et al.*, 2004; Lobachev *et al.*, 2004; Wiltzius *et*

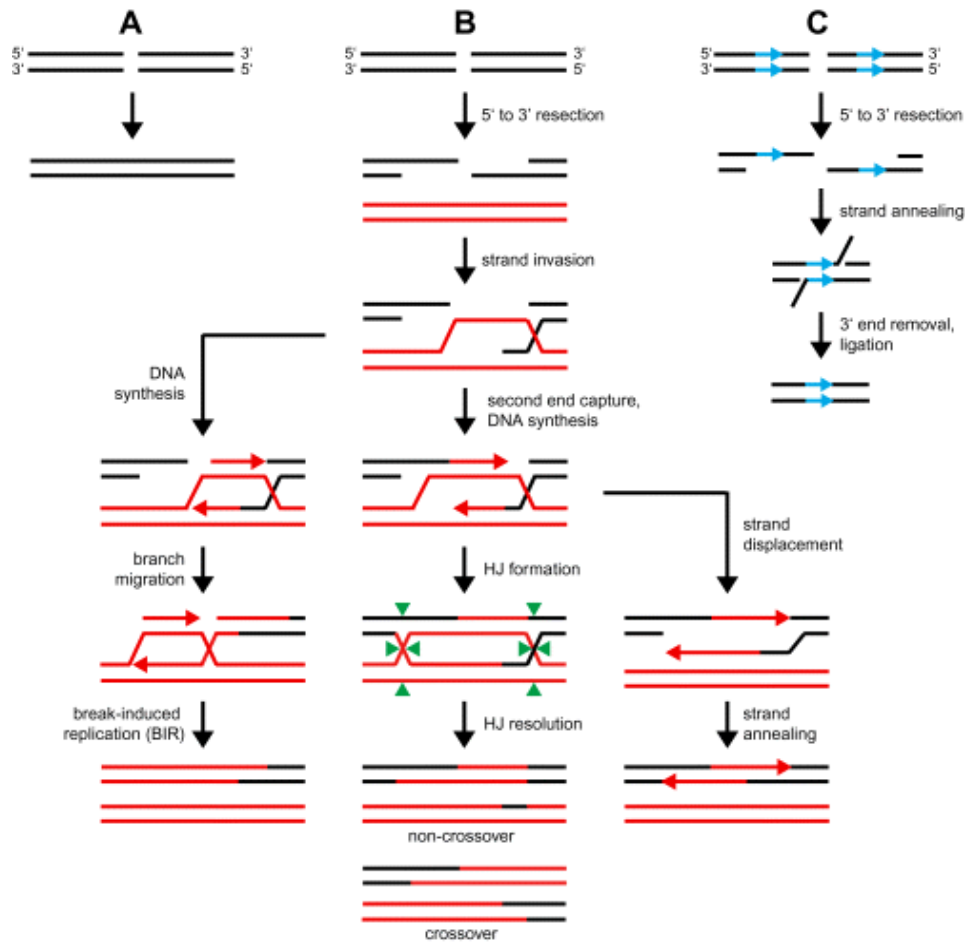
*al.*, 2005). Besides the tethering function, MRX might have a direct role in assisting yeast Ku (Yku) and Dnl4/Lif1, as suggested by the findings that MRX stimulates *in vitro* ligation by the Dnl4/Lif1 complex (Chen *et al.*, 2001) and interacts with Yku and Lif1 (Palmbos *et al.*, 2005).

All HR processes initiate with extensive 5' to 3' end-processing (a process referred to as 5'-3' resection) of the broken ends to yield 3'-ended single-stranded DNA (ssDNA) tails, which are bound by Replication Protein A (RPA) (Longhese *et al.*, 2010). RPA is then displaced by Rad51 to form nucleoprotein filaments that can catalyse homologous pairing and strand invasion (White and Haber, 1990; Sun *et al.*, 1991). Resection of the DSB ends is initiated by the MRX/MRN complex together with the Sae2/CtIP/Ctp1 protein, that remove oligonucleotides from the 5' strand. The resulting partially resected 5' DNA end can be further processed by the 5'-3' exonuclease Exo1, or by the combined activities of the Sgs1-Top3-Rmi1 complex and Dna2 (Mimitou and Symington, 2008; Zhu *et al.*, 2008; Cejka *et al.*, 2010; Niu *et al.*, 2010).

When DSB ends are resected by 5'-3' exonucleases to generate 3'-ended ssDNA tails, repair mechanisms are channeled into different homology-dependent recombination pathways (reviewed in (Krogh and Symington, 2004)). In the major pathway, the 3'-ended ssDNA tail invades the duplex homologous DNA region on a sister chromatid or on a homologous chromosome (**Figure 4B**). Following strand invasion, the 3' end is extended by DNA synthesis and can generate an unidirectional replication fork that migrates along the template chromosome, copying the genetic information in a process called break-induced replication (BIR) (**Figure 4B, left**). Alternatively, if the 3'-ended ssDNA tail at the other side of the DSB is captured (second end capture), subsequent

extension and ligation result in the formation of a double Holliday junction (HJ) intermediate, which can be resolved to give either crossover or noncrossover products depending on how the junctions are cut (**Figure 4B, center**). However, the newly synthesized invading strand can also be displaced from its template and anneal with the 3' ssDNA end at the other side of the DSB in a process called synthesis-dependent strand-annealing (SDSA) (**Figure 4B, right**). Finally, when a DSB occurs between direct repeats, the 3'-ended ssDNA tails can undergo intrachromosomal single-strand annealing (SSA), which results in DSB repair with concomitant deletion of one repeat and of the intervening sequence (**Figure 4C**). Genetic studies in *S. cerevisiae* have established that efficient HR requires MRX, RPA complex and proteins of the *RAD52* epistasis group (reviewed in (Krogh and Symington, 2004)).





**Figure 4. Mechanisms of DSB repair.**

A. When a DSB occurs, NHEJ machinery can rejoin the two chromosomal ends. Whereas ligation of blunt or compatible ends does not require end processing, nucleolytic removal or filling in by polymerases could be required to ligate DNA ends with partially or non-complementary overhangs. B and C. When DSB ends are resected to generate 3'-ended ssDNA tails, repair mechanisms can be channeled into different homology-dependent recombination pathways. (B, left) In the BIR model, 3'-ended ssDNA tail invades the duplex homologous DNA sequence (red lines) and establishes a replication fork that can proceed either to the chromosome terminus or until it meets a converging replication fork. (B, centre) Capture of the second ssDNA end, DNA synthesis and ligation create a double HJ, whose resolution can occur in either plane at

both junctions (green triangles) to generate crossover or non-crossover products. (B, right) In the SDSA model, the newly synthesized strand is displaced and anneals with the 3' ssDNA tail at the other end of the DSB. DNA synthesis completes repair. (C) Single strand annealing repair: when a DSB occurs between direct repeats (blue arrows) and DSB resection is sufficient to uncover the duplicated sequences, the latter can anneal with each other, leaving single-stranded tails that are removed by nucleases, while the resulting gaps/nicks are filled in by DNA repair synthesis and ligation, leading to deletion of one repeat and the intervening region. Red arrows indicate the 3' ends of the newly synthesized strands (adapted from (Longhese *et al.*, 2006)).

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**DNA DOUBLE-STRAND BREAK REPAIR PATHWAY CHOICE.** Several attempts have been made to understand the mechanism of DSB repair pathway choice. It has been proposed that NHEJ serves as an immediate repair pathway and thus temporally precedes HR, in both yeast (Frank-Vaillant and Marcand, 2002) and mammals (Kim *et al.*, 2005). However, whereas in mammals NHEJ and HR have equivalent roles, in yeast only a minority of induced DSB are repaired by NHEJ even when HR is eliminated by deleting the homologous donor sequences (Valencia *et al.*, 2001; Ira *et al.*, 2004). This suggests that NHEJ is not as efficient as HR to repair a DSB in yeast as in mammals.

If the DSB is not repaired by NHEJ, initiation of 5' end resection allows formation of 3' ssDNA tails and engagement into HR. Unlike NHEJ, HR accuracy is dependent upon its ability to find an appropriate donor. The ideal donor can be the sister chromatid to which a chromosome is paired following DNA replication or the homologous chromosome in diploid cells, suggesting that NHEJ and HR utilization should be optimized as a function of ploidy and of the cell cycle phase. Thus, DSB channeling into different repair pathways during the cell cycle is linked to cyclin-dependent kinase (Cdk) activities, whose sequential action determines cell cycle progression. In particular, in *S. cerevisiae* haploid cells, NHEJ is preferentially used in G1, when Cdk1 activity is low, whereas HR

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occurs during the S and G2/M cell cycle phases, when Cdk1 activity is high and an intact sister chromatid is available as a repair template (Aylon *et al.*, 2004; Ira *et al.*, 2004). NHEJ is also allowed in S/G2, but it should be completed before resection takes over, thus committing the break to HR. Such cell cycle-dependent regulation of NHEJ and HR is conserved also in fission yeast, where Cdk activity is essential for the recruitment of the Rad51 recombinase to DSBs induced by ionizing radiation (Caspari *et al.*, 2002). Furthermore, Cdk activity is required for early steps of HR also in human cells (Jazayeri *et al.*, 2006).

Cdk1 activity stimulates HR by promoting DSB end resection and therefore generation of the 3' ssDNA tails, which are necessary for HR and inhibitory for NHEJ (Aylon *et al.*, 2004; Ira *et al.*, 2004; Zhang *et al.*, 2009). Cdk1 triggers DSB resection by both counteracting the inhibitory effect of the NHEJ proteins and stimulating the activity of DSB resection machinery. In fact, in *S. cerevisiae*, deletion of *YKU70* or *YKU80*, as well as that of the NHEJ genes *DNL4* or *LIF1*, allows resection of the DSB ends independently of Cdk1 activity (Clerici *et al.*, 2008; Zierhut and Diffley, 2008; Barlow and Rothstein, 2009). These findings suggest that Cdk1 activity can promote DSB resection by relieving the inhibitory effect exerted by Yku and the NHEJ machinery. The absence of Yku has a stronger effect in promoting 5' DSB ends degradation in G1 than loss of either Dnl4 or Lif1 (Clerici *et al.*, 2008; Zierhut and Diffley, 2008), suggesting that both the end binding function of Yku and NHEJ-mediated religation of the DSB ends contribute to protect the DSB ends from degradation in G1. In any case, DSB resection appears to be propagated to DNA regions far away from the break less efficiently in the absence of Yku than in the presence of high Cdk activity

(Clerici *et al.*, 2008), suggesting that Cdk1 has an active role in regulating extensive resection independently of Yku.

In fact, Cdk1 promotes DSB resection by phosphorylating the Ser267 residue of Sae2 (Huertas *et al.*, 2008), a mechanism that is conserved in the Sae2 vertebrate homolog CtIP (Huertas and Jackson, 2009). Mutation of Sae2 Ser267 to a nonphosphorylatable residue impairs DSB processing in a manner similar to a *sae2* null mutation (Huertas *et al.*, 2008). This defect is likely due to the inability of Cdk1 to phosphorylate Sae2 Ser267, since it is not observed in *sae2-S267E* cells where Ser267 is replaced by a glutamic residue mimicking constitutive phosphorylation. How does Cdk1-dependent phosphorylation of Sae2 promote resection at chromosome ends? In both budding and fission yeasts, the DNA damage sensitivity of *sae2Δ* and *mre11Δ* cells is suppressed by *YKU* deletion and this suppression requires both Exo1 and Sgs1 (Tomita *et al.*, 2003; Limbo *et al.*, 2007; Wasko *et al.*, 2009; Mimitou and Symington, 2010), suggesting that Cdk1-dependent phosphorylation of Sae2 is needed to remove Yku from DSB ends in order to allow the action of Exo1 and Sgs1. Interestingly, removal of the Yku-mediated inhibition of resection appears to require the physical presence of MRX, but not its nuclease activity (Longhese *et al.*, 2010; Shim *et al.*, 2010), suggesting that Yku removal does not depend on the initial processing of the DSB ends by MRX-Sae2. As the lack of MRX leads to increased amounts of Yku associated to DNA ends (Zhang *et al.*, 2007; Wu *et al.*, 2008), one possibility is that Sae2 phosphorylation by Cdk1 promotes MRX-dependent removal of Yku in G2 by enhancing MRX-Sae2 access to DNA. Alternatively (or in addition), Cdk1 might override the resection block imposed by Yku by decreasing its ability to bind DNA ends. It has been recently shown that Cdk1

phosphorylates Lif1, a component of the NHEJ machinery. Although this modification has been shown to promote the stable binding of Lif1 to DSBs and some imprecise NHEJ events, it is still unknown whether it plays a role in regulating DSB resection (Matsuzaki *et al.*, 2012).

However, NHEJ- or Yku-deficient G1 cells are able to resect a relatively small amount of DNA (Clerici *et al.*, 2008; Zierhut and Diffley, 2008), suggesting that Cdk1 activates and/or inhibits additional targets in order to stimulate extensive resection. Two likely candidates for such a role are Dna2 and Rad9, which respectively activate and inhibit DSB resection and are known to be phosphorylated by Cdk1 (Ubersax *et al.*, 2003). Dna2, together with Exo1, generates long ssDNA tails at DSBs by acting in concert with the Sgs1-Top3-Rmi1 complex, and its phosphorylation by Cdk1 stimulates both Dna2 recruitment to DSBs and DSB end resection (Chen *et al.*, 2011). On the other hand, loss of Rad9 increases DNA end-resection even when Cdk1 is not active (Lazzaro *et al.*, 2008), suggesting that also this DNA damage checkpoint protein could be a target of the Cdk1-dependent regulation of resection.



## ***RESULTS***





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**Distinct Cdk1 requirements during single-strand  
annealing, noncrossover, and crossover  
recombination**

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DNA double-strand breaks (DSBs) occur spontaneously during DNA replication and after exposure to certain genotoxic chemicals or ionizing radiation. Efficient repair of DSBs can be accomplished by non-homologous end joining (NHEJ), which directly rejoins broken DNA ends, or by homologous recombination (HR), which utilizes a homologous DNA template to restore the genetic information lost at the break site (reviewed in (Pâques and Haber, 1999; Krogh and Symington, 2004; San Filippo *et al.*, 2008)). Failure to repair DSBs can lead to genome instability and cell death.

HR is initiated by 5'-3' nucleolytic degradation of the DSB ends to yield 3'-ended single-stranded DNA (ssDNA) tails. Replication protein A (RPA) binds to the ssDNA tails to remove their secondary DNA structures, but is then replaced by Rad51 aided by Rad52. Once formed, the Rad51 nucleofilaments search for homologous sequences and then promote invasion of the ssDNA into homologous donor double-stranded DNA to form a joint molecule with a displaced strand (D-loop) (reviewed in (Pâques and Haber, 1999; Krogh and Symington, 2004; San Filippo *et al.*, 2008)). Following strand invasion, the 3' end of the invading strand primes DNA synthesis using the donor sequence as a template, thus restoring those residues that were lost by resection (Li *et al.*, 2009).

According to the canonical double-strand break repair (DSBR) model (Szostak *et al.*, 1983), the displaced strand of the D-loop can anneal with the complementary sequence on the other side of the break (second end capture) to form a double Holliday junction (dHJ) intermediate. Random cleavage of the

two HJs is expected to yield an equal number of noncrossover and crossover products. This DSBR model predicts that both crossover and noncrossover products derive from dHJ resolution. However, the finding that most DSB repair in somatic cells is not associated with crossovers (Bzymek *et al.*, 2010) led to alternative models for noncrossover generation. In one of them, the action of helicases mediates the convergent branch migration of the two HJs, thus producing a hemicatenane structure that is decatenated to form exclusively noncrossover products (Ira *et al.*, 2003; Wu and Hickson, 2003; Lo *et al.*, 2006). A second mechanism, termed synthesis-dependent strand annealing (SDSA), leads to displacement of the invading strand that has been extended by DNA synthesis and that anneals with the complementary sequences exposed by 5'-3' resection (Strathern *et al.*, 1982; Nassif *et al.*, 1994; Ferguson and Holloman, 1996). Because no HJ is formed, only noncrossover products are made. Interestingly, during meiotic recombination, where dHJ resolution into crossovers is essential to drive segregation of homologs to opposite poles, most crossovers are thought to arise via dHJ resolution, whereas noncrossovers form mostly by the SDSA pathway (Allers and Lichten, 2001; Hunter and Kleckner, 2001).

When a DSB is flanked by direct repeats, its repair primarily occurs by single-strand annealing (SSA). Here, the resected DSB ends anneal with each other instead of invading a homologous DNA sequence (reviewed in (Pâques and Haber, 1999; Krogh and Symington, 2004; San Filippo *et al.*, 2008)). Subsequent nucleolytic removal of the protruding single-stranded tails results in deletion of the intervening DNA sequence and one of the repeats. In principle, such a break can also be repaired by break-induced replication (BIR), where the repeat

closer to the cut site can strand-invade the repeat that is further away and set up a recombination-dependent replication fork to copy all the distal sequences. However, SSA usually out-competes BIR, which is a kinetically slow process (Jain *et al.*, 2009).

All the above HR pathways require 5'-3' nucleolytic degradation of DNA ends and the strand-annealing activity of Rad52. In addition, DSBR, SDSA and BIR require the Rad51 protein, which is dispensable for SSA that does not involve strand invasion (Ivanov *et al.*, 1996).

In *Saccharomyces cerevisiae* haploid cells, mitotic HR is generally restricted to the S and G2 phases of the cell cycle, when DNA has been replicated and a sister chromatid is available as an appropriate donor (Aylon *et al.*, 2004; Ira *et al.*, 2004). This cell-cycle specificity depends on cyclin-dependent kinases (Cdks; Cdk1 in *S. cerevisiae*), which promote resection of the 5' DSB ends to yield 3'-ended ssDNA tails that are necessary to initiate HR (Aylon *et al.*, 2004; Ira *et al.*, 2004). End resection occurs through a biphasic mechanism: first the MRX complex and Sae2 clip 50–100 nucleotides from the 5' DNA ends; then Exo1 or Sgs1-Top3-Rmi1 and Dna2 process the early intermediate to form extensive regions of ssDNA (reviewed in (Longhese *et al.*, 2010; Mimitou and Symington, 2011)). The Sae2 protein has been shown to be a Cdk1 target in promoting ssDNA generation at DNA ends during both mitosis and meiosis (Huertas *et al.*, 2008; Manfrini *et al.*, 2010). However, as Sae2 only resects a relatively small amount of DNA and other nucleases and helicases are required for efficient DSB resection, Cdk1 likely has additional targets in promoting this event.

Indeed, DSB end resection is also negatively regulated by the Yku heterodimer (Lee *et al.*, 1998; Clerici *et al.*, 2008) and by the checkpoint protein Rad9 (Lydall

and Weinert, 1995; Lazzaro *et al.*, 2008). Interestingly, the ends of an endonuclease-induced DSB are resected in the G1 phase of the cell cycle (low Cdk1 activity) when Yku is lacking (Clerici *et al.*, 2008). Moreover, *RAD9* deletion allows DSB resection in G2 cells that display low Cdk1 activity due the overexpression of the Cdk1 inhibitor Sic1 (Lazzaro *et al.*, 2008). These findings indicate that Cdk1 requirement for DSB resection is bypassed when the inhibitory function of either Yku or Rad9 is relieved.

Whether Cdk1 promotes other HR events is unknown. Some evidence suggests that HR steps other than DSB resection might be regulated by Cdk1 activity. For example, formation of Rad52 foci after ionizing radiation (IR) is less efficient in G1 than in G2, suggesting that Cdk1 might control Rad52 recruitment to DSBs (Barlow and Rothstein, 2009). Furthermore, Cdk1 targets the Srs2 helicase to dismantle D-loop structures, possibly by counteracting unscheduled Srs2 sumoylation (Saponaro *et al.*, 2010). Proteins implicated in late HR events have also been identified as potential Cdk substrates in other eukaryotes. In particular, human BRCA2 is phosphorylated by Cdks, and this phosphorylation has been proposed to negatively regulate Rad51 recombination activity (Esashi *et al.*, 2005). Moreover, Cdk1-dependent phosphorylation of the fission yeast checkpoint protein Crb2 stimulates resolution of HR intermediates by the topoisomerase Top3 and the ReqQ helicase Rqh1 (Caspari *et al.*, 2002).

Here, we investigate the role of Cdk1 in homology-dependent repair of a DSB. We show that generation of 3'-ended ssDNA at the DSB ends bypasses Cdk1 requirement for the repair of a DSB by either SSA or noncrossover recombination, indicating that Cdk1 is dispensable for these repair events if DSB resection occurs. By contrast, resection is not sufficient to bypass Cdk1

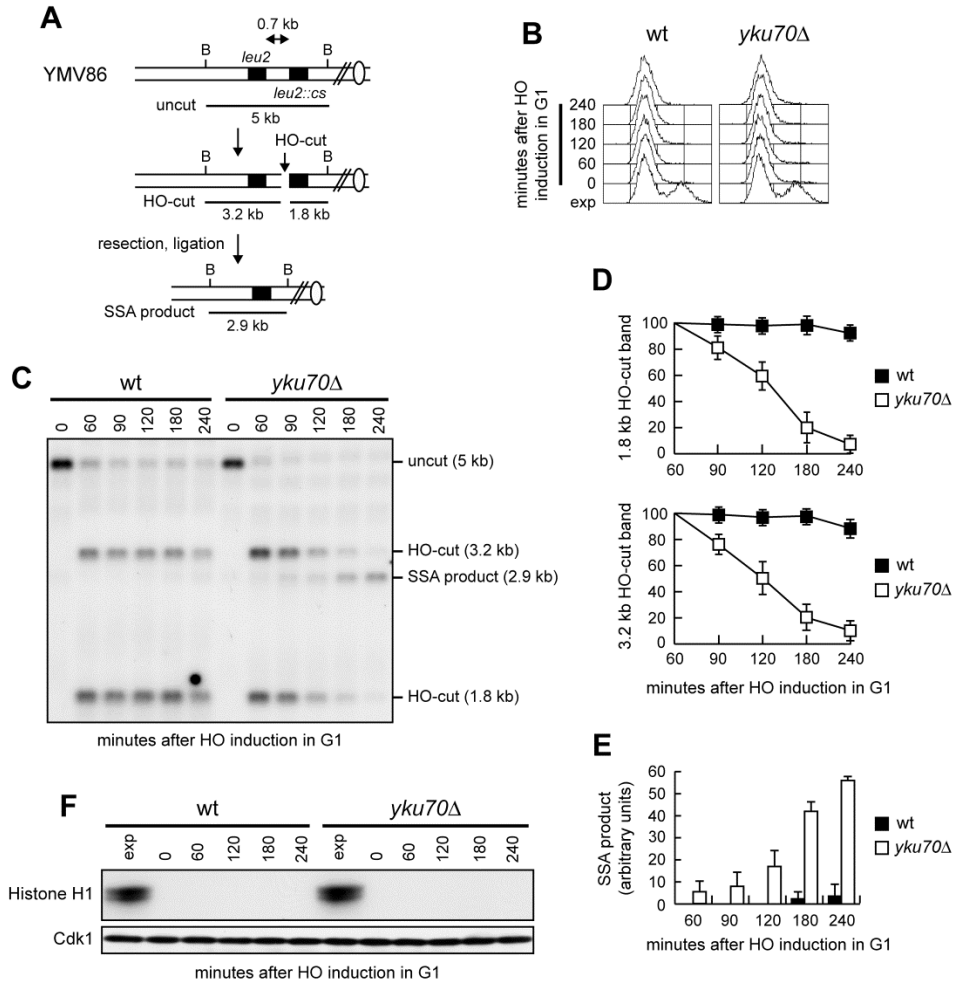
requirement for generating crossover products. Thus, Cdk1 promotes SSA- and noncrossover-mediated recombination by regulating essentially the resection step, while Cdk1 controls further HR steps in order to allow crossover outcomes.

**THE LACK OF Yku70 ALLOWS DSB REPAIR BY SSA IN G1.** HR is inhibited in G1 when Cdk1 activity is low, whereas it occurs during S and G2/M cell cycle phases when Cdk1 activity is high (Aylon *et al.*, 2004; Ira *et al.*, 2004). Although it is well known that Cdk1 promotes resection of DSB ends (Aylon *et al.*, 2004; Ira *et al.*, 2004; Huertas *et al.*, 2008), it is still unclear if other HR steps are regulated by Cdk1. To investigate whether DSB resection is the only step controlled by Cdk1 in HR-mediated DSB repair, we asked if generation of ssDNA at the DSB ends is sufficient to allow HR when Cdk1 activity is low. As DSB resection in G1 is inhibited by the Yku heterodimer and *YKU70* deletion allows ssDNA generation at DSB ends in G1 cells (Clerici *et al.*, 2008), we asked if *yku70Δ* cells are capable to carry out HR in G1.

Homology-dependent repair of a DSB made between tandem DNA repeats occurs primarily by SSA (Jain *et al.*, 2009), which requires DSB resection and re-annealing of RPA-covered ssDNA by the Rad52 protein (Fishman-Lobell *et al.*, 1992; Pâques and Haber, 1999). This process does not involve strand invasion and is therefore independent of Rad51 (Ivanov *et al.*, 1996). We deleted *YKU70* in a strain where tandem repeats of the *LEU2* gene are 0.7 kb apart and one of them (*leu2::cs*) is adjacent to a recognition site for the HO endonuclease (**Figure 5A**) (Vaze *et al.*, 2002). The strain also harbors a *GAL-HO* construct that provides regulated *HO* expression. Since homology is restricted to only one DSB

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end (Figure 5A), the HO-induced break cannot be repaired by gene conversion, making SSA the predominant repair mode. HO was expressed by galactose addition to  $\alpha$ -factor-arrested cells that were kept arrested in G1 with  $\alpha$ -factor for the subsequent 4 hours. Galactose was maintained in the medium in order to permanently express HO, which can recurrently cleave the HO sites eventually reconstituted by NHEJ-mediated DSB repair. Kinetics of DSB repair was evaluated by Southern blot analysis with a *LEU2* probe that also allowed following 5'-end resection on each side of the break by monitoring the disappearance of the HO-cut DNA bands. The quality and persistence of the cell cycle arrest was assessed by FACS analysis (Figure 5B) and by measuring Cdk1 kinase activity (Figure 5F). Consistent with the requirement of Cdk1 activity for DSB resection and repair, both the 1.8 kb and 3.2 kb HO-cut band signals remained high throughout the experiment in wild type G1 cells (Figure 5C and 5D), where the 2.9 kb SSA repair product was only barely detectable (Figure 5C and 5E). By contrast, the SSA repair product accumulated in *yku70 $\Delta$*  G1 cells (Figure 5C and 5E), where both the 1.8 kb and 3.2 kb HO-cut band signals decreased (Figure 5C and 5D). The ability of *yku70 $\Delta$*  cells to carry out SSA does not require Cdk1. In fact, Cdk1 activity, which was present in exponentially growing wild type and *yku70 $\Delta$*  cells, dropped to undetectable levels after G1 arrest (time 0) and remained undetectable in both cultures throughout the experiment (Figure 5F). Thus, the lack of Yku allows DSB repair by SSA in G1, suggesting that ssDNA generation is sufficient to bypass Cdk1 requirement for SSA.



**Figure 5. SSA-mediated DSB repair in *yku70Δ* G1 cells.**

(A) Map of the YMV86 chromosome III region where the HO-cut site is flanked by homologous *leu2* sequences that are 0.7 kb apart. HO-induced DSB formation results in generation of 3.2 kb and 1.8 kb DNA fragments (HO-cut) that can be detected by Southern blot analysis of BglIII-digested genomic DNA with a *LEU2* probe. DSB repair by SSA generates a product of 2.9 kb (SSA product). B, BglIII. (B–E) Exponentially growing YEP+raf (exp) cell cultures of wild type YMV86 and its *yku70Δ* derivative strain were arrested in G1 with  $\alpha$ -factor (time zero) and transferred to YEP+raf+gal in the presence



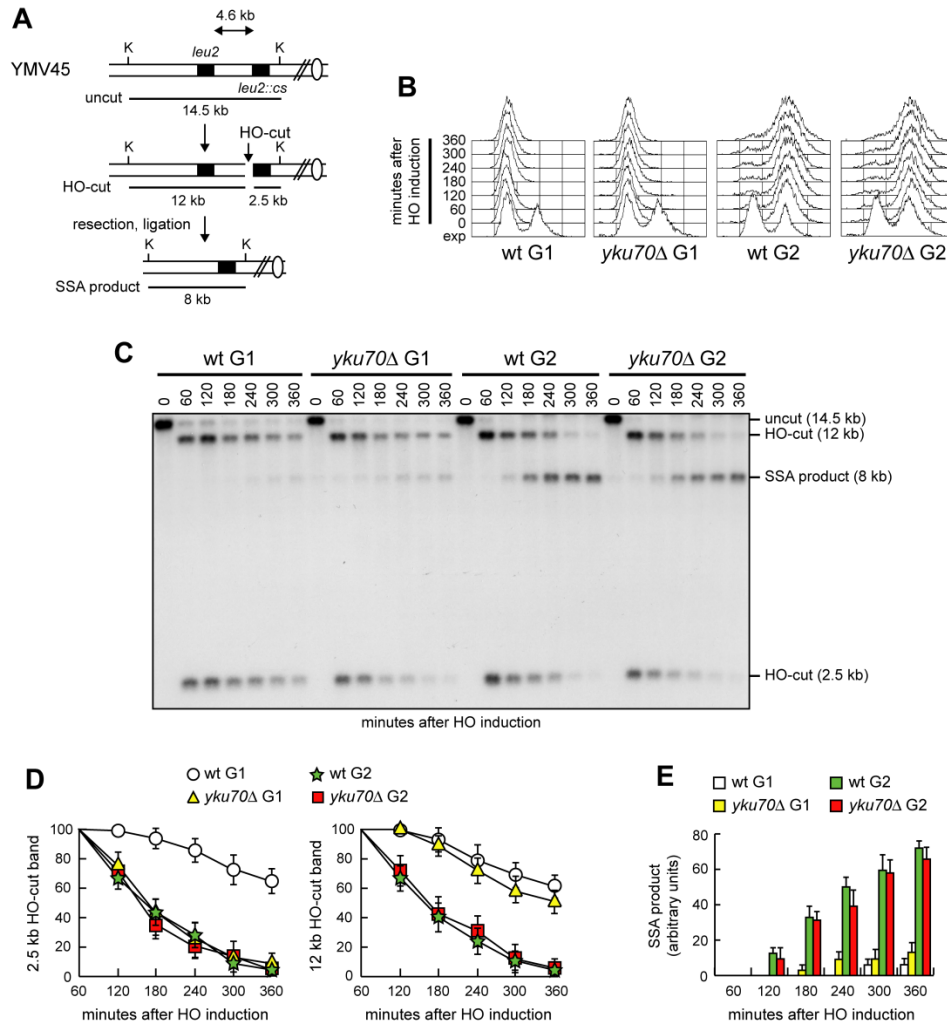
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of  $\alpha$ -factor. (B) FACS analysis of DNA content. (C) Southern blot analysis of BglII-digested genomic DNA. (D, E) Densitometric analysis of the HO-cut (D) and the SSA (E) band signals. Plotted values are the mean value  $\pm$ SD from four independent experiments as in (C), enclosing that described in (F). The intensity of each band was normalized with respect to a loading control. (F) YMV86 derivative strains with the indicated genotypes and expressing fully functional Cdc28-HA were treated as in (B–E). Cell samples were collected at the indicated times to assay Cdk1 kinase activity in anti-HA immunoprecipitates by using histone H1 as substrate (top row) and to determine Cdk1 levels by western blot analysis with anti-HA antibody (bottom row).

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SSA-based DNA repair requires degradation of the 5' DSB ends to reach the complementary DNA sequences that can then anneal. If SSA in *yku70 $\Delta$*  G1 cells depends on generation of 3'-ended ssDNA at DSB ends, then failure of resection to reach the homologous distal *leu2* sequence should prevent SSA. Interestingly, Cdk1-independent resection takes place in *yku70 $\Delta$*  cells, but it is confined to DNA regions closed to the DSB site (Clerici *et al.*, 2008), suggesting that other proteins limit extensive DSB resection in the absence of Yku. We therefore asked whether increasing the distance between the complementary *leu2* sequences prevented DSB repair by SSA in *yku70 $\Delta$*  G1 cells. To this end, we monitored SSA-mediated repair of an HO-induced DSB in a strain where the donor *leu2* sequence was positioned 4.6 kb away from the HO recognition site at *leu2::cs* (Figure 6A) (Vaze *et al.*, 2002). HO expression was induced in  $\alpha$ -factor-arrested cells that were kept blocked in G1 with  $\alpha$ -factor in the presence of galactose (Figure 6B). Consistent with previous findings (Clerici *et al.*, 2008), resection in *yku70 $\Delta$*  G1 cells was restricted to DNA regions closed to the break site. In fact, the 2.5 kb HO-cut signal decreased more efficiently in *yku70 $\Delta$*  than in wild type G1 cells, whereas similar amounts of the 12 kb HO-cut signal were detectable in both wild type and *yku70 $\Delta$*  G1 cells (Figure 6C and 6D). Thus, 5'-3'

nucleolytic degradation in *yku70Δ* G1 cells failed to proceed beyond the distal *leu2* hybridization region. The inability of resection to uncover the homologous distal *leu2* sequence prevented DSB repair by SSA in *yku70Δ* G1 cells. In fact, the 8 kb SSA repair product was only barely detectable in both wild type and *yku70Δ* G1 cells throughout the experiment (**Figure 6C** and **6E**). By contrast, when a similar experiment was performed in G2-arrested cells (**Figure 6B**), where the inhibitory function of Yku on DSB resection is relieved (Bonetti *et al.*, 2010; Shim *et al.*, 2010), the 8 kb SSA repair product was clearly detectable in wild type and *yku70Δ* cells (**Figure 6C** and **6E**), which both showed also a decrease of the 12 kb HO-cut signals compared to the same strains arrested in G1 (**Figure 6C** and **6D**). Thus, the ability of *yku70Δ* G1 cells to repair a DSB by SSA depends on the extent of resection.



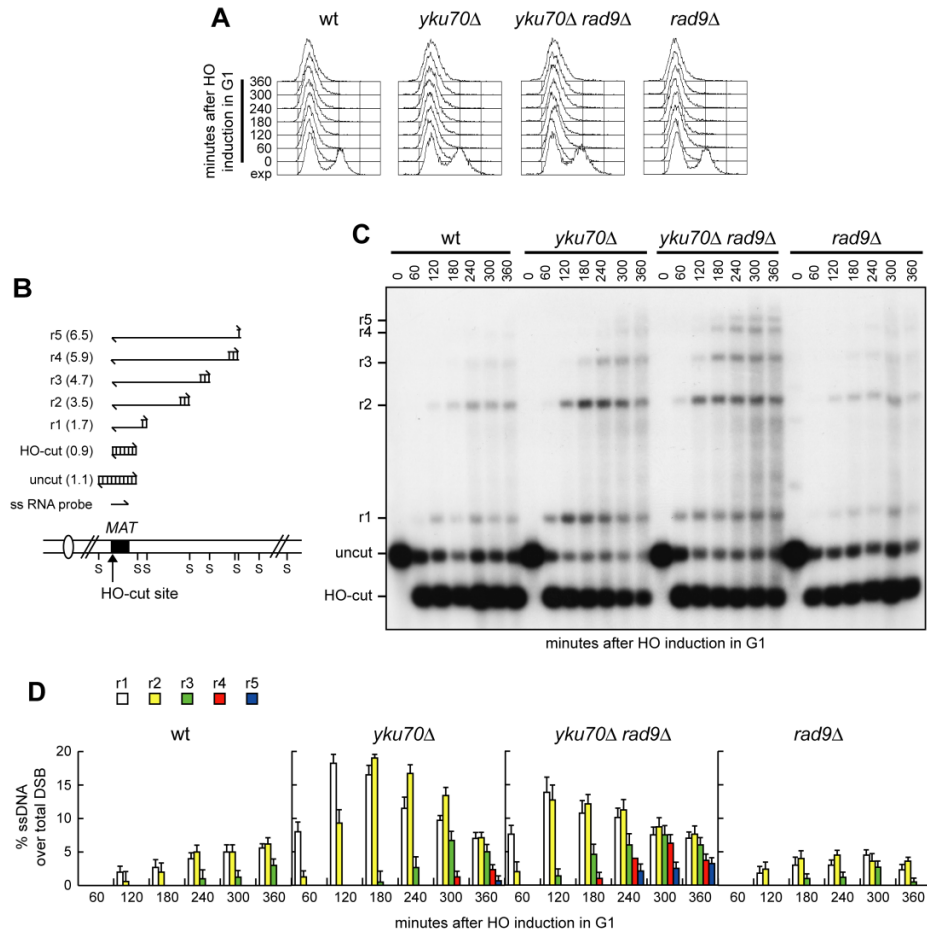
**Figure 6. SSA-mediated DSB repair in *yku70Δ* G1 and G2 cells.**

(A) Map of the YMV45 chromosome III region where the HO-cut site is flanked by homologous *leu2* sequences that are 4.6 kb apart. HO-induced DSB formation results in generation of 12 kb and 2.5 kb DNA fragments (HO-cut) that can be detected by Southern blot analysis of KpnI-digested genomic DNA with a *LEU2* probe. DSB repair by SSA generates a product of 8 kb (SSA product). K, KpnI. (B–E) Exponentially growing YEP+raf (exp) cell cultures of wild type YMV45 and its *yku70Δ* derivative strain were

arrested at time zero in G1 with  $\alpha$ -factor or in G2 with nocodazole and transferred to YEP+raf+gal in the presence of  $\alpha$ -factor or nocodazole, respectively. (B) FACS analysis of DNA content. (C) Southern blot analysis of KpnI-digested genomic DNA. (D, E) Densitometric analysis of the HO-cut (D) and the SSA (E) band signals. Plotted values are the mean value  $\pm$ SD from three independent experiments as in (C). The intensity of each band was normalized with respect to a loading control.

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**THE LACK OF Rad9 ENHANCES RESECTION IN *yku70 $\Delta$*  G1 CELLS.** If ssDNA generation were the limiting step in SSA-mediated DSB repair in G1, then increasing the efficiency/extent of resection should enhance the ability of *yku70 $\Delta$*  cells to carry out SSA in G1. The lack of the checkpoint protein Rad9 has been shown to allow DSB resection in G2 cells that displayed low Cdk1 activity due to high levels of the Cdk1 inhibitor Sic1 (Lazzaro *et al.*, 2008). Thus, we asked whether the lack of Rad9 enhanced the efficiency of DSB resection in *yku70 $\Delta$*  G1 cells. To compare resection efficiency independently of DSB repair, we monitored the appearance of the resection products at an HO-induced DSB generated at the *MAT* locus (Figure 7B) of G1-arrested (Figure 7A) cells, which were not able to repair this DSB because they lacked the homologous donor sequences *HML* and *HMR* (Lee *et al.*, 1998). As expected, wild type cells showed very low levels of the 3'-ended resection products (r1 to r5), which instead clearly accumulated in both *yku70 $\Delta$*  and *yku70 $\Delta$  rad9 $\Delta$*  cells (Figure 7C and 7D). Moreover, the longest r4 and r5 resection products were detectable in *yku70 $\Delta$  rad9 $\Delta$*  cells 120 minutes earlier than in *yku70 $\Delta$*  cells (Figure 7C and 7D), indicating that the lack of Rad9 enhances the resection efficiency of *yku70 $\Delta$*  G1 cells.

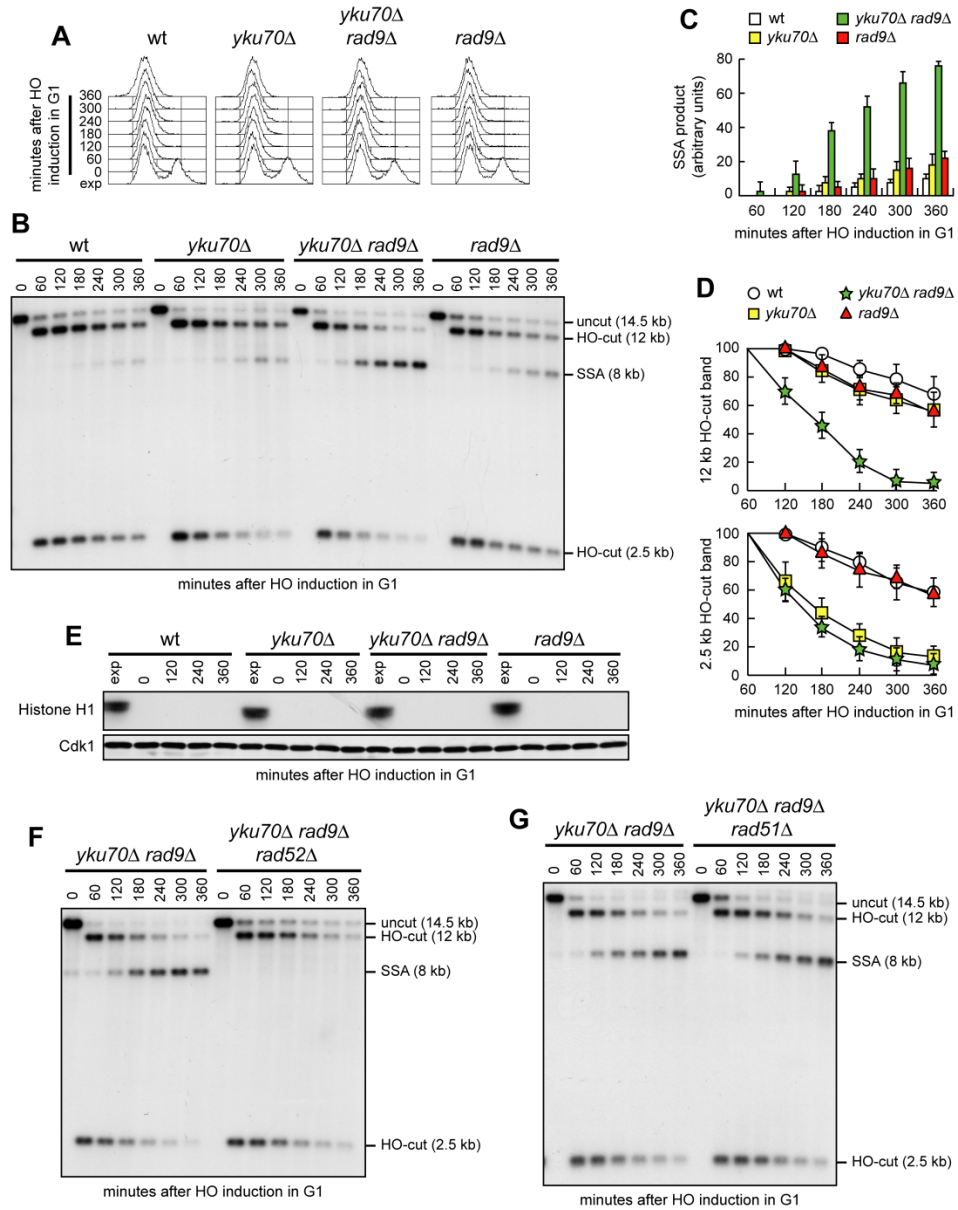


### Figure 7. Rad9 inhibits extensive DSB resection in *yku70*Δ G1 cells.

Exponentially growing YEP+raf (exp) cell cultures of wild type JKM139 and its derivative mutant strains were arrested in G1 with  $\alpha$ -factor (time zero) and transferred to YEP+raf+gal in the presence of  $\alpha$ -factor. (A) FACS analysis of DNA content. (B) System used to detect DSB resection. Gel blots of *SspI*-digested genomic DNA separated on alkaline agarose gel were hybridized with a single-stranded *MAT* probe specific for the unresected strand. 5'-3' resection progressively eliminates *SspI* sites (S), producing larger *SspI* fragments (r1 through r5) detected by the probe. (C) Analysis of ssDNA formation as described in (B). (D) Densitometric analysis of the resection products. Plotted values are the mean value  $\pm$ SD from three independent experiments as in (C). See **Materials and Methods** for details.

Interestingly, although *RAD9* deletion was shown to allow MRX-dependent ssDNA generation in Sic1 overproducing G2 cells (Lazzaro *et al.*, 2008), *rad9Δ* G1 cells did not show increased efficiency of DSB resection compared to wild type cells (**Figure 7C** and **7D**). Thus, Rad9 limits extensive resection in *yku70Δ* cells, but its lack is not sufficient, by itself, to escape the inhibitory effect of Yku on DSB resection in G1.

**THE LACK OF Rad9 ENHANCES SSA IN *yku70Δ* G1 CELLS.** Because DSB resection in G1 was more efficient in *yku70Δ rad9Δ* cells than in *yku70Δ* cells, we asked whether the lack of Rad9 allows efficient SSA-mediated DSB repair in *yku70Δ* G1 cells carrying tandem repeats of the *LEU2* gene 4.6 kb apart. Indeed, the amount of SSA repair products in G1 was much higher in *yku70Δ rad9Δ* cells than in wild type, *yku70Δ* or *rad9Δ* cells (**Figure 8A–8C**). Consistent with DSB resection being more extensive in *yku70Δ rad9Δ* than in *yku70Δ* G1-arrested cells (**Figure 7**), the decrease of the 12 kb HO-cut band signal was much more apparent in *yku70Δ rad9Δ* than in *yku70Δ* G1 cells, whereas the 2.5 kb HO-cut band signal decreased with similar kinetics in both G1 cell cultures (**Figure 8B** and **8D**). Cdk1 kinase activity, which was present in all exponentially growing cells, was not required for accumulation of the repair products in *yku70Δ rad9Δ* cells, as it was undetectable in all G1-arrested cell cultures throughout the experiment (**Figure 8E**).



**Figure 8. *RAD9* deletion increases SSA efficiency in *yku70Δ* G1 cells.**

(A–D) Exponentially growing YEP+raf (exp) cell cultures of wild type YMV45 and its derivative mutant strains were arrested in G1 with  $\alpha$ -factor (time zero) and transferred to YEP+raf+gal in the presence of  $\alpha$ -factor. (A) FACS analysis of DNA content. (B) DSB repair by SSA was analyzed as described in Figure 6. (C, D) Densitometric analysis of the SSA (C) and the HO-cut (D) band signals. Plotted values are the mean value  $\pm$ SD from four independent experiments as in (B), enclosing that described in (E). (E) YMV45 derivative strains with the indicated genotypes and expressing fully functional Cdc28-HA were treated as in (A–D). Cell samples were taken at the indicated times to assay Cdk1 kinase activity (top row) and to determine Cdk1 levels (bottom row) as in Figure 5F. (F, G) Exponentially growing YEP+raf cell cultures of YMV45 derivative strains were arrested in G1 with  $\alpha$ -factor (time zero) and transferred to YEP+raf+gal in the presence of  $\alpha$ -factor. DSB repair by SSA was analyzed as described in Figure 6.

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SSA requires the strand-annealing activity of the Rad52 protein, but it occurs independently of Rad51 (Ivanov *et al.*, 1996). Consistent with the SSA repair mode, formation of the repair products in G1-arrested *yku70Δ rad9Δ* cells was abolished by *RAD52* deletion (Figure 8F), whereas it was unaffected by *RAD51* deletion (Figure 8G). As a DSB flanked by direct repeats could be repaired, at least in principle, also by Rad51-dependent BIR (Jain *et al.*, 2009), the finding that *yku70Δ rad9Δ* and *yku70Δ rad9Δ rad51Δ* G1 cells accumulated the 8 kb repair product with similar kinetics (Figure 8G) indicates that SSA is responsible for this repair event. Thus, we conclude that the lack of Rad9 increases the ability of *yku70Δ* cells to carry out DSB repair by SSA in G1, likely by enhancing the efficiency of DSB resection.

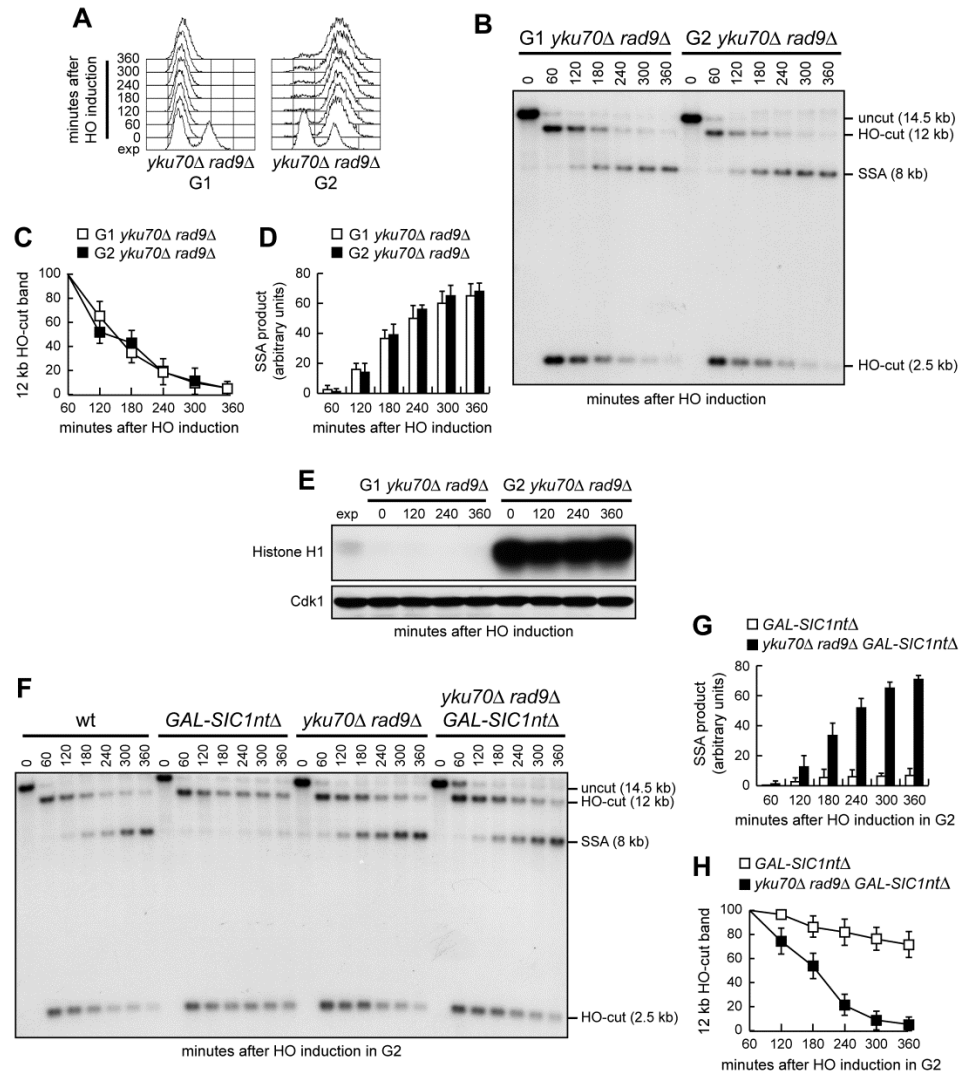
If competence for SSA-mediated DSB repair relies solely on 3'-ended ssDNA generation, then this repair process should take place with similar efficiency in G1- and G2-arrested *yku70Δ rad9Δ* cells. As this expectation is based on the assumption that G1- and G2-arrested *yku70Δ rad9Δ* cells resect DSB ends with similar efficiencies, we compared resection (Figure 9B and 9C) and SSA (Figure



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**9B** and **9D**) in *yku70Δ rad9Δ* cells arrested either in G1 or in G2 (**Figure 9A**) during break induction. Disappearance of the 2.5 kb and 12 kb HO-cut bands occurred with similar kinetics in G1- and G2-arrested *yku70Δ rad9Δ* cells (**Figure 9B** and **9C**), which also accumulated similar amounts of the 8 kb SSA repair product (**Figure 9B** and **9D**). As expected, Cdk1 kinase activity was undetectable in *yku70Δ rad9Δ* cells during the  $\alpha$ -factor arrest, whereas it was high in nocodazole-arrested G2 cells (**Figure 9E**). Thus, DSB resection is the limiting step in DSB repair by SSA.

If SSA is generally restricted to G2 only because high Cdk1 activity allows DSB resection, then inactivation of Cdk1 in G2 should prevent SSA in wild type but not in *yku70Δ rad9Δ* cells, where DSB resection occurs independently of Cdk1. Thus, we compared DSB repair by SSA in G2-arrested wild type and *yku70Δ rad9Δ* cells expressing high levels of a stable version of the mitotic Clb-Cdk1 inhibitor Sic1 (*Sic1ntΔ*) (Desdouets *et al.*, 1998). Consistent with the hypothesis that Cdk1 promotes SSA by regulating the resection step, Sic1 overproduction inhibited SSA repair in G2-arrested wild type cells but not in *yku70Δ rad9Δ* cells. In fact, the 8 kb SSA repair product accumulated in *yku70Δ rad9Δ GAL-SIC1ntΔ* cells (**Figure 9F** and **9G**), which showed a decrease of both the 2.5 kb and 12 kb HO-cut band signals (**Figure 9F** and **9H**). By contrast, the same repair product was only barely detectable in G2-arrested *GAL-SIC1ntΔ* cells, where the HO-cut band signals remained high throughout the experiment (**Figure 9F–9H**).



**Figure 9. DSB resection is the limiting step in DSB repair by SSA.**

(A–D) Exponentially growing YEP+raf (exp) YMV45 *yku70Δ rad9Δ* cells were arrested in G1 with  $\alpha$ -factor or in G2 with nocodazole and transferred to YEP+raf+gal in the presence of  $\alpha$ -factor or nocodazole, respectively. (A) FACS analysis of DNA content. (B) DSB repair by SSA was analyzed as described in Figure 6. (C, D) Densitometric analysis of the 12 kb HO-cut (C) and 8 kb SSA (D) band signals. Plotted values are the mean

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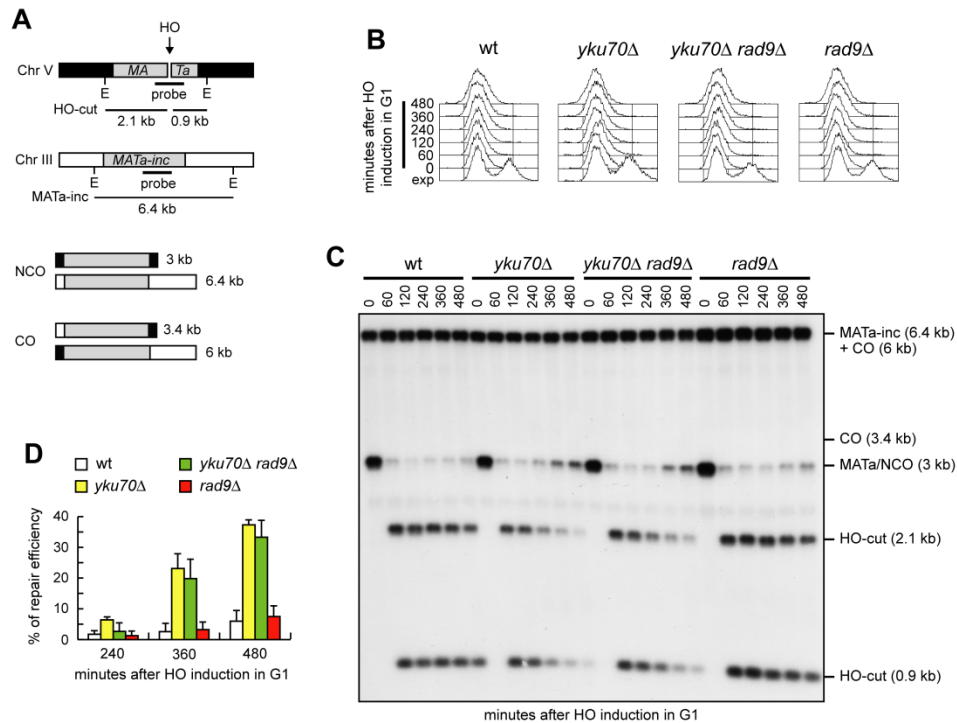
value  $\pm$ SD from four independent experiments as in (B), enclosing that described in (E). The intensity of each band was normalized with respect to a loading control. (E) YMV45 *yku70 $\Delta$  rad9 $\Delta$*  cells expressing fully functional Cdc28-HA were treated as in (A–D). Cell samples were taken at the indicated times to assay Cdk1 kinase activity (top row) and to determine Cdk1 levels (bottom row) as in Figure 5F. (F–H) Exponentially growing YEP+raf YMV45 derivative cells with the indicated genotypes were arrested at time zero in G2 with nocodazole and transferred to YEP+raf+gal in the presence of nocodazole. Cell cycle arrest was verified by FACS analysis (not shown). (F) DSB repair by SSA was analyzed as described in Figure 6. (G, H) Densitometric analysis of the 8 kb SSA (G) and 12 kb HO-cut (H) band signals. Plotted values are the mean value  $\pm$ SD from three independent experiments as in (F). The intensity of each band was normalized with respect to a loading control.

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**THE LACK OF Yku70 ALLOWS NONCROSSOVER RECOMBINATION IN G1.** When both ends of a DSB share homology with an intact DNA sequence, repair by Rad51-dependent recombination pathways leads to the formation of noncrossover or crossover products. We investigated whether generation of 3'-ended ssDNA can bypass Cdk1 requirement also in this process. To detect crossovers and noncrossovers at the molecular level, we used a haploid strain that bears two copies of the *MATa* sequence (Figure 10A) (Prakash *et al.*, 2009; Saponaro *et al.*, 2010). One copy is located ectopically on chromosome V and carries the recognition site for the HO endonuclease, while the endogenous copy on chromosome III carries a single base pair mutation that prevents HO recognition (*MATa-inc*). Upon galactose addition, the HO-induced DSB can be repaired by Rad51-dependent HR using the uncleavable *MATa-inc* sequence as a donor. This repair event can occur either with or without an accompanying crossover (Figure 10A) with the proportion of crossovers being 5–6% among the overall repair events (Prakash *et al.*, 2009; Saponaro *et al.*, 2010). We induced HO expression in  $\alpha$ -factor-arrested cells that were kept arrested in G1 in the presence of galactose (Figure 10B). Galactose was maintained in the

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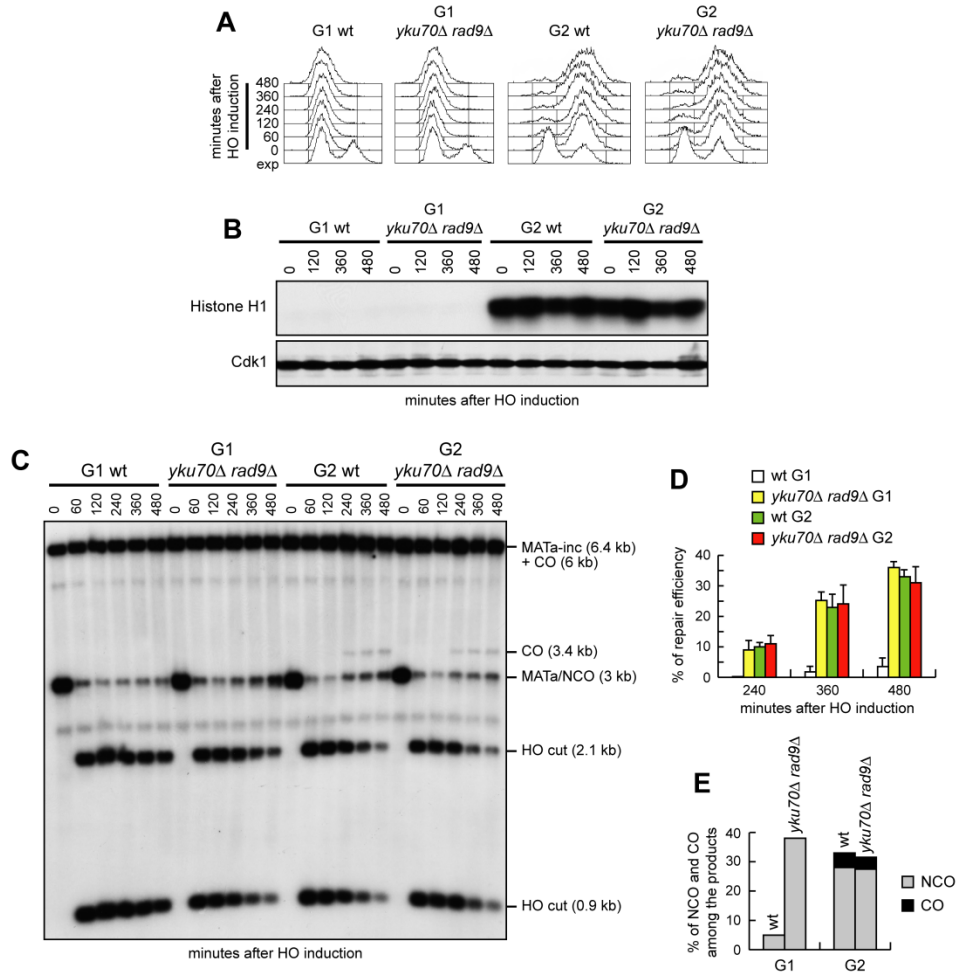
medium to cleave the HO sites that were eventually reconstituted by NHEJ-mediated DSB repair. The 3 kb *MATa* band resulting from recombination events that are not associated to crossovers re-accumulated in both *yku70Δ* and *yku70Δ rad9Δ* G1 cells, but not in wild type and *rad9Δ* G1 cells (Figure 10C and 10D). The repair efficiency in both *yku70Δ* and *yku70Δ rad9Δ* G1 cells was around 40% after 8 hours (Figure 10C and 10D), reaching 80–90% after 24 hours (data not shown). This finding indicates that the absence of Yku is sufficient for noncrossover HR events to take place despite of the low Cdk1 activity.



### Figure 10. Generation of ssDNA bypasses Cdk1 requirement for noncrossover recombination.

(A) In all the strains with the indicated genotypes, galactose-induced HO generates a DSB at a *MATa* DNA sequence inserted on chromosome V, while the homologous *MATa-inc* region on chromosome III cannot be cut by HO and is used as a donor for HR-mediated repair, which can generate both noncrossover (NCO) and crossover (CO) products. The sizes of EcoRI (E) fragments detected by the depicted probe are indicated. (B–D) Exponentially growing YEP+raf (exp) cell cultures were arrested in G1 with  $\alpha$ -factor (time zero) and transferred to YEP+raf+gal in the presence of  $\alpha$ -factor. (B) FACS analysis of DNA content. (C) Southern blot analysis of EcoRI-digested genomic DNA with the *MATa* probe depicted in A. (D) Densitometric analysis of the repair signals. Plotted values are the mean value  $\pm$ SD from three independent experiments as in (C). See **Materials and Methods** for details.

**Cdk1 REQUIREMENT FOR CROSSOVER RECOMBINATION.** Interestingly, the 3.4 kb chromosomal band expected in the experiment above in case of crossover products was not detectable in any G1 cell culture (**Figure 10C**), suggesting a role for Cdk1 in promoting crossover outcomes that is different from its function in DSB resection. We then compared the products of interchromosomal recombination in G1- and G2-arrested wild type and *yku70Δ rad9Δ* cells (**Figure 11A**). As expected, Cdk1 kinase activity remained undetectable in all  $\alpha$ -factor arrested cell cultures, whereas it was high in G2-arrested cells (**Figure 11B**). The overall DSB repair efficiency of G1-arrested *yku70Δ rad9Δ* cells was similar to that of G2-arrested wild type and *yku70Δ rad9Δ* cells (**Figure 11C** and **11D**). However, while no crossover events were detectable in *yku70Δ rad9Δ* G1 cells, ~4–5% of repair events were associated to crossovers in both wild type and *yku70Δ rad9Δ* G2 cells, as indicated by the appearance of the 3.4 kb crossover band (**Figure 11C** and **11E**). Thus, *yku70Δ rad9Δ* G1 cells appear to be specifically defective in generating crossover products. This inability was not due to the absence of Yku and/or Rad9, because similar amounts of crossover products were detectable in wild type and *yku70Δ rad9Δ* G2-arrested cells (high Cdk1 activity) (**Figure 11C** and **11E**). These results suggest that Cdk1 has a function in promoting crossover recombination that is independent of its role in DSB resection.



### Figure 11. Generation of ssDNA does not bypass Cdk1 requirement for crossover recombination.

Exponentially growing YEP+raf (exp) wild type and *yku70Δ rad9Δ* cells carrying the system described in Figure 10A were arrested at time zero in G1 with  $\alpha$ -factor or in G2 with nocodazole, and transferred to YEP+raf+gal in the presence of  $\alpha$ -factor or nocodazole, respectively. (A) FACS analysis of DNA content. (B) Cell samples of strains expressing fully functional Cdc28-HA were taken at the indicated times to assay Cdk1 kinase activity (top row) and to determine Cdk1 levels (bottom row) as in Figure 5F. (C) Southern blot analysis of EcoRI-digested genomic DNA as described in Figure 10. (D) Densitometric analysis of repair band signals (CO+NCO). Plotted values are the mean

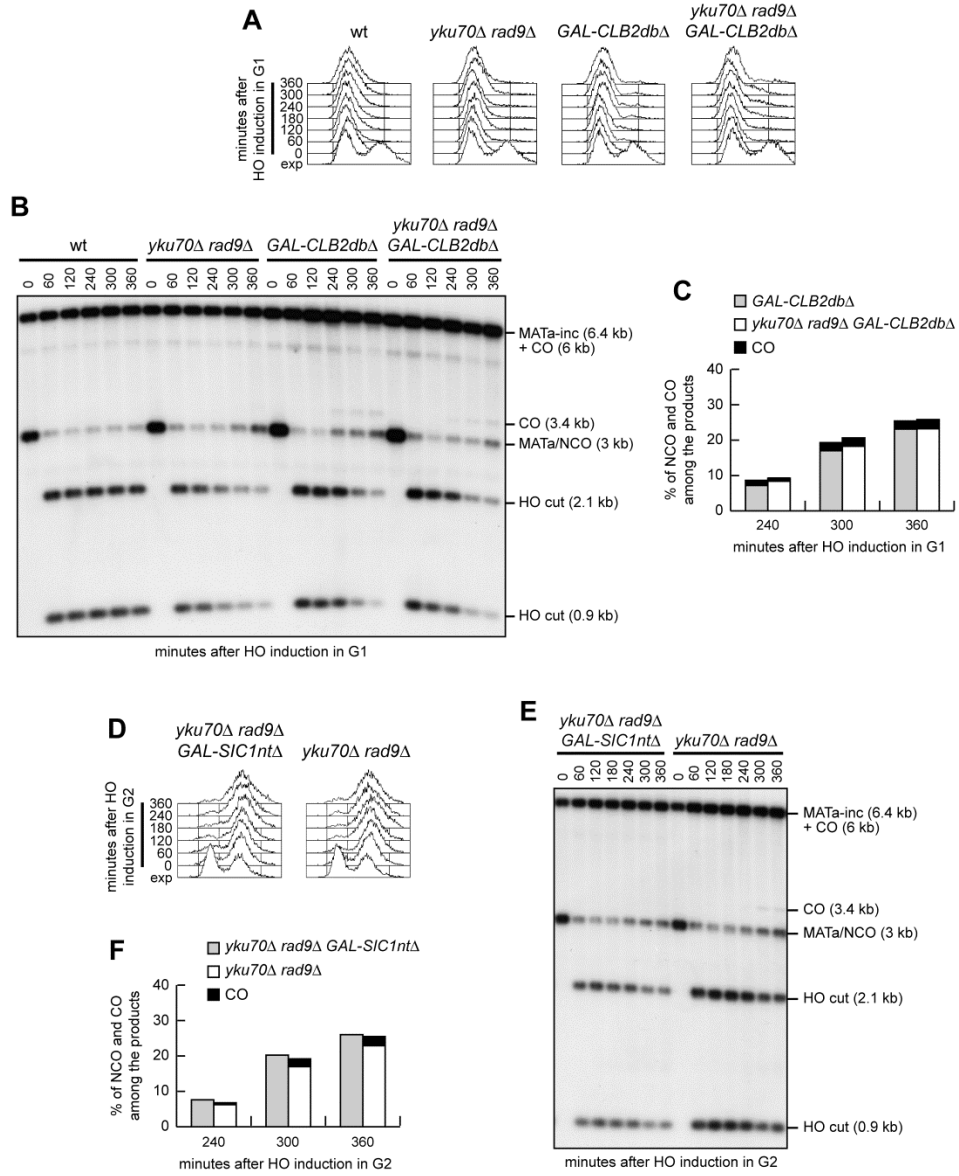
value  $\pm$ SD from four independent experiments as in (C), enclosing that described in (B). (E) Densitometric analysis of CO versus NCO repair bands at 480 minutes from break induction. See **Materials and Methods** for details.

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If the inability to perform crossover recombination in G1 were due to the lack of Cdk1 activation, then ectopic expression of active Cdk1 should allow crossover recombination in G1, whereas Cdk1 inhibition should prevent crossover formation in G2. We then constructed wild type and *yku70 $\Delta$  rad9 $\Delta$*  strains carrying the system in **Figure 10A** and expressing a stable version of the mitotic cyclin *CLB2* under the control of the *GAL* promoter (*GAL-CLB2db $\Delta$* ). This Clb2 variant forms active Clb2-Cdk1 complexes also during G1, because it lacks the destruction box, and therefore it is not subjected to B-type cyclin-specific proteolysis (Amon *et al.*, 1994). Strikingly, when both DSB formation and Clb2db $\Delta$  overproduction were induced in G1-arrested cell cultures by galactose addition (**Figure 12A**), crossover products became detectable in both *GAL-CLB2db $\Delta$*  and *yku70 $\Delta$  rad9 $\Delta$  GAL-CLB2db $\Delta$*  cells, whereas they were not present in wild type and *yku70 $\Delta$  rad9 $\Delta$*  cells under the same conditions (**Figure 12B and 12C**).

To assess whether Cdk1 inhibition prevented crossover formation in G2, we compared the products of interchromosomal recombination in G2-arrested *yku70 $\Delta$  rad9 $\Delta$*  and *yku70 $\Delta$  rad9 $\Delta$  GAL-SIC1nt $\Delta$*  cells (**Figure 12D**), the latter expressing high levels of a stable version of the Cdk1 inhibitor Sic1 (Sic1nt $\Delta$ ) (Desdouets *et al.*, 1998). When both DSB formation and Sic1nt $\Delta$  overproduction were induced in G2-arrested cell cultures by galactose addition, crossover products accumulated, as expected, in *yku70 $\Delta$  rad9 $\Delta$*  cells, but they were undetectable in *yku70 $\Delta$  rad9 $\Delta$  GAL-SIC1nt $\Delta$*  cells (**Figure 12E and 12F**).





**Figure 12. Ectopic Cdk1 activation allows crossovers in G1, whereas Cdk1 inhibition prevents crossover in G2.**

(A–C) Exponentially growing YEP+raf (exp) cultures of cells with the indicated genotypes and carrying the system described in **Figure 10A** were arrested at time zero in G1 with  $\alpha$ -factor and transferred to YEP+raf+gal in the presence of  $\alpha$ -factor. (A) FACS analysis of DNA content. (B) Southern blot analysis of EcoRI-digested genomic DNA as described in **Figure 10**. (C) Densitometric analysis. Plotted values are the mean value from two independent experiments as in (B). (D–F) Exponentially growing YEP+raf cells with the indicated genotypes and carrying the system described in **Figure 10A** were arrested at time zero in G2 with nocodazole and transferred to YEP+raf+gal in the presence of nocodazole. (D) FACS analysis of DNA content. (E) Southern blot analysis of EcoRI-digested genomic DNA as described in **Figure 10**. (F) Densitometric analysis. Plotted values are the mean value from three independent experiments as in (E).

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Thus, Sic1-mediated Cdk1 inhibition prevents generation of crossover products in G2, whereas ectopic Cdk1 activation leads to crossover recombination in G1, supporting the hypothesis that Cdk1 activity is required to promote crossover HR events even when DSB resection is allowed by the absence of Yku and Rad9.

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**G1/S and G2/M cyclin-dependent kinase activities  
commit cells to death in the absence of the S-phase  
checkpoint**

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The integrity of the genome is constantly challenged by DNA damage caused by environmental and intracellular factors. Aberrant DNA replication is a major source of mutations and chromosome rearrangements that can lead to cancer and other diseases in metazoans (reviewed in (Halazonetis *et al.*, 2008)). Replication fork progression can be hampered by exogenous or endogenous DNA damage. Furthermore, faithful replication depends on a balanced supply of deoxyribonucleotides (deoxyribonucleoside triphosphates [dNTPs]), whose levels are maintained during S-phase through the action of the ribonucleotide reductase (RNR) activity that converts the ribonucleotides to dNTPs (reviewed in (Nordlund and Reichard, 2006)). Indeed, replication fork pausing can be experimentally induced by genotoxic drugs, such as hydroxyurea (HU), which reduces dNTP pools by inhibiting RNR activity, and the DNA alkylating agent methyl methanesulfonate (MMS) that causes intra-S damage.

Eukaryotic cells respond to replication interference through a complex signal-transduction pathway, known as the S-phase checkpoint, whose key players in the budding yeast *Saccharomyces cerevisiae* are the Mec1 and Rad53 kinases (reviewed in (Branzei and Foiani, 2010; Zegerman and Diffley, 2009)). Mec1, together with its interacting protein Ddc2, is recruited to stalled forks, where it activates the effector kinase Rad53. Both kinases act in various ways to respond to replication interference. They are needed to complete DNA replication after exposure to HU or MMS (Desany *et al.*, 1998; Tercero and Diffley, 2001) by maintaining the integrity and/or activity of the replication forks (Cobb *et al.*, 2003; Katou *et al.*, 2003; Lucca *et al.*, 2004; De Piccoli *et al.*, 2012).

Furthermore, they stimulate dNTP production (Zhou and Elledge, 1993; Allen *et al.*, 1994; Huang *et al.*, 1998; Zhao *et al.*, 1998) and the transcription of several MCB binding factor (MBF)-regulated genes that are involved in DNA replication (Bastos de Oliveira *et al.*, 2012; Travesa *et al.*, 2012). Finally, they are required for inhibition of late replication origin firing (Santocanale and Diffley, 1998; Shirahige *et al.*, 1998) and for preventing accumulation of aberrant DNA structures, such as reversed forks or excessive single-stranded DNA (ssDNA) (Lopes *et al.*, 2001; Sogo *et al.*, 2002; Feng *et al.*, 2006). Despite their inability to replicate DNA, HU-treated *mec1* and *rad53* mutant cells proceed to elongate the mitotic spindle and to partition unreplicated or partially replicated DNA (Weinert *et al.*, 1994; Desany *et al.*, 1998). This premature chromosome segregation can be the cause of the extensive chromosomal fragmentation that is observed in mammalian cells lacking the Mec1 ortholog ATR (Brown and Baltimore, 2000; de Klein *et al.*, 2000; Canman, 2001), indicating that the S-phase checkpoint ensures that DNA replication is complete before cells divide not only in yeast but also in mammals.

Rad53 and Mec1 kinases are essential for cell viability, but cells lacking either Mec1 or Rad53 can be kept alive by overexpression of the *RNR* genes (Desany *et al.*, 1998) or by the lack of either the Rnr1 inhibitor Sml1 (Zhao *et al.*, 1998) or the transcriptional repressor of the *RNR* genes Crt1 (Huang *et al.*, 1998). Because dNTP pools are limiting even during a normal S phase (Poli *et al.*, 2012), these findings suggest that the essential function of Mec1 and Rad53 is to provide cells with sufficient dNTP levels to support DNA replication. This checkpoint-mediated regulation of dNTP pools is thought to be distinct from the checkpoint-mediated regulation of S-phase progression under replication

stress, because *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells lacking the Sml1-mediated inhibition of RNR activity are still extremely sensitive to agents that cause replication stress, such as HU and MMS.

Given the essential function of these checkpoint kinases in mediating the response to replication stress, a fundamental question to be addressed is which a process(es) regulated by the checkpoint is critical for the maintenance of cell viability. A hypomorphic *mec1* mutant (*mec1-100*) (Paciotti *et al.*, 2001), which does not block late origin firing in HU but is much less HU sensitive than *mec1Δ sml1Δ* cells, argues that regulation of late origin firing plays a relatively minor role in maintaining cell viability after exposure to replication stress (Tercero *et al.*, 2003). Cells lacking Mec1 that are kept viable by *SML1* deletion have been shown to accumulate chromosome breakages during HU treatment as a consequence of not fully replicated chromosomes being under persistent tension exerted by the mitotic spindle (Feng *et al.*, 2009). However, inhibiting spindle formation via nocodazole treatment does not improve viability of *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells during exposure to HU (Desany *et al.*, 1998; Tercero and Diffley, 2001), suggesting that precocious chromosome segregation *per se* is not the reason for the loss of viability of HU-treated *rad53* and *mec1* mutants. This finding has led to the proposal that the DNA replication defects arising when *mec1* and *rad53* mutants experience replication impediments irreversibly commit cells to death during S phase.

To further investigate the role of Mec1 and Rad53 in maintaining cell viability in the presence of replication stress, we searched for extragenic mutations suppressing the hypersensitivity to HU of *mec1Δ sml1Δ* cells. By characterizing one of the identified suppressor mutations, we provide evidence that

decreased activity of the cyclin-dependent kinase (Cdk1 or Cdc28 in yeast) complex suppresses *mec1* and *rad53* cell lethality not only during exposure to replication stress but also during an unchallenged S-phase. Delaying either the G<sub>1</sub>/S transition or spindle elongation improves viability of HU-treated *mec1* and *rad53* mutants and bypasses the Mec1 and Rad53 essential function during an unperturbed S phase. Further investigation of the suppression mechanism suggests that cell death caused by the lack of the S-phase checkpoint may be a consequence of Cdk1 activity forcing unscheduled events, such as the G<sub>1</sub>/S transition and spindle elongation.

**DECREASED Cdk1 ACTIVITY IMPROVES VIABILITY OF *mec1* AND *rad53* MUTANTS BOTH IN THE ABSENCE AND IN THE PRESENCE OF REPLICATION STRESS.** Budding yeast cells lacking Mec1 or Rad53 and kept viable by *SML1* deletion (*mec1Δ sml1Δ* or *rad53Δ sml1Δ*) die even when exposed to very low HU doses (**Figure 13A** and **13B**). To understand the nature of this lethality, we searched for spontaneous mutations that confer increased HU resistance to *mec1Δ sml1Δ* cells (see **Materials and Methods**). Given the extremely high HU sensitivity of *mec1* mutants, this screening was performed using 5 mM HU, which is the minimal HU dose impairing the ability of *mec1Δ sml1Δ* cells to form colonies. One of the suppressors turned out to be a mutation in the *CDC28* gene (see **Materials and Methods**), which encodes for the catalytic subunit Cdc28/Cdk1 of cyclin-dependent kinase. This mutation (*cdc28-sup*) improved viability of *mec1Δ sml1Δ* cells in the presence of either MMS or low HU doses (**Figure 13A**). Suppression was not restricted to the *MEC1* deletion, since *cdc28-sup* also decreased the HU and MMS sensitivity of *ddc2Δ sml1Δ* cells (**Figure**

**13A).** Cells carrying the *cdc28-sup* mutation were also temperature sensitive for growth, but they did not show a uniform terminal phenotype when shifted to 37°C (data not shown).

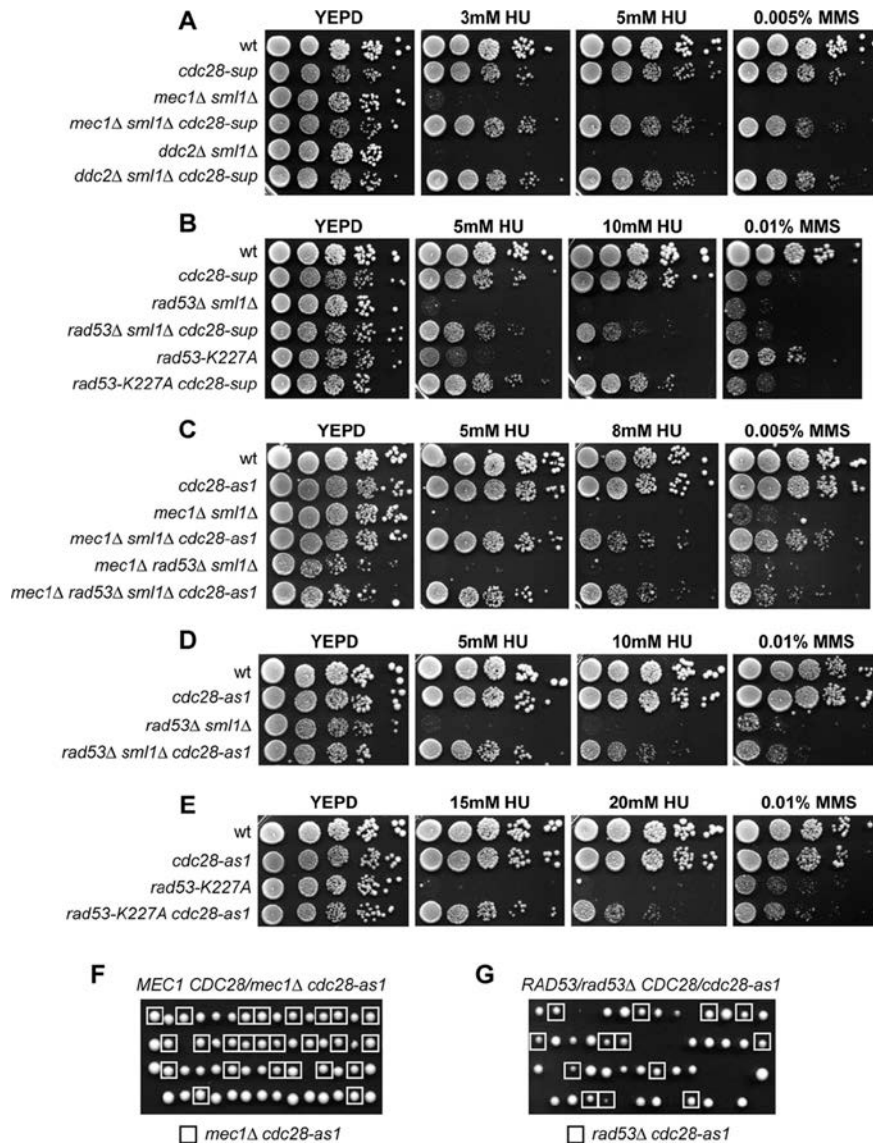
Mec1 might function in supporting cell viability in the presence of HU because it is required to activate the downstream kinase Rad53. However, *mec1Δ sml1Δ* cells are considerably more sensitive to HU and other DNA-damaging agents than are *rad53Δ sml1Δ* cells (Desany *et al.*, 1998), suggesting that Mec1 and Rad53 might have different roles during DNA replication under stress conditions. Thus, we sought to determine whether the *cdc28-sup* mutation could suppress also the HU sensitivity of *rad53Δ sml1Δ* or *rad53-K227A* mutant cells, the latter expressing a Rad53 mutant variant with reduced kinase activity that supports cell viability even in the presence of Sml1 (Sun *et al.*, 1996). The ability of *rad53Δ sml1Δ cdc28-sup* and *rad53-K227A cdc28-sup* cells to form colonies in the presence of HU was higher than that of *rad53Δ sml1Δ* and *rad53-K227A* cells, respectively, indicating that *cdc28-sup* can suppress also the HU sensitivity caused by Rad53 dysfunction (**Figure 13B**). We were unable to determine whether *cdc28-sup* suppressed the MMS sensitivity of *rad53* mutants because the *cdc28-sup* mutation by itself caused cell lethality in the presence of the amount of MMS that was required to impair viability of *rad53* mutants (**Figure 13B**).

To assess whether the Cdk1-dependent lethality in HU of *mec1* and *rad53* cells could be attributed to Cdk1 kinase activity, we constructed *mec1* and *rad53* mutants expressing the *cdc28-as1* allele, which encodes for a kinase with an enlarged ATP-binding pocket, allowing it to bind the nonhydrolyzable ATP analogue 1-NM-PP1. Treatment of *cdc28-as1* cells with 1-NM-PP1 results in



rapid downregulation of Cdc28 kinase activity, but Cdc28-as1 kinase activity is reduced by ~20% compared to wild type even in the absence of 1-NM-PP1 (Bishop *et al.*, 2000). We found that survival to HU of *mec1Δ sml1Δ cdc28-as1* cells was higher than that of *mec1Δ sml1Δ* cells even in the absence of 1-NM-PP1 (**Figure 13C**). Furthermore, the *cdc28-as1* mutation diminished the HU sensitivity of both *rad53Δ sml1Δ* (**Figure 13D**) and *rad53-K227A* cells (**Figure 13E**). Therefore, a reduced Cdk1 activity counteracts cell death in *mec1* and *rad53* mutants exposed to replication impediments. The HU sensitivity of *mec1Δ rad53Δ sml1Δ* cells was similar to that of *mec1Δ sml1Δ* cells (**Figure 13C**), and *cdc28-as1* improved survival in response to HU treatment of *mec1Δ sml1Δ* and *mec1Δ rad53Δ sml1Δ* cells to the same extent (**Figure 13C**). Thus, suppression in *mec1Δ sml1Δ* mutant does not appear to require the activity of Rad53.

Interestingly, although *mec1Δ sml1Δ* cells were more sensitive to MMS than *rad53Δ sml1Δ* cells (compare **Figure 13C** and **13D**), the *cdc28-as1* mutation suppressed the sensitivity to MMS of *mec1Δ sml1Δ* more efficiently to that of both *rad53Δ sml1Δ* and *rad53-K227A* cells (**Figure 13C** to **13E**). These findings suggest that the causes of death in MMS-treated *mec1* and *rad53* mutants are different, supporting previous data showing that Mec1 and Rad53 play different roles in the response to MMS treatment (Segurado and Diffley, 2008).



**Figure 13. Hypomorphic mutations in CDC28 improve viability of *mec1* and *rad53* mutants.**

(A to E) Exponentially growing cultures of strains with the indicated genotypes were serially diluted (1:10), and each dilution was spotted out onto YEPD plates with or without HU and MMS at the indicated concentrations. The plates were then incubated

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at 25°C for 3 days. (F and G) Meiotic tetrads from *MEC1 CDC28/mec1 cdc28-as1* and *RAD53/rad53 CDC28/cdc28-as1* diploid strains were dissected on YEPD plates, which were incubated at 25°C for 3 days. Clones from double-mutant spores are highlighted by squares.

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Although the MMS sensitivity of *mec1Δ rad53Δ sml1Δ* cells was similar to that of *mec1Δ sml1Δ* cells (Figure 13C), *cdc28-as1* improved survival in response to MMS treatment of *mec1Δ sml1Δ* cells more efficiently than that of *mec1Δ rad53Δ sml1Δ* cells (Figure 13C and 13D), indicating that Rad53 contributes to the *cdc28-as1*-mediated suppression of the MMS sensitivity caused by the lack of Mec1.

Rad53 and Mec1 are essential for cell viability, prompting us to ask whether a reduced Cdk1 activity could also bypass the essential function of these checkpoint kinases. Diploid strains heterozygous for *cdc28-as1* and either *mec1Δ* or *rad53Δ* alleles were generated and spore viability was monitored after tetrad dissection. Since the *MEC1* and *CDC28* genes are linked to each other on chromosome II, *mec1Δ* and *cdc28-as1* alleles were expected to cosegregate in most tetrads from the *MEC1 CDC28/mec1Δ cdc28-as1* diploid. As expected, *mec1Δ* and *rad53Δ* spores failed to form colonies, whereas all of the *mec1Δ cdc28-as1* (Figure 13F) and *rad53Δ cdc28-as1* (Figure 13G) double-mutant spores formed colonies of almost wild-type size, indicating that a reduced Cdk1 activity rescues the lethality caused by the lack of either Mec1 or Rad53. Collectively, these data indicate that carrying out a certain Cdk1-dependent event(s) causes cell death in the absence of Mec1 or Rad53 independently of exogenous DNA replication stress.

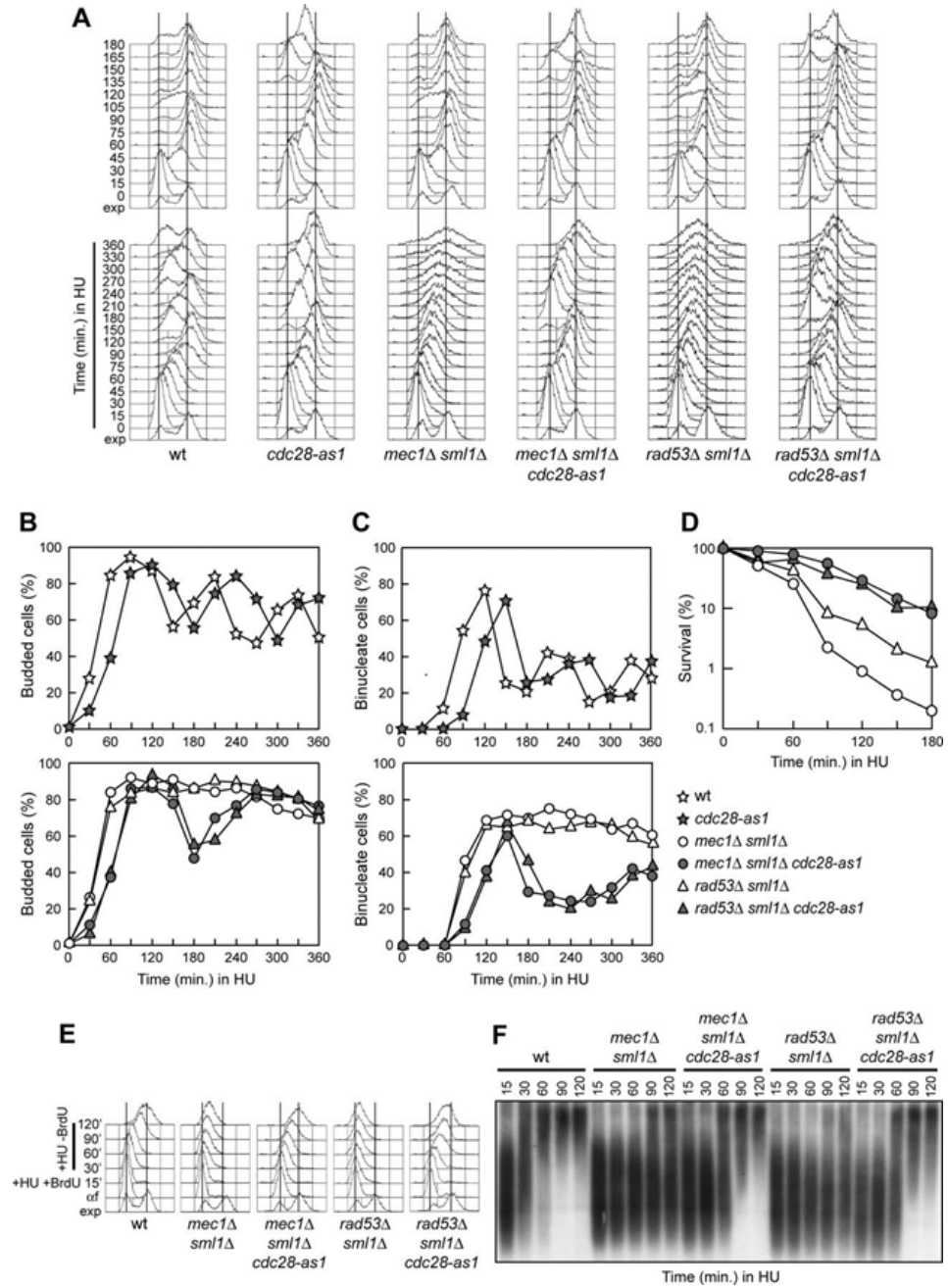
**DECREASED Cdk1 ACTIVITY SUPPRESSES THE DNA REPLICATION DEFECTS OF *mec1* AND *rad53* MUTANTS EXPOSED TO REPLICATION STRESS.**

To understand the molecular mechanisms underlying the suppression described above, we analyzed the effects of the *cdc28-as1* mutation on the kinetics of DNA replication and cell cycle progression of *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells exposed to a low HU dose. Cell cultures were blocked in G<sub>1</sub> with α-factor and released from the G<sub>1</sub> arrest either in the absence or in the presence of 20 mM HU (Figure 14). As expected (Desany *et al.*, 1998), *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells released in the presence of HU were unable to complete DNA replication even at 360 min after α-factor release (Figure 14A). However, most cells partitioned incompletely replicated DNA, undergoing nuclear division at 90 min after the release (Figure 14C). Consistent with the requirement of Cdk1 to perform the G<sub>1</sub>/S and G<sub>2</sub>/M transitions, the presence of *cdc28-as1* delayed bud formation (Figure 14B) and nuclear division (Figure 14C) of wild-type, *mec1Δ sml1Δ*, and *rad53Δ sml1Δ* cells released in the presence of HU. Then, HU-treated *mec1Δ sml1Δ cdc28-as1* and *rad53Δ sml1Δ cdc28-as1* cells exited from mitosis, divided, and initiated a new cell cycle, whereas similarly treated *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells arrested as budded cells with two nuclei, as expected (Figure 14A to 14C). Accordingly, the survival rates in HU of *mec1Δ sml1Δ cdc28-as1* and *rad53Δ sml1Δ cdc28-as1* were higher than those of *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells throughout the experiment (Figure 14D). Thus, reducing Cdk1 activity in HU-treated *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells restores cell cycle progression and cell viability.

Interestingly, *mec1Δ sml1Δ cdc28-as1* and *rad53Δ sml1Δ cdc28-as1* cells seemed to have completed the bulk DNA synthesis at the time of nuclear

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division (**Figure 14A** and **14C**), suggesting that lowering the Cdk1 activity suppresses the DNA replication defects of *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells. To follow more directly the DNA replication kinetics, we performed BrdU pulse-chase experiments. Cells were synchronized in G<sub>1</sub> with  $\alpha$ -factor and released into medium containing 20 mM HU and BrdU for 15 min (**Figure 14E**) to label the nascent DNA. The BrdU was then chased by transferring cells to medium containing thymidine at a high concentration and 20 mM HU (**Figure 14E**). Labeled nascent DNA replication intermediates, which appeared as a smear in all of the strains after 15 min in HU plus BrdU, rapidly increased in size after the chase in wild-type cells (**Figure 14F**). Consistent with a failure of *mec1* and *rad53* mutants to complete DNA replication, the formation of high-molecular-weight molecules of nascent DNA was delayed in *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells. Strikingly, almost all of the incorporated BrdU in *mec1Δ sml1Δ cdc28-as1* and *rad53Δ sml1Δ cdc28-as1* cells was present in the high-molecular-weight fraction by 90 to 120 min after  $\alpha$ -factor release (**Figure 14F**). Altogether, these data suggest that reducing Cdk1 activity increases survival of HU-treated *mec1* and *rad53* mutants by suppressing their DNA replication defects.



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**Figure 14. Effects of *cdc28-as1* on DNA replication and cell cycle progression of HU-treated *mec1* and *rad53* cells.**

(A to D) Exponentially growing cultures of cells with the indicated genotypes were arrested in G<sub>1</sub> with  $\alpha$ -factor and released at 25°C in YEPD in the absence or in the presence of 20 mM HU. At the indicated times after  $\alpha$ -factor release, cell samples were taken for fluorescence-activated cell sorting (FACS) analysis of DNA content (A) and for scoring budding (B) and nuclear division (C). (D) Cell survival in the liquid cultures described in panels A to C was analyzed by evaluating CFU on YEPD after plating proper cell dilutions at the indicated times after  $\alpha$ -factor release, followed by incubation at 25°C for 3 days. (E and F) G<sub>1</sub>-arrested cells ( $\alpha$ f) were released into YEPRG containing 20 mM HU plus 25  $\mu$ M BrdU. After 15 min (+HU +BrdU 15'), the cells were chased with 2 mM thymidine into fresh medium containing 20 mM HU (+HU -BrdU). Cell samples were taken at the indicated times for FACS analysis of DNA content (E) and for detecting BrdU-labeled DNA with anti-BrdU antibodies after gel electrophoresis and transfer to a membrane (F).

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**Cdk1-DEPENDENT LETHALITY OF *mec1* AND *rad53* MUTANTS INVOLVES A Cdk1 FUNCTION AT THE G<sub>1</sub>/S TRANSITION.** Regulation of Cdk1 activity during the cell cycle depends on the interaction of the Cdk1 kinase with different cyclin subunits. In budding yeast, the partially redundant G<sub>1</sub> cyclins Cln1 to Cln3 are required to perform the G<sub>1</sub>/S transition (Richardson *et al.*, 1989). Interestingly, deletion of both *CLN1* and *CLN2* was shown to partially bypass the essential requirement of Mec1, whereas overexpression of *CLN1* or *CLN2* (but not of *CLN3*) exacerbated the growth defects of *mec1* mutants in the absence of genotoxic agents (Vallen and Cross, 1999). We therefore sought to determine whether the Cdk1-dependent lethality of HU-treated *mec1* and *rad53* cells could be attributed to a function of Cdk1 in G<sub>1</sub>. Deletion of *CLN2* improved the viability of *mec1* $\Delta$  *sml1* $\Delta$ , *rad53* $\Delta$  *sml1* $\Delta$ , and *rad53-K227A* mutant cells exposed to moderate HU doses (Figure 15A), whereas *CLN1* deletion did not (data not shown). Similar to what we observed for the *cdc28-as1* allele, the lack of Cln2 suppressed the MMS sensitivity of *mec1* $\Delta$  *sml1* $\Delta$

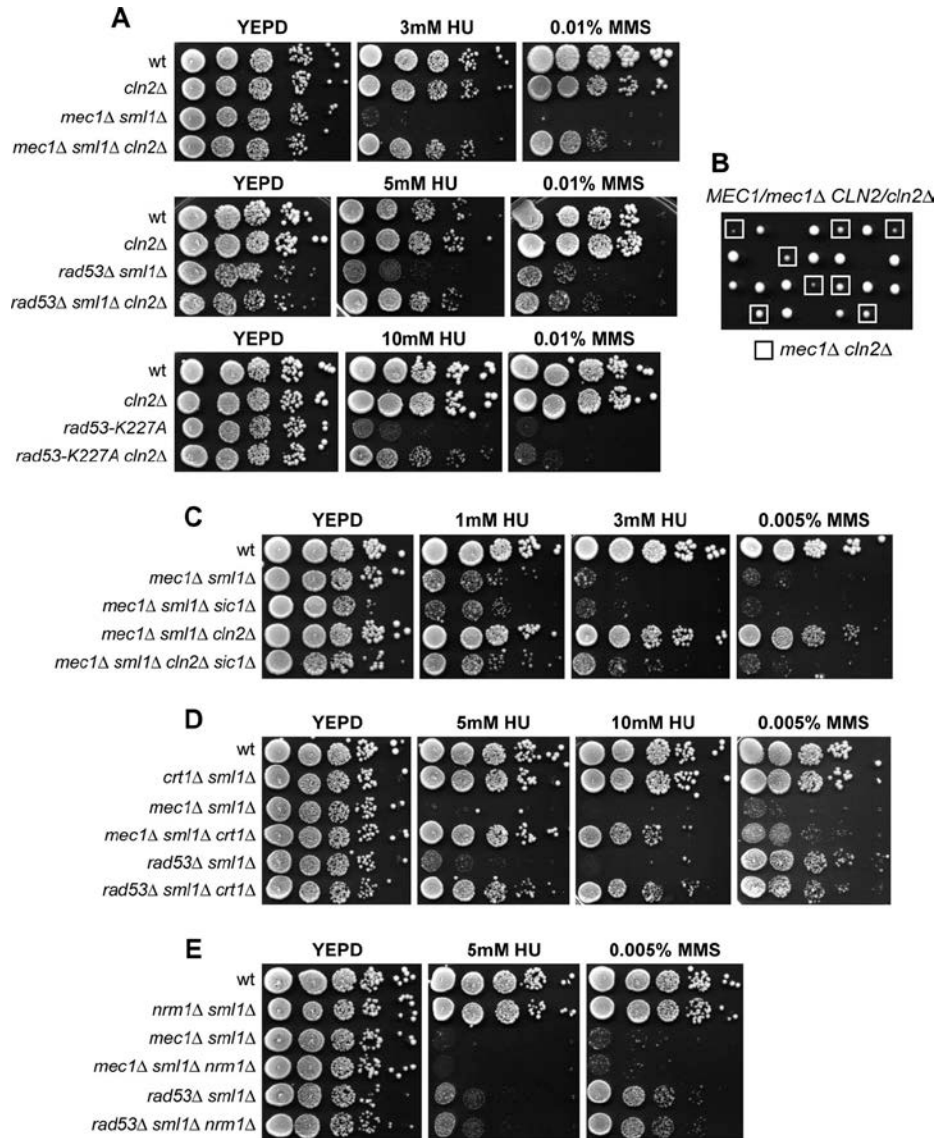
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cells, but only a very weak (if any) suppressor effect was detectable in *rad53Δ sml1Δ* and *rad53-K227A* cells (**Figure 15A**).

We also found that the lack of Cln2 bypasses the essential function of Mec1 and Rad53. In fact, tetrad dissection of diploid strains heterozygous for *cln2Δ* and *mec1Δ* alleles showed that all of the *mec1Δ cln2Δ* double-mutant spores formed colonies, although with a smaller size than wild-type or *cln2Δ* spores (**Figure 15B**). Similar results were obtained by combining the *cln2Δ* and *rad53Δ* alleles (data not shown).

The function of Cln1 and Cln2 in G<sub>1</sub> is to promote entry into S-phase by activating the S-phase Clb5-6/Cdk1 complexes, which in turn trigger replication origin firing. This Cln/Cdk1 function is accomplished through phosphorylation and degradation of the specific Clb/Cdk1 inhibitor Sic1 (Schwob *et al.*, 1994; Verma *et al.*, 1997). At the G<sub>1</sub>/S transition, the S-phase checkpoint induces transcription of the *RNR* genes and of several MBF-regulated genes through the inhibition of transcriptional repressors Crt1 (Huang *et al.*, 1998) and Nrm1 (Bastos de Oliveira *et al.*, 2012; Travesa *et al.*, 2012), respectively. Whether this checkpoint-mediated transcriptional program helps cell survival in the presence of replication stress is not known. Because *CLN2* deletion causes a G<sub>1</sub>/S transition delay (Dirick *et al.*, 1995), one possibility is that this delay provides *mec1* and *rad53* mutant cells with a longer time to amend the amount of dNTPs and/or proteins that are required to support DNA replication under stress conditions. If this were the case, the *cln2Δ*-mediated suppression should be overcome by abrogating the G<sub>1</sub>/S delay.





**Figure 15. The lack of Cln2 improves viability of *mec1* and *rad53* mutants.**

(A and C to E) Exponentially growing cultures of strains with the indicated genotypes were serially diluted (1:10), and each dilution was spotted out onto YEPD plates with or without HU and MMS at the indicated concentrations. The plates were then incubated

at 25°C for 3 days. (B) Meiotic tetrads from a *MEC1/mec1Δ CLN2/cln2Δ* diploid strain were dissected on YEPD plates, which were incubated for 3 days at 25°C. Clones from double-mutant spores are highlighted by squares.

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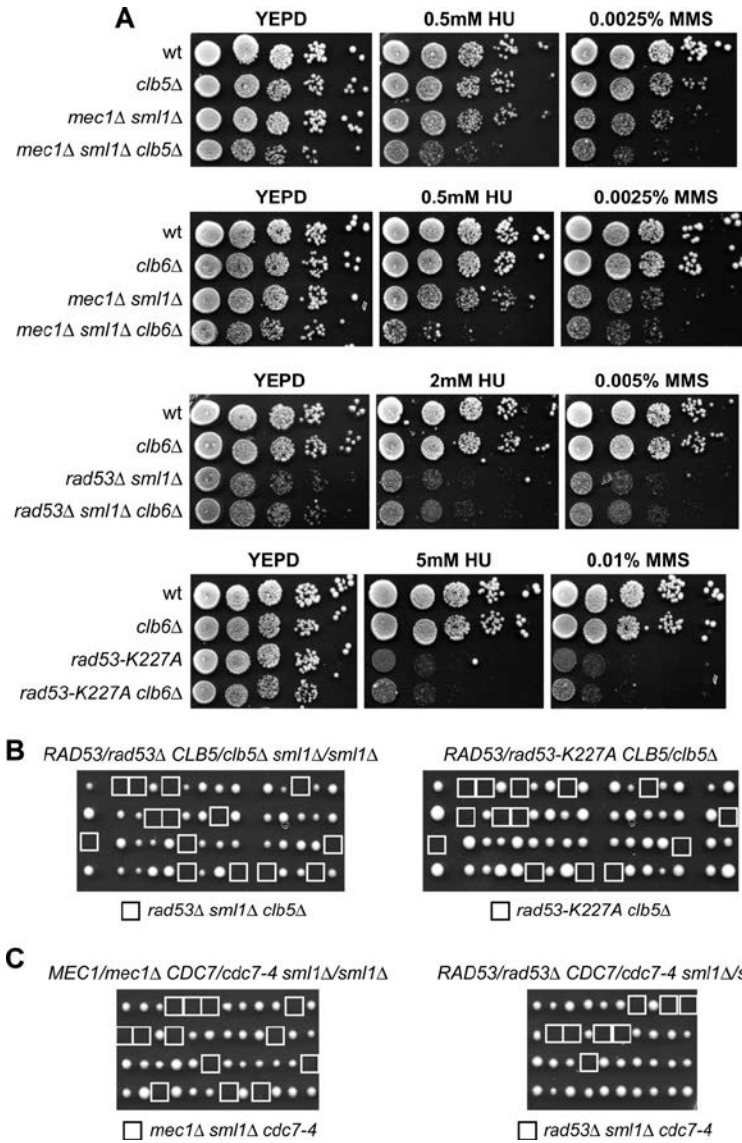
Indeed, deletion of *SIC1*, which is known to abolish the delay in S-phase entry caused by the absence of Cln2 (Dirick *et al.*, 1995), counteracted the ability of *cln2Δ* to suppress the HU and MMS sensitivity of *mec1Δ sml1Δ* cells. In fact, loss of viability of *mec1Δ sml1Δ cln2Δ sic1Δ* mutant cells after plating on HU- and MMS-containing media was similar to that of *mec1Δ sml1Δ sic1Δ* and *mec1Δ sml1Δ* cells (Figure 15C). This finding suggests that *CLN2* deletion can suppress the sensitivity to replication stress of *mec1* and *rad53* mutant cells by delaying entry into S phase.

The data presented above imply that the role of Mec1 and Rad53 in inducing transcription is important to maintain cell survival in the presence of replication stress. To further assess this possibility, we sought to determine whether the lack of the transcriptional repressor Crt1 or Nrm1 improves the survival in response to HU and/or MMS of *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells. Indeed, the lack of Crt1, which was previously shown to bypass the essential function of Mec1 (Huang *et al.*, 1998), decreased the HU and MMS sensitivity of *mec1Δ sml1Δ* cells and the HU sensitivity of *rad53Δ sml1Δ* cells (Figure 15D), whereas the lack of Nrm1 did not (Figure 15E). Thus, checkpoint-mediated inhibition of Crt1 and subsequent transcriptional activation of the *RNR* genes help cells to survive in the presence of replication stress.

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**DECREASING S-PHASE Cdk1 FUNCTION IS DETRIMENTAL IN *mec1* AND *rad53* MUTANTS.** In *S. cerevisiae*, the two B-type cyclins—Clb5 and Clb6—stimulate the function of Cdk1 in promoting replication origin firing, with Clb5 playing the major role (Epstein and Cross, 1992; Kühne and Linder, 1993; Schwob and Nasmyth, 1993). Decreasing Clb5/Clb6-Cdk1 activity might improve the viability of HU- and/or MMS-treated *mec1* and *rad53* mutants by lowering the total number of replication forks and therefore the chance to block them. This does not seem to be the case, since neither *CLB5* deletion nor *CLB6* deletion suppressed the sensitivity to HU or MMS of *mec1* and *rad53* cells (**Figure 16A**). On the contrary, *mec1Δ sml1Δ clb5Δ* and *mec1Δ sml1Δ clb6Δ* cells displayed enhanced sensitivity to HU and MMS compared to each single mutant (**Figure 16A**). Moreover, consistent with previous data (Gibson *et al.*, 2004), *mec1Δ sml1Δ clb5Δ* cells grew less efficiently than *mec1Δ sml1Δ* cells even in the absence of HU or MMS (**Figure 16A**), and the deletion of *CLB5* was lethal in both *rad53Δ sml1Δ* and *rad53-K227A* mutant cells (**Figure 16B**).

Loss of viability of *rad53 clb5Δ* and *mec1 clb5Δ* double-mutant cells even in the absence of the Sml1-mediated inhibition of RNR activity might be due to a diminished number of fired replication origins and therefore of active replication forks that can complete DNA replication before cells divide. Since origin firing throughout S-phase requires also the Cdc7-Dbf4 (DDK) complex (reviewed in (Labib, 2010)), we analyzed the consequences of introducing the *cdc7-4* allele in *mec1Δ sml1Δ* and *rad53Δ sml1Δ* mutants.



**Figure 16. *CLB5* deletion affects viability of *mec1* and *rad53* mutants.**  
 (A) Exponentially growing cultures of strains with the indicated genotypes were serially diluted (1:10), and each dilution was spotted out onto YEPD plates with or without HU and MMS at the indicated concentrations. The plates were then incubated at 25°C for 3

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days. (B) Meiotic tetrads from *RAD53/rad53Δ CLB5/clb5Δ sml1Δ/sml1Δ* and *RAD53/rad53-K227A CLB5/clb5Δ* diploid strains were dissected on YEPD plates, which were incubated for 3 days at 25°C. (C) Meiotic tetrads from *MEC1/mec1Δ CDC7/cdc7-4 sml1Δ/sml1Δ* and *RAD53/rad53Δ CDC7/cdc7-4 sml1Δ/sml1Δ* diploid strains were dissected on YEPD plates, which were incubated for 3 days at 25°C. The squares in panels B and C highlight putative double-mutant spores based on viable spore genotyping.

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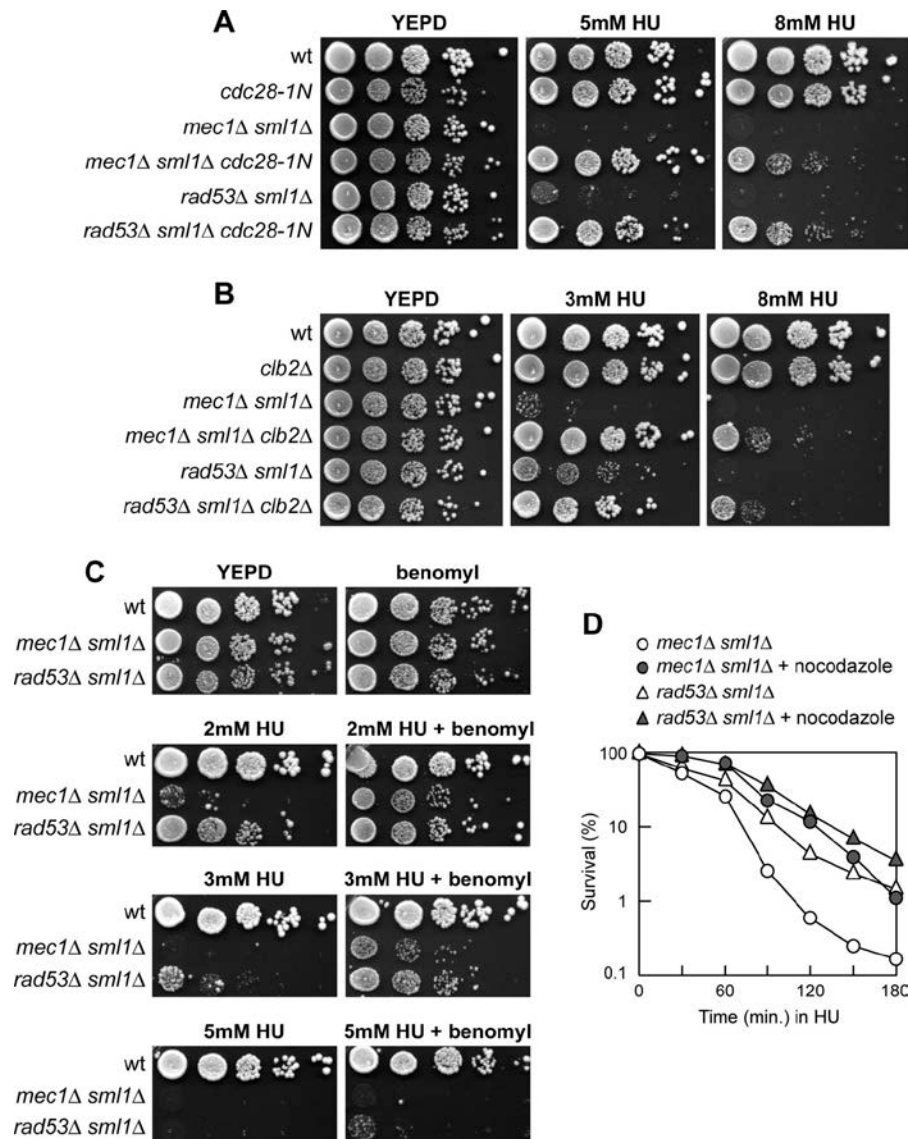
No viable *rad53Δ sml1Δ cdc7-4* and *mec1Δ sml1Δ cdc7-4* spores could be found after tetrad dissection of homozygous *sml1Δ* diploid strains, which were heterozygous for *cdc7-4* and either *mec1Δ* or *rad53Δ*, whereas each kind of single mutant spores was obtained at normal frequency (**Figure 16C**).

Thus, a decreased S-phase function of Cdk1 cannot account for the suppressor effect on the HU sensitivity of *mec1* and *rad53* mutants observed by reducing Cdk1 activity. Instead, disabling S-phase Cdk1 complexes impairs viability of these mutants even in the absence of exogenous replication impediments, possibly because it diminishes the number of active replication forks and therefore the chance to complete DNA replication.

**Cdk1-DEPENDENT LETHALITY OF *mec1* AND *rad53* MUTANTS INVOLVES A G<sub>2</sub>/M FUNCTION OF Cdk1.** To investigate whether the Cdk1-dependent lethality of *mec1* and *rad53* cells could implicate also some mitotic Cdk1 functions, we sought to determine whether the *cdc28-1N* allele, which is specifically defective in the interaction with the mitotic cyclins (Surana *et al.*, 1991), could suppress the HU and MMS sensitivity of *mec1* and *rad53* mutants. Furthermore, because Cdk1 association with the mitotic cyclins Clb1 to Clb4 drives spindle assembly and progression to metaphase (Rahal and Amon, 2008), we also analyzed the consequences of deleting the mitotic cyclin gene

*CLB2*. The *cdc28-1N* allele increased survival to HU of *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells (**Figure 17A**). Furthermore, viability on HU of *mec1Δ sml1Δ clb2Δ* and *rad53Δ sml1Δ clb2Δ* cells was higher than that of *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells, respectively (**Figure 17B**). Thus, a mitotic function of Cdk1 contributes to impair viability of HU-treated *mec1* and *rad53* mutants.

Although *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells are unable to complete DNA replication in the presence of HU, they prematurely elongate the spindles and partition unreplicated or partially replicated chromosomes (Weinert *et al.*, 1994; Desany *et al.*, 1998). Since Cdk1 activity is required to assemble a mitotic spindle (Rahal and Amon, 2008), decreased Cdk1 activity might suppress the sensitivity of *mec1* and *rad53* mutants to replication stress by providing the cells with a longer time to finish replicating the bulk of their chromosomes before they segregate. If this were the case, cell death in these mutants should be suppressed by disrupting kinetochore-microtubule attachment via nocodazole or benomyl treatment. Indeed, benomyl addition to HU-containing medium improved the viability of *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells (**Figure 17C**). Furthermore, the survival rates of G<sub>1</sub>-arrested *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells after release into YEPD medium containing 20 mM HU and nocodazole were higher than those of the same cells released into YEPD containing only 20 mM HU (**Figure 17D**). Interestingly, nocodazole addition suppressed the HU sensitivity of *mec1Δ sml1Δ* cells more efficiently than that of *rad53Δ sml1Δ* cells (**Figure 17D**), suggesting that the DNA replication defects in HU-treated *mec1* and *rad53* mutants do not completely overlap.



**Figure 17. Delaying spindle formation improves the viability of *mec1* and *rad53* mutants during replicative stress.**

(A and B) Exponentially growing cultures of strains with the indicated genotypes were serially diluted (1:10), and each dilution was spotted out onto YEPD plates with or

without HU at the indicated concentrations. Plates were then incubated at 25°C for 3 days. (C) Exponentially growing cultures of strains with the indicated genotypes were serially diluted (1:10), and each dilution was spotted out onto YEPD plates containing the indicated HU concentrations in the presence or absence of 10 µg of benomyl/ml. (D) G1-synchronized cultures were divided in two, and one-half was released into YEPD containing 20 mM HU and 5 µg of nocodazole/ml, whereas the other half was released into YEPD containing only 20 mM HU. Cell survival was analyzed by evaluating CFU on YEPD after plating proper cell dilutions at the indicated times after  $\alpha$ -factor release, followed by incubation at 25°C for 3 days. The experiment was repeated three times with similar results.

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**DELAYING SPINDLE ELONGATION SUPPRESSES THE SENSITIVITY OF *mec1* AND *rad53* MUTANTS TO REPLICATION STRESS.** Premature spindle elongation and DNA partitioning in HU-treated *mec1* and *rad53* mutants is due to upregulation of the major kinesin motor protein Cin8 (Krishnan *et al.*, 2004), which is known to contribute to the formation, stability and extension of the spindles (Hoyt *et al.*, 1992; Roof *et al.*, 1992; Straight *et al.*, 1998). Since Cdk1 activity drives the assembly of mitotic spindles by restraining Cin8 proteolysis (Crasta *et al.*, 2006), delaying spindle extension by deleting *CIN8* might be expected to improve the survival of *mec1* and *rad53* mutants to HU and/or MMS treatment. Indeed, the viability of *mec1* $\Delta$  *sml1* $\Delta$  *cin8* $\Delta$ , *rad53* $\Delta$  *sml1* $\Delta$  *cin8* $\Delta$ , and *rad53*-K227A *cin8* $\Delta$  cells in the presence of HU was considerably higher than that of *mec1* $\Delta$  *sml1* $\Delta$ , *rad53* $\Delta$  *sml1* $\Delta$ , and *rad53*-K227A cells, respectively (Figure 18A), suggesting that premature spindle elongation contributes to kill HU-treated *mec1* and *rad53* mutants. Similar to *cdc28-as1* and *cln2* $\Delta$ , *cin8* $\Delta$  efficiently suppressed the sensitivity to MMS of *mec1* $\Delta$  *sml1* $\Delta$  cells but suppressed very poorly that of *rad53* $\Delta$  *sml1* $\Delta$  cells (Figure 18A). Moreover, inactivation of both Cin8 and Cln2 suppressed the sensitivity to HU and MMS of *mec1* $\Delta$  *sml1* $\Delta$  cells more efficiently than did the single Cin8 or Cln2 inactivation (Figure 18B), in

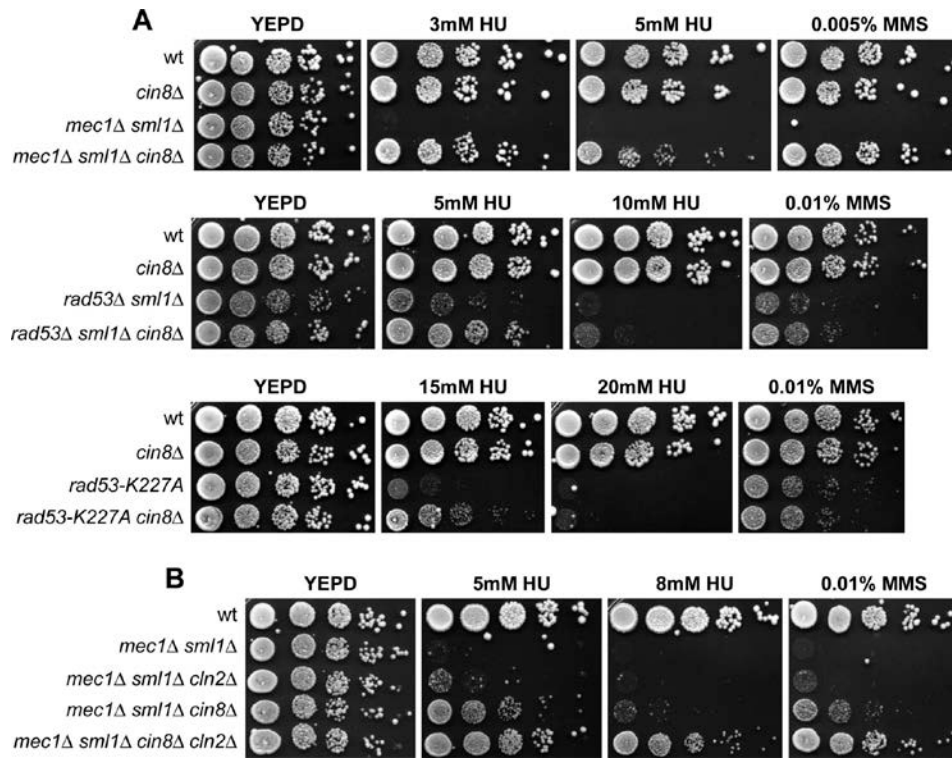
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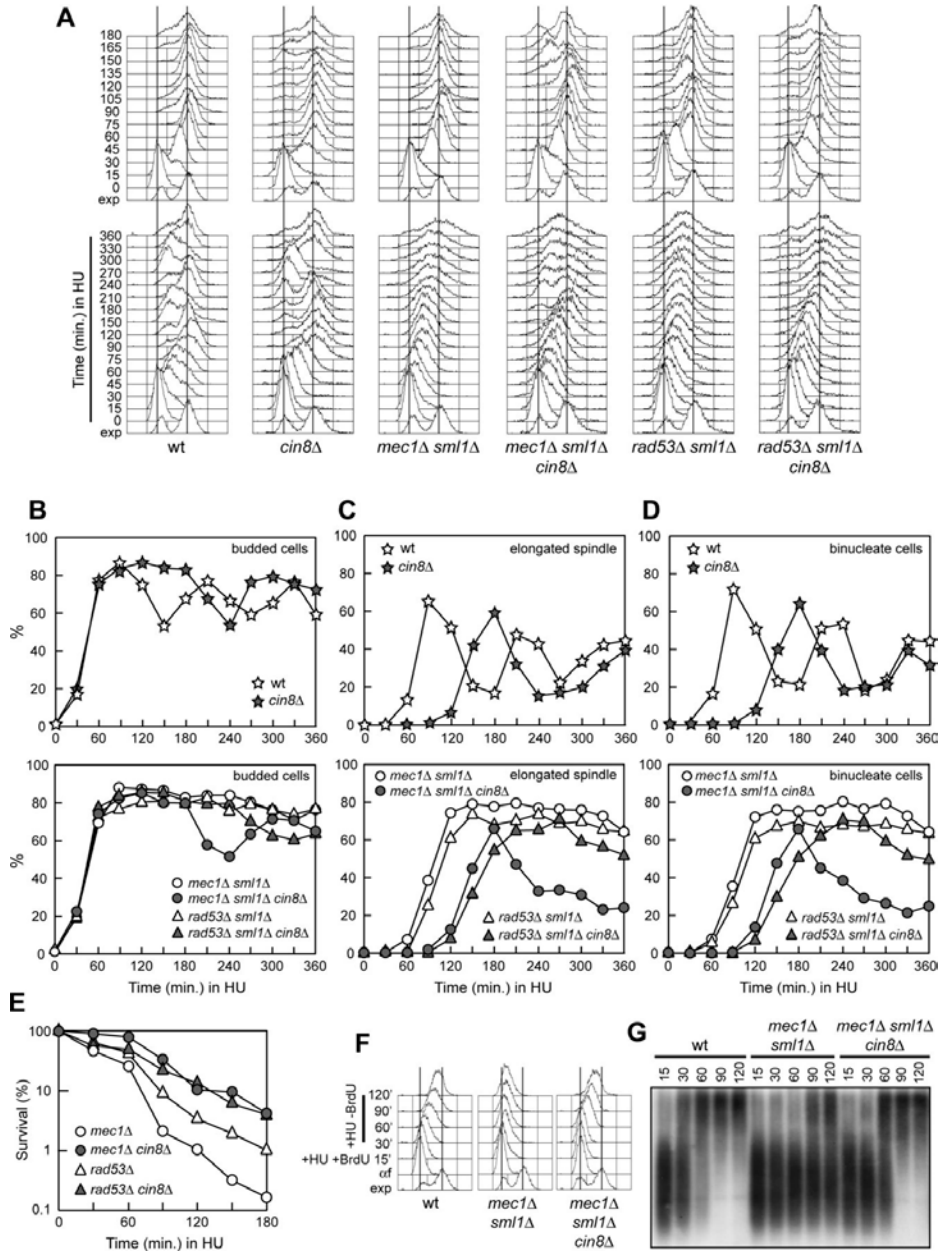
agreement with the notion that both the G<sub>1</sub>/S and the G<sub>2</sub>/M functions of Cdk1 are involved in determining this sensitivity.

To characterize the *cin8Δ*-mediated suppression, cells were arrested in G<sub>1</sub> with  $\alpha$ -factor and released in YEPD in the presence or absence of 20 mM HU. As expected, although HU-treated *mec1Δ sml1Δ* and *rad53Δ sml1Δ* mutants failed to complete DNA replication (**Figure 19A**), a significant proportion of them elongated the spindles (**Figure 19C**), divided the nuclei (**Figure 19D**), and then arrested as binucleate cells with elongated spindles. Deletion of *CIN8* did not affect bud emergence (**Figure 19B**) but slowed down spindle elongation and nuclear division in both *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells (**Figure 19C** and **19D**). Moreover, the survival rates of HU-treated *mec1Δ sml1Δ cin8Δ* and *rad53Δ sml1Δ cin8Δ* were higher than those of *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells throughout the experiment (**Figure 19E**). Consistent with the finding that *CIN8* deletion (as well as nocodazole addition) suppresses the HU sensitivity of *mec1Δ sml1Δ* more efficiently than that of *rad53Δ sml1Δ* cells (**Figure 19E**), most HU-treated *mec1Δ sml1Δ cin8Δ* cells exited from mitosis and resumed cell cycle progression within 210 min after release from G<sub>1</sub> arrest, whereas most *rad53Δ sml1Δ cin8Δ* cells were still arrested after 360 min (**Figure 19A** to **19D**). Interestingly, while spindle elongation and nuclear division in HU-treated *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells took place when DNA was not fully replicated, the bulk of DNA synthesis seemed to be completed at the time of spindle elongation/nuclear division in *mec1Δ sml1Δ cin8Δ* and *rad53Δ sml1Δ cin8Δ* cells under the same conditions (**Figure 19A** to **19D**).



**Figure 18. The lack of Cin8 increases survival of *mec1* and *rad53* mutants under replicative stress.**

Exponentially growing cultures of strains with the indicated genotypes were serially diluted (1:10), and each dilution was spotted out onto YEPD plates with or without HU and MMS at the indicated concentrations. The plates were then incubated at 25°C for 3 days.



**Figure 19. Effect of *CIN8* deletion on cell cycle progression of HU-treated *mec1* and *rad53* mutant cells.**

(A to E) Exponentially growing cultures of cells with the indicated genotypes, all expressing a Tub1-GFP fusion, were arrested in G<sub>1</sub> with  $\alpha$ -factor and released at 25°C in YEPD in the presence or absence of 20 mM HU. Cell samples were taken at the indicated times after  $\alpha$ -factor release for FACS analysis of DNA content (A) and for scoring budding (B), spindle elongation (C), and nuclear division (D). (E) Cell survival of the liquid cultures described in panels A to D was analyzed by evaluating CFU on YEPD after plating proper cell dilutions at the indicated times after  $\alpha$ -factor release, followed by incubation at 25°C for 3 days. (F and G) Immunodetection of BrdU-pulsed DNA. G<sub>1</sub>-arrested cells ( $\alpha$ f) were released into YEPRG containing 20 mM HU plus 25  $\mu$ M BrdU. After 15 min (+HU +BrdU 15'), the cells were chased with 2 mM thymidine into medium containing 20 mM HU (+HU -BrdU). Cell samples were taken at the indicated times after chase for FACS analysis of DNA content (F) and for detecting BrdU-labeled DNA with anti-BrdU antibodies as in Figure 14F (G).

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This indication was confirmed for *mec1* $\Delta$  *sm1* $\Delta$  *cin8* $\Delta$  cells by BrdU pulse-chase experiments during HU exposure. The cells were synchronized in G<sub>1</sub> with  $\alpha$ -factor and released into medium containing 20 mM HU and BrdU for 15 min (Figure 19F) to label the nascent DNA. The BrdU was then chased by transferring cells to medium containing thymidine at a high concentration and 20 mM HU (Figure 19F). As expected, the formation of high-molecular-weight molecules of nascent DNA was delayed in *mec1* $\Delta$  *sm1* $\Delta$  cells compared to wild-type cells (Figure 19G), whereas *mec1* $\Delta$  *sm1* $\Delta$  *cin8* $\Delta$  cells contained almost all of the incorporated BrdU into the high-molecular-weight fraction by 90 to 120 min after  $\alpha$ -factor release (Figure 19G). Collectively, these data indicate that delaying spindle elongation suppresses the DNA replication defects of *mec1* and *rad53* mutants exposed to a mild HU dose, possibly by providing additional time for completing DNA synthesis before spindle elongation and chromosome segregation take place.

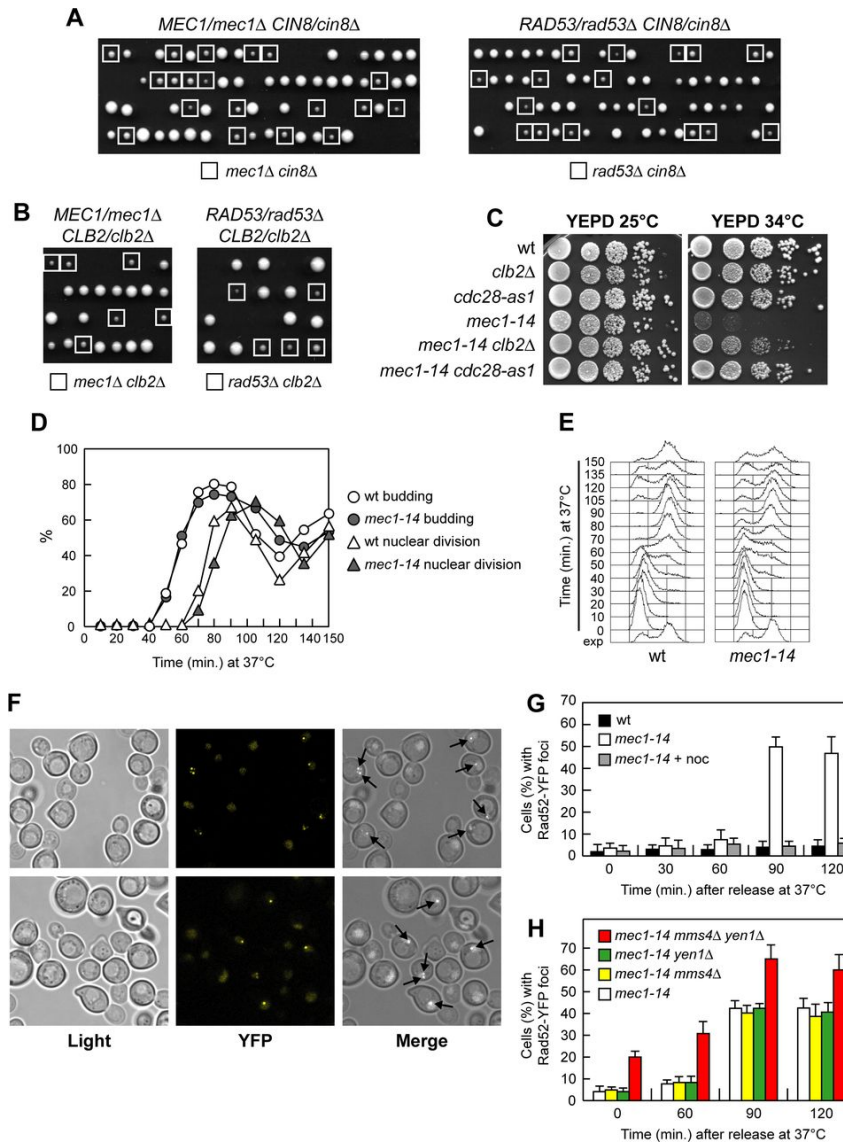
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**DELAYING SPINDLE ELONGATION BYPASSES THE ESSENTIAL FUNCTION OF Mec1 AND Rad53.** Cells lacking Mec1 or Rad53, but carrying wild-type *SML1*, die even in the absence of exogenous replication stress because they replicate their DNA with suboptimal dNTP levels (Zhao *et al.*, 1998). As HU depletes the dNTP pools, the cause of death in *mec1Δ SML1* and *rad53Δ SML1* cells might mimic that of *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells replicating their DNA in the presence of HU. Thus, we sought to determine whether delaying nuclear division by *CLB2* or *CIN8* deletion could bypass the essential function of Mec1 and Rad53. Tetrad dissection of diploid strains heterozygous for *cin8Δ* and either *mec1Δ* or *rad53Δ* alleles showed that most *mec1Δ cin8Δ* and *rad53Δ cin8Δ* double-mutant spores formed colonies, although with a smaller size than wild-type or *cin8Δ* spores (**Figure 20A**). Similar results were obtained by combining the *mec1Δ* or *rad53Δ* mutation with the *clb2Δ* mutation (**Figure 20B**). Thus, delaying spindle elongation not only suppresses the sensitivity of *mec1* and *rad53* to replication stress but also bypasses their essential function, suggesting that loss of viability in *mec1Δ SML1* and *rad53Δ SML1* cells is at least partially due to segregation of incompletely replicated chromosomes.

Segregation of partially replicated chromosomes may contribute to cell death because it causes lethal DNA breaks. Since DNA breaks are subjected to the action of the recombination protein Rad52 (Lisby *et al.*, 2004), we investigated whether conditional inactivation of Mec1 in the presence of wild-type *SML1* leads to the formation of Rad52 foci. We failed to efficiently deplete Mec1 by using repressible promoters or inducible degradation systems, and therefore we conditionally inactivated Mec1 by using the temperature-sensitive *mec1-14* allele that we identified previously (Longhese *et al.*, 1996). Consistent with our

finding that the *cdc28-as1* and *clb2Δ* alleles bypass the essential function of Mec1, both the *cdc28-as1* and *clb2Δ* mutations improved the viability of *mec1-14* cells at 34°C (**Figure 20C**). When wild-type and *mec1-14* cell cultures were arrested in G<sub>1</sub> with α-factor at 25°C and released into the cell cycle at 37°C, both cell types showed similar kinetics of bud emergence (**Figure 20D**). However, *mec1-14* cells delayed completion of DNA replication compared to wild-type cells, indicating a DNA replication defect (**Figure 20E**). Moreover, *mec1-14* cells dramatically accumulated Rad52 foci about 90 min after α-factor release at 37°C, concomitantly with nuclear division (**Figure 20F and 20G**), suggesting that lethal chromosome breaks occur when *mec1-14* cells enter mitosis. These Rad52 foci could arise as a consequence of the action of endonucleases, which process the replication intermediates that persist until mitosis in *mec1-14* cells. Alternatively, segregation of incompletely replicated chromosomes can be the cause of Rad52 focus formation in Mec1-deficient cells. Since the endonucleases Mms4 and Yen1, which are known to resolve recombination intermediates, are activated at the G<sub>2</sub>/M transition (Matos *et al.*, 2011), we analyzed their contribution in the generation of Rad52 foci in *mec1-14* cells. We found that the lack of Mms4, Yen1, or both did not impair Rad52 focus formation in *mec1-14* cells (**Figure 20H**). Rather, the lack of both Mms4 and Yen1 caused an increase of Rad52 foci in *mec1-14* cells even at 25°C (time zero in **Figure 20H**), possibly because these two nucleases are involved in repairing the double-strand breaks that arise in *mec1-14* cells.



**Figure 20. Delaying chromosome segregation bypasses the essential function of Mec1 and Rad53.**

(A and B) Meiotic tetrads from *MEC1/mec1Δ CIN8/cin8Δ* and *RAD53/rad53Δ CIN8/cin8Δ* diploid strains (A) and from *MEC1/mec1Δ CLB2/clb2Δ* and *RAD53/rad53Δ CLB2/clb2Δ* diploid strains (B) were dissected on YEPD plates, which were then incubated for 3 days at 25°C. Clones from double-mutant spores are highlighted by squares. (C) Exponentially growing cultures of strains with the indicated genotypes were serially diluted (1:10), and each dilution was spotted out onto YEPD plates, which were then incubated at the indicated temperatures. (D to G) Cultures of wild-type and *mec1-14* cells expressing a Rad52-YFP fusion were arrested in G1 with  $\alpha$ -factor at 25°C and then released into fresh medium at 37°C. Cell samples were taken at the indicated times after  $\alpha$ -factor release for scoring budding and nuclear division (D), for FACS analysis of DNA content (E), and for visualizing Rad52-YFP foci by fluorescence microscopy (F and G). (F) Rad52-YFP foci in *mec1-14* cells after 90 min at 37°C. Arrowheads in the merge mark Rad52 foci relative to cells. (G) Quantitation of Rad52-YFP foci. Half of the *mec1-14* G1-arrested cell culture was also released into fresh medium at 37°C in the presence of 5  $\mu$ g of nocodazole/ml (*mec1-14* + noc), and samples were taken to determine the fraction of cells containing Rad52-YFP. (H) G1-arrested cell cultures were released into fresh medium at 37°C, and Rad52-YFP foci were quantified at the indicated times after  $\alpha$ -factor release. The plotted values in panels G and H are mean values  $\pm$  the standard deviations from three independent experiments. At least 200 cells were counted for each cell culture at each time point.

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Interestingly, when *mec1-14* cells were released from a G<sub>1</sub> block at 37°C in the presence of nocodazole, Rad52 focus formation was greatly reduced (Figure 20G), supporting the hypothesis that premature chromosome segregation can be the cause of DNA break formation in Mec1-deficient cells.



## ***DISCUSSION***



**DISTINCT Cdk1 REQUIREMENTS DURING SINGLE-STRAND ANNEALING, NONCROSSOVER, AND CROSSOVER RECOMBINATION**

HR is highly coordinated with the cell cycle: it takes place predominantly during the S and G2 phases, when the presence of a sister chromatid provides a donor template and high Cdk1 activity promotes DSB end resection to expose ssDNA that is necessary to initiate HR (Caspari *et al.*, 2002; Aylon *et al.*, 2004; Ira *et al.*, 2004; Huertas *et al.*, 2008). To study whether Cdk1 plays additional role(s) in HR, we asked whether generation of ssDNA at the DSB ends is sufficient to bypass Cdk1 requirement for HR. Because the lack of either Yku or Rad9 allows Cdk1-independent generation of 3'-ended ssDNA at DSB ends (Clerici *et al.*, 2008; Lazzaro *et al.*, 2008), we investigated whether cells lacking Yku and/or Rad9 could repair a DSB by HR when Cdk1 activity is low. We found that DSB repair by SSA can take place in G1-arrested *yku70Δ* cells. The ability of these cells to carry out SSA in G1 depends on Cdk1-mediated generation of 3'-ended ssDNA at the DSB ends. In fact, the lack of Rad9 increases efficiency of both resection and SSA in *yku70Δ* G1 cells. Furthermore, Cdk1 inhibition prevents SSA in G2 wild type cells, but not in *yku70Δ rad9Δ* G2 cells, where DSB resection occurs independently of Cdk1. We also found that G1-arrested *yku70Δ* and *yku70Δ rad9Δ* cells can undergo interchromosomal recombination events that are not accompanied by crossovers. Thus, Cdk1 requirement for carrying out SSA and noncrossover recombination is bypassed by DSB resection, indicating

that Cdk1 promotes these HR events essentially by regulating the resection step.

Rad52 is essential for both SSA and noncrossover recombination events, while only the latter require the assembly of Rad51 nucleoprotein filaments, which promote homologous pairing and strand exchange (reviewed in (Pâques and Haber, 1999; Krogh and Symington, 2004; San Filippo *et al.*, 2008)). As the function of Cdk1 in DSB repair by SSA and noncrossover recombination is primarily the regulation of the resection step, neither Rad51 nor Rad52 appear to require Cdk1 activity to exert their biochemical activities.

Interestingly, although *RAD9* deletion was shown to allow MRX-dependent DSB resection in G2 cells that overproduced the Cdk1 inhibitor Sic1 (Lazzaro *et al.*, 2008), the lack of Rad9 did not increase DSB resection or HR-mediated DSB repair in G1 compared to wild type cells. Thus, although Rad9 provides a barrier to resection in *yku70Δ* G1 cells, its lack is not sufficient, by itself, to escape the inhibitory effect of Yku on DSB resection in G1. This finding is consistent with previous data showing that the resection block imposed by Yku is relieved in G2 (Bonetti *et al.*, 2010; Shim *et al.*, 2010). It also indicates that Rad9 prevents DSB resection in all cell cycle phases, but its inhibitory effect in G1 becomes apparent only in the absence of Yku.

Surprisingly, we found that G1-arrested *yku70Δ rad9Δ* cells are specifically impaired in the formation of crossovers by interchromosomal recombination. Expression of an activated form of Cdk1 allows crossover recombination in both wild type and *yku70Δ rad9Δ* G1 cells, whereas inhibition of Cdk1 activity in G2-arrested *yku70Δ rad9Δ* cells prevents crossover formation without affecting noncrossover outcomes. These findings are consistent with a role of Cdk1 in

promoting crossover recombination that is independent of its function in DSB resection.

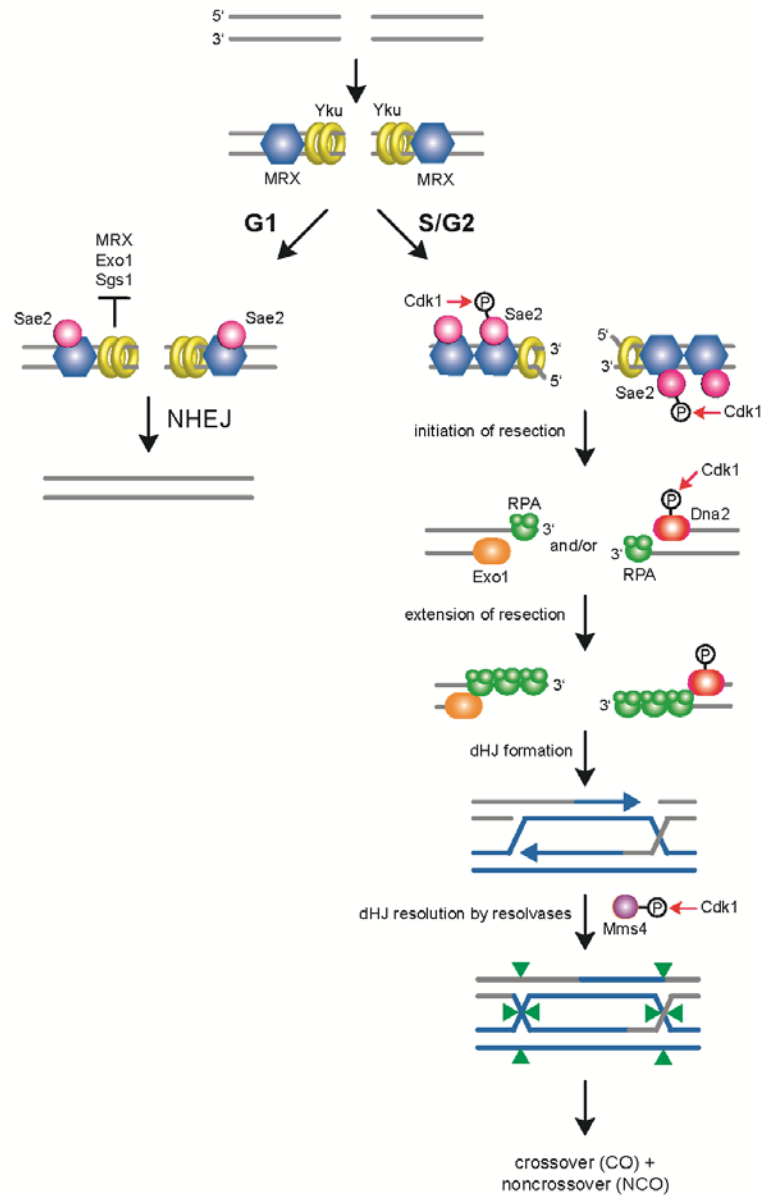
How does Cdk1 promote crossover outcomes? The choice between crossover and noncrossover is tightly regulated (Martini *et al.*, 2006). Meiotic recombination results frequently in crossovers (Youds and Boulton, 2011), while DSB repair in mitotic cells is rarely associated with crossovers (~5%) (Bzymek *et al.*, 2010). An explanation of these differences could be that specific mechanisms limit crossovers during mitotic homologous recombination. Indeed, dissociation of the D-loop intermediates gives rise to noncrossover products, and this process is promoted by the helicases Srs2 and Mph1 (Ira *et al.*, 2003; Robert *et al.*, 2006; Prakash *et al.*, 2009; Saponaro *et al.*, 2010). Furthermore, noncrossover outcomes can arise also from the dissolution of dHJ intermediates that requires the combined activity of the BLM/Sgs1 helicase, which drives migration of the constrained dHJs, and the Top3-Rmi1 complex, which decatenates the interlinked strands between the two HJs (Ira *et al.*, 2003; Wu and Hickson, 2003; Lo *et al.*, 2006). One possibility is that Cdk1 promotes crossover recombination by inhibiting proteins specifically involved in limiting crossover generation (i.e. Sgs1, Top3-Rmi1, Srs2 and Mph1). A similar mechanism seems to act during meiotic recombination, where proteins required for homologous chromosome synapsis have been proposed to antagonize the anti-crossover activity of Sgs1 (Jessop *et al.*, 2006). However, none of the above anti-crossover proteins have been reported to undergo Cdk1-dependent inhibitory phosphorylation. Alternatively, as dHJ formation requires a transition from D-loop to second-end capture (Hunter and Kleckner, 2001), Cdk1 might favour this transition by promoting DNA synthesis and

therefore by stabilizing the D-loop intermediates. Finally, Cdk1 might stimulate the activities of Mus81-Mms4, Slx1-Slx4, Yen1 and/or Rad1-Rad10 resolvases that generate crossover products by cleaving the dHJs (reviewed in (Svendsen and Harper, 2010)). Consistent with this last hypothesis, the Yen1 and Mms4 resolvases appear to be phosphorylated by Cdk1 (Ubersax *et al.*, 2003) and the nuclease activity of mammalian Mus81-Mms4 and Yen1 are tightly regulated throughout the mitotic cell cycle (Matos *et al.*, 2011). Furthermore, *S. cerevisiae* Mms4 undergoes Cdk1-dependent phosphorylation, and this phosphorylation allows its activation as a nuclease (Gallo-Fernández *et al.*, 2012).

In conclusion, Cdk1 controls primarily DSB resection to allow SSA and noncrossover recombination, while crossover outcomes appear to require additional Cdk1-promoted events. As mitotic crossovers have the potential for deleterious genome rearrangements, their Cdk1-dependent regulation can provide an additional safety mechanism, ensuring that the rare mitotic recombination events accompanied by crossing over at least occur in S/G2, when a sister chromatid is available as appropriate donor.

According to the data collected to date we propose a model for the cell cycle-dependent regulation of the DSB repair pathway choice (**Figure 21**). Cdk1 activity promotes DSB repair through HR by stimulating DSB end resection, which is necessary for HR and inhibitory for NHEJ (Aylon *et al.*, 2004; Ira *et al.*, 2004; Zhang *et al.*, 2009). In particular, Cdk1 triggers DSB resection by both counteracting the inhibitory effect of the NHEJ proteins (see **Introduction**) and stimulating the activity of DSB resection machinery. In fact, it has been

demonstrated that Cdk1 phosphorylates and therefore activates Sae2 (Huertas *et al.*, 2008), which is involved in the removal of Yku from DSB ends and in the initiation of DSB resection, and Dna2 (Chen *et al.*, 2011), that drives extensive DSB resection. This first level of Cdk1-dependent regulation on DSB repair is the only one required to carry out SSA and noncrossover recombination. However, in order to generate crossover recombination products, other levels of Cdk1-dependent regulation are required. For instance, Mms4 resolvase, that gives rise to crossover product formation by cleaving dHJ, has been recently demonstrated to be target of Cdk1 (Matos *et al.*, 2011; Gallo-Fernández *et al.*, 2012). Whether Cdk1 regulates other steps during HR in order to generate crossover products is still unknown.





**Figure 21. DSB repair pathways during the cell cycle.**

The MRX complex and Yku bind to DSB ends. In G1 (left panel), Yku and MRX mediate recruitment of the NHEJ proteins (Lif1, Dnl4 and Nej1), which allow NHEJ-mediated religation of the DSB ends. Both Yku and the NHEJ proteins prevent initiation of resection by MRX. When the DSB ends are not bound by MRX, Yku still prevents Exo1- and Sgs1-mediated resection. In S/G2 (right panel), Cdk1 enhances Sae2/MRX function in resection by phosphorylating Sae2, thus channelling DNA repair into HR. Then, MRX and Sae2 catalyze the initial processing of the 5' strand, resulting in generation of short ssDNA stretches. Sae2 phosphorylation also promotes removal of Yku to allow further nucleolytic resection by the concerted action of Exo1 and Dna2 (together with Sgs1) at the DSB ends. Also Dna2 action requires phosphorylation by Cdk1. The 3'-ended ssDNA invades the homologous DNA sequence and the displaced strand anneals with the ssDNA on the other end of the break, forming a double Holliday Junction (dHJ). Resolution of the dHJ through nucleolytic cleavage by resolvases gives rise to crossover or noncrossover products. Among the resolvases, Mms4 is a known target of Cdk1 in promoting HR.

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## G1/S AND G2/M CYCLIN-DEPENDENT KINASE ACTIVITIES COMMIT CELLS TO DEATH IN THE ABSENCE OF THE S-PHASE CHECKPOINT

Why cells deficient for the S-phase checkpoint die in the presence of replication stress is a long-standing question. Here we show that reducing Cdk1 activity suppresses the loss of viability and the DNA replication defects of *mec1Δ sml1Δ* and *rad53Δ sml1Δ* mutant cells that are treated with low HU doses. Furthermore, reducing the Cdk1 activity bypasses the essential function of Mec1 and Rad53 kinases during an unchallenged S phase. Thus, the execution of some Cdk1-dependent events is detrimental when the S phase checkpoint is not functional independently of the presence of exogenous replication impediments, suggesting that the essential function of Mec1 and Rad53 is not necessarily separated from the function they exert under replicative stress conditions. Although our work has been carried out in budding yeast, lowering Cdk1 activity appears to counteract the deleterious effects caused by a deficient DNA damage checkpoint also in mammalian cells. In fact, downregulation of the Cdk activator CDC25A rescues the replicative stress occurring after inhibition of the checkpoint proteins Chk1 and ATR (Beck *et al.*, 2010; Toledo *et al.*, 2011; Sørensen and Syljuåsen, 2012).

The Cdk1-dependent lethality of *mec1* and *rad53* mutants involves a function of Cdk1 in G1. In fact, the lack of the G1 cyclin Cln2, which causes a G1/S delay by impairing proteolysis of the Clb-Cdk1 specific inhibitor Sic1 (Dirick *et al.*, 1995), suppresses the sensitivity of *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells to low HU doses. Accordingly, the deletion of *SIC1*, which abrogates the G1/S delay caused

by the *cln2Δ* mutation (Dirick *et al.*, 1995), counteracts the ability of *cln2Δ* to suppress the HU sensitivity of *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells. Thus, a delay in S-phase entry appears to support cell survival to replication stress in the absence of Mec1 or Rad53. This finding is complementary to earlier observations in mammalian systems, where ATR and Chk1 inhibitors were shown to be particularly toxic for p53-deficient cells, which lack the G1 checkpoint and therefore experience a less restrictive S-phase entry in the presence of DNA damage (Reaper *et al.*, 2011; Toledo *et al.*, 2011).

It has been shown that Mec1 and Rad53 stimulate production of dNTPs by inducing the expression of the *RNR* genes at the G1/S transition via inactivation of the transcriptional repressor Crt1 (Huang *et al.*, 1998). Indeed, the lack of Crt1 not only bypasses the essential function of Mec1 and Rad53 (Huang *et al.*, 1998) but also improves survival in response to HU treatment of *mec1Δ sml1Δ* and *rad53Δ sml1Δ* mutant cells (**Figure 15D**). Therefore, the role of Mec1 and Rad53 in inducing transcription of the *RNR* genes contributes to maintain cell viability also under replication stress. In this scenario, the S-phase delay in *cln2Δ* cells could provide *mec1* and *rad53* mutants with a longer time to reach high enough dNTP levels to support DNA replication. Consistent with previous data showing that increased dNTP pools promote replication of MMS-damaged DNA (Chabes *et al.*, 2003; Poli *et al.*, 2012), the lack of Cln2 or Crt1 improves survival of *mec1* mutants also to MMS treatment.

The Cdk1-dependent lethality of *mec1* and *rad53* mutants cannot be entirely ascribed to the G1 function of Cdk1. In fact, the lack of the mitotic cyclin Clb2 also improves the viability of HU treated *mec1Δ sml1Δ* and *rad53Δ sml1Δ* mutants. Although *mec1* and *rad53* mutants are unable to complete DNA

replication in the presence of HU, they prematurely undergo spindle elongation due to upregulation of the kinesin motor protein Cin8 (Krishnan *et al.*, 2004). Because Cdk1 activity is required to assemble a mitotic spindle by restraining Cin8 proteolysis (Crasta *et al.*, 2006), a reduced Cdk1 activity might suppress the sensitivity of *mec1* and *rad53* mutants to replication stress by delaying spindle elongation. Indeed, we found that both restraining spindle extension by *CIN8* deletion and disrupting kinetochore-microtubule attachments by nocodazole improve the survival in response to HU of *mec1* and *rad53* mutants. Interestingly, the lack of Cin8 allows *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells to complete DNA synthesis in the presence of HU, suggesting that the DNA replication defects in these mutants can be overcome by providing cells with additional time to complete DNA replication before chromosome segregation takes place. This hypothesis implies that the segregation of incompletely replicated DNA contributes to kill HU-treated *mec1* and *rad53* mutants. Because HU treated *mec1Δ sml1Δ* cells can duplicate their centromeres (Feng *et al.*, 2009) and therefore are proficient for bipolar attachment that generates tension within the spindle, this precocious spindle elongation can lead to lethal chromosome breaks due to disjunction of incompletely replicated chromosomes (Feng *et al.*, 2009). Although the force exerted by a bipolar spindle might be insufficient to generate chromosome breakage, the presence of ssDNA in HU-treated *mec1* and *rad53* mutants (Sogo *et al.*, 2002; Feng *et al.*, 2006) may cause spindle-induced breakage.

Interestingly, the lack of Cin8 suppresses the sensitivity to HU of *mec1* and *rad53* cells more efficiently than nocodazole addition. Indeed, nocodazole disrupts the kinetochore-microtubule attachment, thus causing the release of

the kinetochores from the spindle pole bodies to which they were first attached. It has been shown that premature spindle elongation caused by Cin8 deregulation in nocodazole-treated cells leads to mis-segregation of sister chromatids because it impairs the recapture and biorientation of microtubules after nocodazole removal (Liu *et al.*, 2008; Chai *et al.*, 2010). Thus, one possibility is that the unrestrained Cin8-dependent spindle elongation in HU-treated *mec1* and *rad53* mutants limits the ability of nocodazole to rescue *mec1* and *rad53* cell lethality in HU.

Noteworthy, although *mec1Δ sml1Δ* cells are more sensitive to HU and MMS than *rad53Δ sml1Δ* cells, both nocodazole addition and *CIN8* deletion improve survival to HU of *mec1* cells more efficiently than that of *rad53* cells. Furthermore, delaying spindle elongation suppresses the MMS sensitivity of *mec1Δ sml1Δ* cells, but it only slightly reduces that of *rad53Δ sml1Δ* cells. Although HU slows down DNA synthesis by depleting dNTPs, MMS-induced lesions block the progression of DNA replication forks because replicative polymerases cannot accommodate 3-methyl-adenine in their catalytic sites (Paulovich and Hartwell, 1995; Green and Lehmann, 2005). Interestingly, deletion of the *EXO1* nuclease gene has no effect on the sensitivity of *mec1* mutants to MMS, whereas it suppresses cell lethality and the fork progression defects of MMS-treated *rad53Δ sml1Δ* cells (Segurado and Diffley, 2008). It is therefore tempting to propose that, while the Mec1 requirement in supporting replication of MMS-damaged DNA can be bypassed by providing additional time to complete DNA replication, the lack of Rad53 results in defects at the replication forks that become substrates for irreversible Exo1-dependent replication fork breakdown or resection events. In line with this view, it has

been shown that Exo1 can process aberrant DNA intermediates in *rad53*-deficient cells (Cotta-Ramusino *et al.*, 2005) and that Rad53-dependent phosphorylation of Exo1 may act to limit ssDNA accumulation (Morin *et al.*, 2008).

In any case, delaying spindle elongation can rescue the HU induced lethality of *mec1* and *rad53* mutants only during exposure to low HU doses. This finding is consistent with previous data showing that the addition of nocodazole does not improve viability of *rad53* and *mec1* cells following transient exposure to a high HU dose (200 mM) (Desany *et al.*, 1998; Tercero and Diffley, 2001). Thus, high HU levels seem to irreversibly commit checkpoint mutants to death, possibly because they induce the stalling of the replication forks that are defective in restarting replication (De Piccoli *et al.*, 2012) and/or can undergo irreversible pathological transitions, such as the replisome dissociation from the nascent DNA chains (Cobb *et al.*, 2003; Katou *et al.*, 2003; Lucca *et al.*, 2004).

Is the essential function of the S-phase checkpoint related to its function in supporting DNA replication under mild replication stress? Mec1 and Rad53 are essential for cell viability, and their essential function can be bypassed by increasing dNTP levels through *SML1* deletion (Zhao *et al.*, 1998), suggesting that the lethality of *mec1*- and *rad53*-null mutants is due to DNA replication occurring in the absence of adequate dNTP accumulation. We found that, like the lack of Cln2 (Vallen and Cross, 1999) (**Figure 15B**), the lack of Clb2 or Cin8 not only suppresses the sensitivity of *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells to replication stress but also bypasses the essential function of Mec1 and Rad53. Therefore, cells lacking *MEC1* or *RAD53*, but carrying wild-type *SML1*, appear to die through a mechanism similar to that killing *mec1Δ* and *rad53Δ* cells exposed

to low HU doses in the absence of the Sml1-mediated inhibition of RNR activity. In both cases, cells experience defective DNA replication caused by a condition of nucleotide depletion, and cell death can be overcome by providing cells with additional time to accumulate dNTPs and accomplish DNA replication before chromosome segregation takes place. As proposed for HU-treated *mec1Δ sml1Δ* and *rad53Δ sml1Δ* cells (Feng *et al.*, 2009), partition of incompletely replicated DNA in *mec1Δ SML1* and *rad53Δ SML1* cells could give rise to lethal chromosome breaks. Consistent with this hypothesis, conditional inactivation of Mec1 by temperature-sensitive *mec1* alleles gives rise to the accumulation of chromosome breakage (Cha and Kleckner, 2002) and of Rad52 foci that can be reversed by nocodazole addition (**Figure 20G**).

In summary, we demonstrate that Cdk1-driven events impair cell viability when the S-phase checkpoint is not functional. Given that some cancer cells (such as those expressing oncogenes or lacking tumor suppressors) undergo high levels of replicative stress and that Cdk inhibitors are considered relevant candidates as anticancer drugs, this link between the S-phase checkpoint and Cdk1 activity may be important in developing new targeted strategies improving the efficiency of cancer treatments.

In conclusion, our results suggest that Cdk1 influences the DDR through multiple mechanisms. Indeed, Cdk1 is required for DSB-induced checkpoint activation, DSB repair by homologous recombination, and crossover formation. On the other hand, Cdk1 activity must be carefully regulated, because too much Cdk1 activity can affect genome integrity, at least when the checkpoint is not functional.





## ***MATERIALS AND METHODS***



## YEAST AND BACTERIAL STRAINS

YEAST STRAINS. Yeast strains used for this work are listed in Table 3.

Table 3. *Saccharomyces cerevisiae* strains used in this study.

Strain	Relevant genotype	Source
YMV86	<i>ho hmlΔ::ADE1 mataΔ::hisG hmrΔ::ADE1 ade3::GAL-HO ade1 lys5 ura3-52 trp1 leu2::leu2-NATMX-HOcs</i>	(Vaze <i>et al.</i> , 2002)
YLL2756	YMV86 <i>yku70Δ::URA3</i>	This study
YLL3047	YMV86 <i>CDC28-3HA::TRP1</i>	This study
YLL3048	YMV86 <i>yku70Δ::URA3 CDC28-3HA::TRP1</i>	This study
YMV45 <i>bar1Δ</i>	<i>ho hmlΔ::ADE1 mataΔ::hisG hmrΔ::ADE1 leu2::leu2(Asp718-Sall)-URA3-HOcs ade3::GAL-HO ade1 lys5 ura3-52 trp1 bar1Δ::HPHMX</i>	(Vaze <i>et al.</i> , 2002)
YLL2912	YMV45 <i>bar1Δ::HPHMX yku70Δ::NATMX</i>	This study
YLL2910	YMV45 <i>bar1Δ::HPHMX rad9Δ::KANMX4</i>	This study
YLL2903	YMV45 <i>bar1Δ::HPHMX yku70Δ::NATMX rad9Δ::KANMX4</i>	This study
YLL3036	YMV45 <i>bar1Δ::HPHMX CDC28-3HA::TRP1</i>	This study
YLL3049	YMV45 <i>bar1Δ::HPHMX yku70Δ::NATMX CDC28-3HA::TRP1</i>	This study
YLL3050	YMV45 <i>bar1Δ::HPHMX rad9Δ::KANMX4 CDC28-3HA::TRP1</i>	This study
YLL3037	YMV45 <i>bar1Δ::HPHMX yku70Δ::NATMX rad9Δ::KANMX4 CDC28-3HA::TRP1</i>	This study
YLL2956	YMV45 <i>bar1Δ::HPHMX yku70Δ::NATMX rad9Δ::KANMX4 rad52Δ::TRP1</i>	This study
YLL3043	YMV45 <i>bar1Δ::HPHMX yku70Δ::NATMX rad9Δ::KANMX4 rad51Δ::TRP1</i>	This study
YLL3044	YMV45 <i>bar1Δ::HPHMX trp1::GAL-SIC1ntΔ::TRP1</i>	This study
YLL3045	YMV45 <i>bar1Δ::HPHMX yku70Δ::NATMX rad9Δ::KANMX4 trp1::GAL-SIC1ntΔ::TRP1</i>	This study
JKM139 <i>bar1Δ</i>	<i>MATa ho hmlΔ::ADE1 hmrΔ::ADE1 ade1-100 leu2-3;112 lys5 trp1::hisG ura3-52 ade3::GAL-HO bar1Δ::HPHMX</i>	(Lee <i>et al.</i> , 1998)
YLL2892	JKM139 <i>bar1Δ::HPHMX yku70Δ::URA3</i>	This study
YLL2962	JKM139 <i>bar1Δ::HPHMX rad9Δ::KANMX4</i>	This study
YLL2978	JKM139 <i>bar1Δ::HPHMX yku70Δ::URA3 rad9Δ::KANMX4</i>	This study

tGI354 <i>bar1Δ</i>	<i>ho hmlΔ::ADE1 MATa-inc hmrΔ::ADE1 ade1 leu2-3;112 lys5 trp1::hisG ura3-52 ade3::GAL::HO arg5,6::MATa::HPHMX bar1Δ::TRP1</i>	(Saponaro <i>et al.</i> , 2010)
YLL2954	tGI354 <i>bar1Δ::TRP1 yku70Δ::URA3</i>	This study
YLL2980	tGI354 <i>bar1Δ::TRP1 rad9Δ::KANMX4</i>	This study
YLL2970	tGI354 <i>bar1Δ::TRP1 yku70Δ::URA3 rad9Δ::KANMX4</i>	This study
YLL3019	tGI354 <i>bar1Δ::TRP1 yku70Δ::NATMX rad9Δ::KANMX4 ura3::GAL-SIC1ntΔ-MYC-HIS::URA3</i>	This study
YLL3051	tGI354 <i>bar1Δ::TRP1 CDC28-3HA::URA3</i>	This study
YLL3052	tGI354 <i>bar1Δ::TRP1 yku70Δ::NATMX rad9Δ::KANMX4 CDC28-3HA::URA3</i>	This study
YLL3038	tGI354 <i>bar1Δ::TRP1 ura3::GAL-CLB2dbΔ::URA3</i>	This study
YLL3039	tGI354 <i>bar1Δ::TRP1 yku70Δ::NATMX rad9Δ::KANMX4 ura3::GAL-CLB2dbΔ::URA3</i>	This study
W303	<i>MATa or MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad5-535</i>	
YLL490	W303 <i>MATa mec1Δ::HIS3 sml1Δ::KANMX4</i>	(Longhese <i>et al.</i> , 2000)
YLL509	W303 <i>MATa rad53Δ::HIS3 sml1Δ::KANMX4</i>	Longhese <i>et al.</i> , 2000)
DMP2760/3B	W303 <i>MATa rad53-K227A::KANMX4</i>	Longhese <i>et al.</i> , 2000)
DMP5237/4B	W303 <i>MATa cdc28-sup</i>	This study
DMP5237/6D	W303 <i>MATa cdc28-sup mec1Δ::HIS3 sml1Δ::KANMX4</i>	This study
DMP2995/1B	W303 <i>MATa ddc2Δ::KANMX4 sml1Δ::KANMX4</i>	(Paciotti <i>et al.</i> , 2000)
DMP5283/6C	W303 <i>MATa cdc28-sup ddc2Δ::HIS3 sml1Δ::KANMX4</i>	This study
DMP5255/5C	W303 <i>MATa cdc28-sup rad53Δ::HIS3 sml1Δ::KANMX4</i>	This study
DMP5256/9C	W303 <i>MATa cdc28-sup rad53-K227A::KANMX4</i>	This study
SP1791	W303 <i>MATa TUB1-GFP</i>	S. Piatti
DMP5393/50B	W303 <i>MATa cdc28-as1::TRP1 TUB1-GFP</i>	This study
DMP5377/11D	W303 <i>MATa rad53-K227A::KANMX4 TUB1-GFP</i>	This study
DMP5376/7B	W303 <i>MATa mec1Δ::HIS3 sml1Δ::KANMX4 TUB1-GFP</i>	This study
DMP5379/1B	W303 <i>MATa rad53Δ::HIS3 sml1Δ::KANMX4 TUB1-GFP</i>	This study
DMP5391/20B	W303 <i>MATa cdc28-as1::TRP1 mec1Δ::HIS3 sml1Δ::KANMX4 TUB1-GFP</i>	This study

*Materials and Methods*

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DMP5410/6B	W303 MATa <i>cdc28-as1::TRP1 rad53Δ::HIS3 sml1Δ::KANMX4 TUB1-GFP</i>	This study
DMP5393/49C	W303 MATa <i>cdc28-as1::TRP1 rad53-K227A::KANMX4 TUB1-GFP</i>	This study
DMP5585/18C	W303 MATα <i>mec1Δ::HIS3 rad53Δ::HIS3 sml1Δ::KANMX4</i>	This study
DMP5583/14A	W303 MATa <i>mec1Δ::HIS3 rad53Δ::HIS3 sml1Δ::KANMX4 cdc28-as1::TRP1</i>	This study
2664	W303 MATa <i>trp1::TRP1 GAL-dNK leu2::LEU2 GAL-hENT</i>	J. Diffley
DMP5347/9A	W303 MATa <i>trp1::TRP1 GAL-dNK leu2::LEU2 GAL-hENT mec1Δ::HIS3 sml1Δ::KANMX4</i>	This study
DMP5494/22D	W303 MATa <i>trp1::TRP1 GAL-dNK leu2::LEU2 GAL-hENT mec1Δ::HIS3 sml1Δ::KANMX4 cdc28-as1::TRP1</i>	This study
DMP5537/5B	W303 MATa <i>trp1::TRP1 GAL-dNK leu2::LEU2 GAL-hENT rad53Δ::HIS3 sml1Δ::KANMX4</i>	This study
DMP5538/4D	W303 MATa <i>trp1::TRP1 GAL-dNK leu2::LEU2 GAL-hENT rad53Δ::HIS3 sml1Δ::KANMX4 cdc28-as1::TRP1</i>	This study
DMP5414/10D	W303 MATa <i>cln2Δ::HPHMX</i>	This study
DMP5415/6B	W303 MATa <i>cln2Δ::HPHMX mec1Δ::HIS3 sml1Δ::KANMX4</i>	This study
DMP5412/3A	W303 MATa <i>cln2Δ::HPHMX rad53Δ::HIS3 sml1Δ::KANMX4</i>	This study
DMP5414/9D	W303 MATa <i>cln2Δ::HPHMX rad53-K227A::KANMX4</i>	This study
DMP5468/6B	W303 MATa <i>sic1Δ::HIS3 mec1Δ::HIS3 sml1Δ::KANMX4</i>	This study
DMP5475/4A	W303 MATa <i>sic1Δ::HIS3 cln2Δ::HPHMX mec1Δ::HIS3 sml1Δ::KANMX4</i>	This study
YLL3070.7	W303 MATa <i>crt1Δ::NATMX sml1Δ::KANMX4</i>	This study
DMP5535/2C	W303 MATa <i>crt1Δ::NATMX mec1Δ::HIS3 sml1Δ::KANMX4</i>	This study
DMP5536/4A	W303 MATa <i>crt1Δ::NATMX rad53Δ::HIS3 sml1Δ::KANMX4</i>	This study
YLL3215.1	W303 MATα <i>nrm1Δ::NATMX sml1Δ::KANMX4</i>	This study
DMP5501/3D	W303 MATa <i>nrm1Δ::NATMX mec1Δ::HIS3 sml1Δ::KANMX4</i>	This study
DMP5502/7D	W303 MATa <i>nrm1Δ::NATMX rad53Δ::HIS3 sml1Δ::KANMX4</i>	This study
DMP5348/8B	W303 MATa <i>clb5Δ::HIS3</i>	This study

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DMP5365/6A	W303 MATa <i>clb5Δ::HIS3 mec1Δ::HIS3 sml1Δ::KANMX4</i>	This study
DMP5349/22B	W303 MATa <i>clb6Δ::LEU2</i>	This study
DMP5366/1B	W303 MATa <i>clb6Δ::LEU2 mec1Δ::HIS3 sml1Δ::KANMX4</i>	This study
DMP5369/13B	W303 MATa <i>clb6Δ::LEU2 rad53Δ::HIS3 sml1Δ::KANMX4</i>	This study
DMP5367/9A	W303 MATa <i>clb6Δ::LEU2 rad53-K227A::KANMX4</i>	This study
SP65	W303 MATa <i>cdc28-1N</i>	S. Piatti
DMP5238/3A	W303 MATa <i>cdc28-1N mec1Δ::HIS3 sml1Δ::KANMX4</i>	This study
DMP5260/3C	W303 MATa <i>cdc28-1N rad53Δ::HIS3 sml1Δ::KANMX4</i>	This study
DMP5351/18D	W303 MATa <i>clb2Δ::LEU2</i>	This study
DMP5351/3D	W303 MATa <i>clb2Δ::LEU2 mec1Δ::HIS3 sml1Δ::KANMX4</i>	This study
DMP5352/17B	W303 MATa <i>clb2Δ::LEU2 rad53Δ::HIS3 sml1Δ::KANMX4</i>	This study
DMP5384/23B	W303 MATa <i>cin8Δ::TRP1 TUB1-GFP</i>	This study
DMP5383/20C	W303 MATa <i>cin8Δ::TRP1 mec1Δ::HIS3 sml1Δ::KANMX4 TUB1-GFP</i>	This study
DMP5384/34C	W303 MATa <i>cin8Δ::TRP1 rad53Δ::HIS3 sml1Δ::KANMX4 TUB1-GFP</i>	This study
DMP5385/17C	W303 MATa <i>cin8Δ::TRP1 rad53-K227A::KANMX4 TUB1-GFP</i>	This study
DMP5442/18A	W303 MATa <i>cin8Δ::TRP1 cln2Δ::HPHMX mec1Δ::HIS3 sml1Δ::KANMX4</i>	This study
DMP5495/16C	W303 MATa <i>trp1::TRP1 GAL-dNK leu2::LEU2 GAL-hENT cin8Δ::TRP1 mec1Δ::HIS3 sml1Δ::KANMX4</i>	This study
DMP2696/3D	W303 MATa <i>mec1-14</i>	(Longhese <i>et al.</i> , 2000)
DMP5533/3D	W303 MATa <i>mec1-14 clb2Δ::LEU2</i>	This study
DMP5520/17A	W303 MATa <i>mec1-14 cdc28-as1::TRP1</i>	This study
DMP5528/9C	W303 MATa <i>RAD52-YFP ADE2</i>	This study
DMP5528/25A	W303 MATa <i>mec1-14 RAD52-YFP ADE2</i>	This study
DMP5593/18A	W303 MATa <i>mec1-14 RAD52-YFP ADE2 mms4Δ::NATMX</i>	This study
YLL3260.37	W303 MATa <i>mec1-14 RAD52-YFP ADE2 yen1Δ::KANMX4</i>	This study
DMP5595/7A	W303 MATa <i>mec1-14 RAD52-YFP ADE2 mms4Δ::NATMX yen1Δ::KANMX4</i>	This study

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(Trovesi *et al.*, 2011) Strains JKM139, YMV86 and YMV45 were kindly provided by J. Haber (Brandeis University, Waltham, USA). Strains YMV86 and YMV45 are isogenic to YFP17 (*matΔ::hisG hmlΔ::ADE1 hmrΔ::ADE1 ade1 lys5 ura3-52 trp1 ho ade3::GAL-HO leu2::cs*) except for the presence of a *LEU2* fragment inserted, respectively, 0.7 kb or 4.6 kb centromere-distal to *leu2::cs* (Vaze *et al.*, 2002). Strain tGI354 was kindly provided by G. Liberi (IFOM, Milano, Italy) and J. Haber (Saponaro *et al.*, 2010). To induce a persistent G1 arrest with  $\alpha$ -factor, all strains used in this study carried the deletion of the *BAR1* gene, which encodes a protease that degrades the  $\alpha$ -factor. Deletions of the *YKU70*, *RAD9*, *RAD51*, *RAD52* and *BAR1* genes were generated by one-step PCR disruption method. YMV86, YMV45 and tGI354 derivatives strains carrying a fully functional *CDC28-HA* allele at the *CDC28* chromosomal locus were generated by one-step PCR tagging method. A plasmid carrying the *GAL-CLB2dbΔ* allele was kindly provided by R. Visintin (IEO, Milan, Italy) and was used to integrate the *GAL-CLB2dbΔ* fusion at the *URA3* locus in the tGI354 derivative strains. Strain YLL3019, carrying the *GALSIC1ntΔ* allele integrated at the *URA3* locus, was obtained by transforming strain tGI354 *rad9Δ yku70Δ* with *Apal*-digested plasmid pLD1, kindly provided by J. Diffley (Clare Hall Laboratories, South Mimms, United Kingdom). The *GAL-SIC1ntΔ* fusion was cloned into a *TRP1*-based integrative plasmid that was used to integrate the fusion at the *TRP1* locus in the YMV45 derivative strains. Integration accuracy was verified by Southern blot analysis. Cells were grown in YEP medium (1% yeast extract, 2% bacto-peptone) supplemented with 2% raffinose (YEP+raf) or 2% raffinose and 3% galactose (YEP+raf+gal).

(Manfrini *et al.*, 2012) All yeast strains (see Table 3) were derivatives of W303 (*ade2-1, trp1-1, leu2-3,112, his3-11,15, ura3, and rad5-535*). Gene deletions were generated by one-step gene replacement. The *cdc28-as1* mutant, kindly provided by R. Kolodner (San Diego, CA), was backcrossed three times with W303. The *cdc28-1N* mutant and the strain expressing green fluorescent protein (GFP)-tagged Tub1 were kindly provided by Simonetta Piatti (Montpellier, France). The strain expressing both the nucleoside transporter hENT and the herpes simplex virus thymidine kinase used for bromodeoxyuridine (BrdU) incorporation was kindly provided by J. Diffley (South Mimms, United Kingdom). All of the strains expressing the *RAD52-YFP* fusion were derivatives of strain W3749/4C, kindly provided by R. Rothstein (New York, NY). Cells were grown in either synthetic minimal medium supplemented with the appropriate nutrients or YEP (1% yeast extract, 2% Bacto peptone, 50 mg of adenine/liter) medium supplemented with 2% glucose (YEPD), 2% raffinose (YEPR), or 2% raffinose and 2% galactose (YEPRG). Benomyl and nocodazole were used at 10 and 5  $\mu\text{g/ml}$ , respectively, in 1% dimethyl sulfoxide (DMSO). Unless otherwise indicated, the experiments were performed at 25°C.

***E. coli* STRAIN.** *E. coli* DH5 $\alpha^{\text{TM}}$  strain (*F*<sup>-</sup>,  $\phi$ 80 *dlacZM15, D(lacZTA-argF) U169, deoR, recA1, endA1, hsdR17, (rK-,mK+) phoA supE44,  $\lambda$ -, thi-1, gyrA96, relA1*) is used as bacterial host for plasmid manipulation and amplification. *E. coli* DH5 $\alpha^{\text{TM}}$  competent cells are purchased from Invitrogen.



## GROWTH MEDIA

### *S. cerevisiae* MEDIA

**YEP (Yeast-Extract Peptone)** is the standard rich media for *S. cerevisiae* and contains 10 g/L yeast extract, 20 g/L peptone and 50 mg/L adenine. YEP must be supplemented with 2% glucose (YEPD), 2% raffinose (YEP+raf) or 2% raffinose and 2% galactose (YEP+raf+gal) as carbon source. YEP-based selective media are obtained including 400 µg/mL G418, 300 µg/mL hygromycin-B or 100 µg/mL nourseotricin. Solid media are obtained including 2% agar. Stock solutions are 50% glucose, 30% raffinose, 30% galactose, 80 mg/mL G418, 50 mg/mL hygromycin-B and 50 mg/mL nourseotricin. YEP and glucose stock solution are autoclave-sterilized and stored at RT. Sugars and antibiotics stock solutions are sterilized by micro-filtration and stored at RT and 4°C respectively.

**S.C. (Synthetic Complete)** is the minimal growth media for *S. cerevisiae* and contains 1.7 g/L YNB (without aminoacids), 5 g/L ammonium sulphate, 200µM inositol, 25 mg/L uracil, 25 mg/L adenine, 25 mg/L histidine, 25 mg/L leucine, 25 mg/L tryptophan. S.C. can be supplemented with drop-out solution (20 mg/L arginine, 60 mg/L isoleucine, 40 mg/L lysine, 10 mg/L methionine, 60 mg/L phenylalanine, 50 mg/L tyrosine) based on yeast strains requirements. Different carbon sources can be used as in rich media (2% glucose, 2% raffinose or 2% raffinose and 1% galactose). Different carbon sources can be used as in rich media. One or more aminoacid/base can be omitted to have S.C.-based selective media (e.g. S.C.-ura is S.C. lacking uracil). To obtain G418 or NAT S.C. selective medium the 5 g/L ammonium sulphate are replaced with 1 g/L monosodic glutamic acid. Solid media are obtained by including 2% agar. Stock

solutions are 17 g/L YNB + 50 g/L ammonium sulphate (or 10g/L monosodic glutamic acid), 5 g/L uracil, 5 g/L adenine, 5 g/L histidine, 5 g/L leucine, 5 g/L tryptophan, 100X drop out solution (2 g/L arginine, 6 g/L isoleucine, 4 g/L lysine, 1 g/L methionine, 6 g/L phenylalanine, 5 g/L tyrosine), 20mM inositol. All of these solutions are sterilized by micro-filtration and stored at 4°C.

**VB sporulation medium** contains 13.6 g/L sodium acetate, 1.9 g/L KCl, 0.35 g/L MgSO<sub>4</sub>, 1.2 g/L NaCl. pH is adjusted to 7.0. To obtain solid medium include 2% agar. pH is adjusted to 7.0. Sterilization by autoclavation.

#### ***E. coli* MEDIA**

**LD** is the standard growth medium for *E. coli*. LD medium contains 10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl. Solid medium is obtained by including 1% agar. LD+Amp selective medium is obtained including 50 µg/mL ampicillin. LD is autoclave-sterilized and stored at RT. Ampicillin stock solution (2.5 g/L) is sterilized by micro-filtration and stored at 4°C.

#### **CONSERVATION AND STORAGE OF *S. cerevisiae* AND *E. coli* STRAINS**

Yeast cells are grown 2-3 days at 30°C on YEPD plates, resuspended in 15% glycerol and stored at -80°C. Bacteria are grown o/n at 37°C on LD+Amp plates, resuspended in 50% glycerol and stored at -80°C. Yeast and bacteria cells can be stored for years in these conditions.

## **MOLECULAR BIOLOGY TECHNIQUES**

### **AGAROSE GEL ELECTROPHORESIS**

Agarose gel electrophoresis is the most easy and common way of separating and analyzing DNA molecules. This technique allows the separation of DNA fragments based on their different molecular weight (or length in kb). The purpose of this technique might be to visualize the DNA, to quantify it or to isolate a particular DNA fragment. The DNA is visualized by the addition in the gel of ethidium bromide, which is a fluorescent dye that intercalates between bases of nucleic acids. Ethidium bromide absorbs UV light and transmits the energy as visible orange light, revealing the DNA molecules to which is bound.

To pour a gel, agarose powder is mixed with TAE (0.04M Tris-Acetate 0.001M EDTA) to the desired concentration, and the solution is microwaved until completely melted. Most gels are made between 0.8% and 2% agarose. A 0.8% gel will show good resolution of large DNA fragments (5-10 Kb) and a 2% gel will show good resolution for small fragments (0.2-1 Kb). Ethidium bromide is added to the gel at a final concentration of 1 µg/mL to facilitate visualization of DNA after electrophoresis. After cooling the solution to about 60°C, it is poured into a casting tray containing a sample comb and allowed to solidify at RT or at 4°C. The comb is then removed and the gel is placed into an electrophoresis chamber and just covered with the buffer (TAE). Sample containing DNA mixed with loading buffer are then pipetted into the sample wells. The loading buffer contains 0.05% bromophenol blue and 5% glycerol, which give color and density to the sample. A marker containing DNA fragments of known length and concentration is loaded in parallel to determine size and quantity of DNA

fragments in the samples. Then current is applied and DNA will migrate toward the positive electrode. When adequate migration has occurred, DNA fragments are visualized by placing the gel on a UV transilluminator.

#### **DNA EXTRACTION FROM AGAROSE GELS (PAPER STRIP METHOD)**

This method allow to isolate a DNA fragment of interest. Using a scalpel blade cut a slit immediately in front of the band to be extracted. Cut a piece of GF-C filter to size to fit inside the slit. Place the paper strip in the slit and switch on the current for 1-2 minutes at 150 V. The DNA runs onward into the paper and is delayed in the smaller mesh size of the paper. Remove the strip of paper and place it into a 0.5 mL microcentrifuge tube. Make a tiny hole in the bottom of the tube using a syringe needle, place the 0.5 mL tube inside a 1.5 mL tube and spin for 30 seconds. Buffer and DNA are retained in the 1.5 mL tube. Extract the DNA with 1 volume of phenol/chloroform and precipitate the DNA with 100mM sodium acetate and 3 volumes of 100% ethanol. After microcentrifugation re-dissolve DNA in an appropriate volume of water, TRIS (10mM Tris HCl pH 8.5) or TE (10mM Tris HCl 1mM EDTA pH7.4) buffer.

#### **RESTRICTION ENDONUCLEASES**

Type II endonucleases (also known as restriction endonucleases or restriction enzymes) cut DNA molecules at defined positions close to their recognitions sequences in a reaction known as enzymatic digestion. They produce discrete DNA fragments that can separated by agarose gel electrophoresis, generating distinct gel banding patterns. For these reasons they are used for DNA analysis and gene cloning. Restriction enzymes are generally stored at -20°C in a

solution containing 50% glycerol, in which they are stable but not active. Glycerol concentration in the reaction mixture must be below 5% in order to allow enzymatic reaction to occur. They generally work at 37°C with some exceptions (e.g. Apal activity is maximal at 25°C) and they must be supplemented with a reaction buffer provided by the manufacturer, and in some cases with Bovin Serum Albumin. We use restriction endonucleases purchased from NEB and PROMEGA.

#### **LIGATION**

DNA is previously purified from agarose gel with the paper strip method, phenol/chloroform extracted, ethanol precipitated and resuspended in the appropriate volume of water or TE buffer. The ligation reaction is performed in the following conditions: DNA fragment and vector are incubated overnight at 16°C with 1 µl T4 DNA Ligase (PROMEGA) and T4 DNA Ligase Buffer (PROMEGA). The ligation reaction is then used to transform competent *E. coli* cells. Plasmids are recovered from Amp<sup>+</sup> transformants and subjected to restriction analysis.

#### **PREPARATION OF YEAST GENOMIC DNA FOR POLYMERASE CHAIN REACTION**

Resuspend yeast cells in 200 µL Yeast Lysis Buffer (2% TRITON X100, 1% SDS, 100mM NaCl, 10mM Tris HCl pH 8, 1mM EDTA pH 8), add 200 µL glass beads, 200 µL phenol/chloroform and vortex 3 minutes. Ethanol precipitate the aqueous phase obtained after 5 minutes centrifugation. Resuspend DNA in the appropriate volume of water and use 1 µL as a template for PCR.

### **POLYMERASE CHAIN REACTION (PCR)**

PCR allows to obtain high copy number of a specific DNA fragment of interest starting from very low quantity of DNA fragment. The reaction is directed to a specific DNA fragment by using a couple of oligonucleotides flanking the DNA sequence of interest. These oligonucleotides work as primers for the DNA polymerase. The reaction consist of a number of polymerization cycles which are based on 3 main temperature-dependent steps: denaturation of DNA (which occur over 90°C), primer annealing to DNA (typically take place at 45-55°C depending on primer characteristic), synthesis of the DNA sequence of interest by a thermophilic DNA polymerase (which usually works at 68 or 72°C). Different polymerases with different properties (processivity, fidelity, working temperature, etc) are commercially available and suitable for different purpose. Taq polymerase works at 72°C and is generally used for analytical PCR. Polymerases with higher fidelity like Pfx and VENT polymerases, which work respectively at 68 and 72°C, are generally employed when 100% polymerization accuracy is required.

The typical 50 µL PCR mixture contains 1µL of template DNA, 0.5 µM each primer, 200µM dNTPs, 5 µL of 10X Reaction Buffer, 1mM MgCl<sub>2</sub>, 1-2 U DNA polymerase and water to 50 µL. The typical cycle-program for a reaction is: 1. 3' denaturation at 94-95°C; 2. 30" denaturation at 94-95°C; 3. 30" annealing at primers T<sub>m</sub> (melting temperature); 4. 1' polymerization per Kb at 68 or 72°C (depending on polymerase); 5. repeat 30 times from step 2; 6. 5-10' polymerization at 68-72°C. The choice of primer sequences determines the working T<sub>m</sub>, which depends on the length (L) and GC% content of the

oligonucleotides and can be calculated as follows:  $T_m = 59.9 + 0.41(\text{GC}\%) - 675/L$ .

#### **PLASMID DNA EXTRACTION FROM *E. coli* (I): MINIPREPS BOILING**

*E. coli* cells (2mL overnight culture) are harvested by centrifugation and resuspended in 500  $\mu\text{L}$  STET buffer (8% sucrose, 5% TRITON X-100, 50mM EDTA, 50mM Tris-HCl, pH 8). Bacterial cell wall is digested boiling the sample for 2 minutes with 1 mg/mL lysozyme. Cellular impurities are removed by centrifugation and DNA is precipitated with isopropanol and resuspended in the appropriate volume of water or TE.

#### **PLASMID DNA EXTRACTION FROM *E. coli* (II): MINIPREPS WITH QIAGEN COLUMNS**

This protocol allows the purification of up to 20  $\mu\text{g}$  high copy plasmid DNA from 1-5 mL overnight *E. coli* culture in LD medium. Cells are pelleted by centrifugation and resuspended in 250  $\mu\text{L}$  buffer P1 (100  $\mu\text{g}/\text{mL}$  RNase, 50mM Tris HCl pH 8, 10mM EDTA pH 8). After addition of 250  $\mu\text{L}$  buffer P2 (200mM NaOH, 1% SDS) the solution is mixed thoroughly by inverting the tube 4-6 times, and the lysis reaction occur in 5 minutes at RT. 350  $\mu\text{L}$  N3 buffer (QIAGEN) are added to the solution, which is then centrifuged for 10 minutes. The supernatant is applied to a QIAprep spin column which is washed once with PB buffer (QIAGEN) and once with PE buffer (QIAGEN). The DNA is eluted with EB buffer (10mM Tris HCl pH 8.5) or water.

#### **TRANSFORMATION OF *E.coli* DH5 $\alpha$**

DH5 $\alpha$  competent cells are thawed on ice. Then, 50-100  $\mu$ L cells are incubated 30 minutes in ice with 1  $\mu$ L plasmid DNA. Cells are then subjected to heat shock at 37°C for 30 seconds and then incubated on ice for 2 minutes. Finally, 900  $\mu$ L LD are added to the tube and cells are incubated 30 minutes at 37°C to allow expression of ampicillin resistance. Cells are then plated on LD+amp and overnight incubated at 37°C.

#### **TRANSFORMATION ON *S. cerevisiae***

YEPD exponentially growing yeast cells are harvested by centrifugation and washed with 1 mL 1M lithium acetate (LiAc) pH 7.5. Cells are then resuspended in 1M LiAc pH 7.5 to obtain a cells/LiAc 1:1 solution. 12  $\mu$ L cells/LiAc are incubated 30-45 minutes at RT with 45  $\mu$ L 50% PEG (PolyEthyleneGlycol) 3350, 4  $\mu$ L carrier DNA (salmon sperm DNA) and 1-4  $\mu$ L DNA of interest (double each quantity when transform with PCR products). After addition of 6  $\mu$ L 60% glycerol cells are incubated at RT for 30-45 minutes, heat-shocked at 42°C for 5-10 minutes and plated on appropriate selective medium.

#### **EXTRACTION OF YEAST GENOMIC DNA (TEENY YEAST DNA PREPS)**

Yeast cells are harvested from overnight cultures by centrifugation, washed with 1 mL of 0.9M sorbytol 0.1M EDTA pH 7.5 and resuspended in 0.4 mL of the same solution supplemented with 14mM  $\beta$ -mercaptoethanol. Yeast cell wall is digested by 45 minutes incubation at 37°C with 0.4 mg/mL 20T zimoliase. Spheroplasts are harvested by 30 seconds centrifugation and resuspended in 400  $\mu$ L TE. After addition of 90  $\mu$ L of a solution containing EDTA pH 8.5, Tris base and SDS, spheroplasts are incubated 30 minutes at 65°C. Samples are kept



on ice for 1 hour following addition of 80  $\mu$ L 5M potassium acetate. Cell residues are eliminated by 15 minutes centrifugation at 4°C. DNA is precipitated with chilled 100% ethanol, resuspended in 500  $\mu$ L TE and incubated 30 minutes with 25  $\mu$ L 1 mg/mL RNase to eliminate RNA. DNA is then precipitated with isopropanol and resuspended in the appropriate volume (typically 50  $\mu$ L) of TE.

#### **SOUTHERN BLOT ANALYSIS**

Yeast genomic DNA prepared with standard methods is digested with the appropriate restriction enzyme(s). The resulting DNA fragments are separated by agarose gel electrophoresis in a 0.8% agarose gel. When adequate migration has occurred, gel is washed 40 minutes with a denaturation buffer (0.2N NaOH, 0.6M NaCl), and 40 minutes with a neutralization buffer (1.5M NaCl, 1M Tris HCl, pH 7.4). DNA is blotted onto a positively charged nylon membrane by overnight capillary transfer with 10X SSC buffer (20X SSC: 3M sodium chloride, 0.3M sodium citrate, pH 7.5). Membrane is then washed with 4X SSC and UV-crosslinked. Hybridization is carried out by incubating membrane for 5 hours at 50°C with pre-hybridization buffer (50% formamide, 5X SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 2% Blocking reagent) following by o/n incubation at 50°C with pre-hybridization buffer + probe. The probe is obtained by random priming method (DECAprime™ kit by Ambion) on a suitable DNA template and with <sup>32</sup>P d-ATP. Filter is then washed (45'+15') at 55°C with a washing solution (0.2M sodium phosphate buffer pH 7.2, SDS 1%, water), air dried and then exposed to an autoradiography film.

#### **DENATURING GEL ELECTROPHORESIS AND SOUTHERN BLOT ANALYSIS TO VISUALIZE ssDNA**

A 0.8% agarose gel (in H<sub>2</sub>O) is submerged in a gel box containing a 50mM NaOH, 1mM EDTA solution for 30 minutes to equilibrate. Ethidium bromide is omitted because it does not efficiently bind to DNA under these conditions. After digestion with the appropriate restriction enzyme(s), DNA samples are prepared by adjusting the solution to 0.3M sodium acetate and 5mM EDTA (pH 8.0) following by addition of 2 volumes of ethanol to precipitate DNA. After chilling (o/n) and centrifuging the samples (15 minutes, possibly at 4°C), pellet is resuspended in alkaline gel loading buffer (1X buffer: 50mM NaOH, 1mM EDTA pH 8.5, 2.5% Ficoll (Type 400) and 0.025% bromophenol blue). After loading the DNA in the gel, a glass plate can be placed on the gel to prevent the dye from diffusing from the agarose during the course of the run. Because of the large currents that can be generated with denaturing gels, gels are usually run slowly at lower voltages (e.g. 30 V o/n). After the DNA has migrated far enough, the gel can be stained with 0.5 µg/ml ethidium bromide in 1X TAE electrophoresis buffer (1 hour). The DNA will be faint because the DNA is single stranded. Gel is then soaked in 0.25N HCl for 7 minutes with gentle agitation, rinsed with water and soaked in 0.5M NaOH, 1.5M NaCl for 30 minutes with gentle agitation. Gel is then rinsed briefly with water and DNA is blotted by capillary transfer onto neutral nylon membrane using 10X SSC. Hybridization is carried out by incubating membrane for 5 hours at 42°C with pre-hybridization buffer (50% formamide, denhardtts solution + 4X BSA, 6% dextran sulphate, 100 µg/mL salmon sperm DNA, 200 µg/mL tRNA carrier) following by o/n incubation at 42°C with pre-hybridization buffer + ssRNA probe. The ssRNA

probe is obtained by *in vitro* transcription using Promega Riboprobe System-T7 and a pGEM-7Zf-based plasmid as a template. Following hybridization, membrane is washed twice with 5X SSPE (20X SSPE = 3M NaCl, 200 $\mu$ M NaH<sub>2</sub>PO<sub>4</sub>, 20 $\mu$ M EDTA, pH 7.4) at 42°C for 15 minutes, 30 minutes with 1X SSPE 0.1% SDS at 42°C, 30 minutes with 0.1X SSPE 0.1% SDS at 42°C, 15 minutes with 0.2X SSPE 0.1% SDS at 68°C and 5 minutes with 0.2X SSPE at RT. Finally membrane is exposed to a X-ray film.

## SYNCHRONIZATION OF YEAST CELLS

### SYNCHRONIZATION OF YEAST CELLS WITH $\alpha$ -FACTOR

$\alpha$ -factor allows to synchronize a population of yeast cells in G1 phase. This pheromone activates a signal transduction cascade which arrests yeast cells in G1 phase. Only *MATa* cells are responsive to  $\alpha$ -factor. To synchronize in G1 a population of exponentially growing yeast cells in YEPD, 2  $\mu$ g/mL  $\alpha$ -factor is added to  $6 \times 10^6$  cells/mL culture. As the percentage of budded cells will fall below 5% cells are considered to be G1-arrested. Cells are then washed and resuspended in fresh medium with or without 3  $\mu$ g/mL  $\alpha$ -factor to keep cells G1-arrested or release them into the cell cycle respectively. At this time cell cultures can be either treated with genotoxic agents or left untreated. If cells carry the deletion of *BAR1* gene, that encodes a protease that degrades the  $\alpha$ -factor, 0.5  $\mu$ g/mL  $\alpha$ -factor is sufficient to induce a G1-arrest that lasts several hours.

### **SYNCHRONIZATION OF YEAST CELLS WITH NOCODAZOLE**

Nocodazole allows to synchronize a population of yeast cells in G2 phase. This drug causes the depolymerization of microtubules, thus activating the mitotic checkpoint which arrests cells at the metaphase to anaphase transition (G2 phase). To synchronize in G2 a population of exponentially growing yeast cells in YEPD, 0.5 µg/mL nocodazole is added to  $6 \times 10^6$  cells/mL culture together with DMSO at a final concentration of 1% (use a stock solution of 100X nocodazole in 100% DMSO). As the percentage of dumbbell cells will reach 95% cells are considered to be G2-arrested. Cells are then washed and resuspended in fresh medium with or without 1.5 µg/mL nocodazole to keep cells G2-arrested or release them into the cell cycle respectively. At this time cell cultures can be either treated with genotoxic agents or left untreated.

## **OTHER TECHNIQUES**

### **FACS ANALYSIS OF DNA CONTENTS**

FACS (Fluorescence-Activated Cell Sorting) analysis allow to determine the DNA content of every single cell of a given population of yeast cells.  $6 \times 10^6$  cells are harvested by centrifugation, resuspended in 70% ethanol and incubated at RT for 1 hour. Cells are then washed with 1 mL 50mM Tris pH 7.5 and incubated overnight at 37°C in the same solution with 1 mg/mL RNase. Samples are centrifuged and cells are incubated at 37°C for 30 minutes with 5 mg/mL pepsin in 55mM HCl, washed with 1 mL FACS Buffer and stained in 0.5 mL FACS buffer with 50 µg/mL propidium iodide. 100 µL of each sample are diluted in 1 mL 50mM Tris pH 7.5 and analyzed with a Becton-Dickinson FACS-Scan. The same

samples can also be analyzed by fluorescence microscopy to score nuclear division.

#### **TOTAL PROTEIN EXTRACTS**

Total protein extracts were prepared from  $10^8$  cells collected from exponentially growing yeast cultures. Cells are harvested by centrifugation and washed with 20% trichloroacetic acid (TCA) in order to prevent proteolysis and resuspended in 50  $\mu$ L 20% TCA. After addition of 200  $\mu$ L of glass beads, cells are disrupted by vortexing for 8 minutes. Glass beads are washed with 400  $\mu$ L 5% TCA, and the resulting extract are centrifuged at 3000 rpm for 10 minutes. The pellet is resuspended in 70  $\mu$ L Laemmli buffer (0.62M Tris, 2% SDS, 10% glycine, 0.001% Bfb, 100mM DTT), neutralized with 30  $\mu$ L 1M Tris base, boiled for 3 minutes, and finally clarified by centrifugation.

#### **SDS-PAGE AND WESTERN BLOT ANALYSIS**

Protein extracts are loaded in 10% polyacrylamide gels (composition). Proteins are separated based on their molecular weight by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE). When adequate migration has occurred proteins are blotted onto nitrocellulose membrane. Membrane is saturated by 1 hour incubation with 4% milk in TBS containing 0.2% TRITON X-100 and incubated for 2 hours with primary antibodies. Membrane is washed three times with TBS for 10 minutes, incubated for 1 hour with secondary antibodies and again washed with TBS. Detection is performed with ECL (Enhanced ChemiLuminescence – GE Healthcare) and X-ray films according to the manufacturer. Primary polyclonal

rabbit anti-Rad53 antibodies are kindly provided by John Diffley (Clare Hall Laboratories, London). Primary monoclonal 12CA5 anti-HA and 9E10 anti-MYC antibodies are purchased at GE Healthcare, as well as peroxidase conjugated IgG anti-rabbit and anti-mouse secondary antibodies.

**KINASE ASSAY** (Trovesi *et al.*, 2011)

For Cdk1 kinase assays, protein extracts were prepared as described previously (Schwob *et al.*, 1994). HA-tagged Cdk1 was immunoprecipitated with anti-HA antibody from 150 µg of protein extracts and the kinase activity in the immunoprecipitates was measured on histone H1 (Surana *et al.*, 1993).

**DSB RESECTION AND REPAIR** (Trovesi *et al.*, 2011)

DSB formation and repair in YMV86 and YMV45 strains were detected by Southern blot analysis using an Asp718-Sall fragment containing part of the *LEU2* gene as a probe. DSB end resection at the *MAT* locus in JKM139 derivative strains was analyzed on alkaline agarose gels as described in (Clerici *et al.*, 2008), by using a single-stranded probe complementary to the unresected DSB strand. This probe was obtained by *in vitro* transcription using Promega Riboprobe System-T7 and plasmid pML514 as a template. Plasmid pML514 was constructed by inserting in the pGEM7Zf EcoRI site a 900-bp fragment containing part of the *MAT* $\alpha$  locus (coordinates 200870 to 201587 on chromosome III). Quantitative analysis of DSB resection was performed by calculating the ratio of band intensities for ssDNA and total amount of DSB products. DSB repair in tGI354 strain was detected as described in (Saponaro *et al.*, 2010). To determine the amount of noncrossover and crossover products, the normalized intensity of the corresponding bands at different time points

after DSB formation was divided by the normalized intensity of the uncut *MATa* band at time zero before HO induction (100%). The repair efficiency (NCO+CO) was normalized with respect to the efficiency of DSB formation by subtracting the value calculated 2 hours after HO induction (maximum efficiency of DSB formation) from the values calculated at the subsequent time points after galactose addition.

**SCREENING FOR SUPPRESSORS OF THE HU SENSITIVITY OF *mec1Δ sml1Δ* CELLS**  
(Manfrini *et al.*, 2012)

We searched for spontaneous extragenic mutations suppressing the HU sensitivity of *mec1Δ sml1Δ* cells. Since 5 mM HU was the minimal HU dose impairing the ability of *mec1Δsml1Δ* cells to form colonies, we plated *mec1Δ sml1Δ* (YLL490) cells on yeast extract-peptone-dextrose (YEPD) plates containing 5 mM HU and searched for clones able to form colonies. This analysis allowed us to identify 20 independent clones able to grow on 5 mM HU. By crossing these clones with a *MEC1 sml1Δ* strain, we found that the suppressor phenotype for two of them was due to a single-gene recessive mutation. One of these two clones was also temperature sensitive for growth, and this phenotype segregated tightly linked to the suppressor phenotype. We cloned the corresponding gene by transforming the original mutant clone with a yeast genomic DNA library constructed in a *LEU2* centromeric plasmid and searching for recombinant plasmids able to inhibit the mutant ability to form colonies on 5 mM HU. Analysis of several positive transformant clones revealed that the minimal complementing region was restricted to a DNA fragment containing

the *CDC28* gene. Further genetic analysis allowed us to demonstrate that *CDC28* was indeed the gene identified by the suppressor mutation.

**MICROSCOPY** (Manfrini *et al.*, 2012)

To visualize the mitotic spindle, cells expressing *TUB1-GFP* were fixed in 100% ethanol at the time points of interest and then washed in 10 mM Tris (pH 8.0) pending microscopic analysis. The GFP fluorophore was visualized using a band-pass GFP filter. To visualize Rad52-YFP foci, cells expressing *RAD52-YFP* were grown in synthetic medium supplemented with adenine to minimize autofluorescence. The cells were washed in 0.1 M potassium phosphate buffer at the time points of interest and analyzed immediately at the microscope. Cells were imaged on concanavalin A-coated slides. Microscopy was performed on a Leica TCS resonant STED DMI6000 CS microscope equipped with a multiline argon ion laser. Images of the YFP-stained yeast cells were acquired by collecting between 530 and 600 nm the fluorescence excited by the 27- $\mu$ W output of the 514-nm line of the argon laser. Both the emission and the transmitted light images have been recorded at 400-Hz scan speed through a 100X HXC PL APO oil objective (numerical aperture = 1.4) after identification of the cellular focal plane by 1- $\mu$ m step z-scan measurements. Microscopy images were analyzed by using ImageJ.

**PULSE-CHASE BrdU EXPERIMENT** (Manfrini *et al.*, 2012)

The pulse-chase BrdU experiment and immunodetection of BrdU-labeled DNA were performed as described previously (Vernis *et al.*, 2003; Segurado and Diffley, 2008).



**DROP TEST** (Manfrini *et al.*, 2012)

For spot assays, exponentially growing overnight cultures were counted, and 10-fold serial dilutions of equivalent cell numbers were spotted onto plates containing the indicated media. Experiments involving G1 synchronization were carried out by incubating exponentially growing cells in appropriate media containing 5 µg of α-factor/ml at 25°C for 2 h.



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