Published in final edited form as:

Nat Struct Mol Biol. 2013 May ; 20(5): 598–603. doi:10.1038/nsmb.2550.

Regulation of Mus81-Eme1 Holliday junction resolvase in response to DNA damage

Pierre-Marie Dehé^{1,2,6}, Stéphane Coulon^{1,2,6}, Sarah Scaglione^{1,2}, Paul Shanahan³, Arato Takedachi^{1,2}, James A. Wohlschlegel^{4,5}, John R. Yates 3rd⁴, Bertrand Llorente¹, Paul Russell³, and Pierre-Henri L. Gaillard^{1,2}

¹Centre National de la Recherche Scientifique, Unité Mixte de Recherche 7258, Inserm-Unité 1068, Centre de Recherche en Cancérologie de Marseille, Institut Paoli-Calmettes, F-13009, France

²Aix-Marseille Université, F-13284, Marseille, France

³Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037, USA

⁴Department of Chemical Physiology, The Scripps Research Institute, La Jolla, CA 92037, USA

Abstract

Structure-specific DNA endonucleases have critical roles during DNA replication, repair and recombination, yet they also harbor the potential for causing genome instability. Controlling these enzymes may be essential to ensure efficient processing of *ad hoc* substrates and to prevent random, unscheduled processing of other DNA structures, but it is unknown whether structure-specific endonucleases are regulated in response to DNA damage. Here, we uncover DNA damage-induced activation of Mus81-Eme1 Holliday junction resolvase in fission yeast. This novel regulation requires both Cdc2^{CDK1} and Rad3^{ATR}-dependent phosphorylations of Eme1. Mus81-Eme1 activation prevents gross chromosomal rearrangements in cells lacking the BLM-related DNA helicase Rqh1. We propose that linking Mus81-Eme1 DNA damaged-induced activation to cell cycle progression ensures efficient resolution of Holliday junctions that escape dissolution by Rqh1-TopIII while preventing unnecessary DNA cleavages.

Homologous recombination repair of damaged replication forks and double-strand breaks can lead to the formation of Holliday junctions (HJs). These 4-way DNA structures must be resolved to allow chromosome segregation during anaphase. Whereas the canonical bacterial HJ resolvase RuvC strongly prefers to process HJs, all three known eukaryotic nuclear HJ resolvases, Mus81-Eme1^{Mms4}, Gen1^{Yen1} and Slx1-Slx4, efficiently process *in vitro* a variety of DNA structures that contain junctions between single-strand and double-strand DNA^{1–7}. Given the multiplicity of DNA repair and recombination mechanisms that can generate these substrates, nuclear HJ resolvases must be tightly regulated *in vivo*^{8,9}. HJ resolution activity

Correspondence should be addressed to P-H.L.G. (pierre-henri.gaillard@inserm.fr).

⁵Current address: Department of Biological Chemistry, David Geffen School of Medicine, University of California Los Angeles, CA 90095, USA

⁶These authors contributed equally to this work

Author Contributions. PMD, SC, SS, AT, PS, JW, PHLG designed and performed experiments. PHLG, PR, BL, JRY designed experiments and supervised the work and PHLG wrote the paper. Experiments in Figure 1, 2 and 4 were performed by PMD and SS with initial input from PHLG, PS and SC in strain engineering. Experiments in Figure 3 were all performed by PMD with initial input from PHLG and PS in strain engineering 3a,b. In Figures 5a,b experiments were performed by PMD. PHLG performed experiments in 5c,d. Experiments in Figures 6a,b were carried out by SC.

Competing financial interests

The authors declare no competing financial interests.

is particularly important in absence of Bloom (BLM)-related helicases, which are proposed to dissolve double Holliday junctions (HJs) in a non-endonucleolytic process that prevents sister-chromatid exchange^{10–12}. In absence of BLM-related helicases, HJ resolvases are essential for viability^{1,11,13–16}, but this comes at the expense of an increased risk of genomic instability and loss of heterozygosity due to crossovers. Thus, while crossovers are required for proper chromosome segregation and promoting genetic variation during meiosis, HJ resolving enzymes in vegetative cells need to be kept under tight control so that HJs are preferentially processed by BLM-related helicases. It was recently shown that *Saccharomyces cerevisiae* Mus81-Mms4^{Eme1} is activated during G2/M through phosphorylation of Mms4^{Eme1} by Cdc5^{PLK1} kinase^{17,18}. This regulation may act as a failsafe mechanism to ensure resolution of HJs formed by the occasional spontaneous collapse of replication forks. However, it is unclear whether Mus81-Eme1 is independently activated in response to DNA lesions caused by external DNA damaging agents or by the accumulation of HJs in mitotic cells lacking functional BLM-related helicases. To gain insight into this fundamental question we undertook to investigate the importance of regulating Mus81-Eme1 in absence of BLM-related helicases in the fission yeast *Schizosaccharomyces pombe*, where Mus81-Eme1 is the primary HJ resolvase and Rqh1 the major BLM-related helicase.

RESULTS

Stimulation of Mus81-Eme1 in response to DNA damage

To determine whether Mus81-Eme1 is regulated in response to DNA damage, we first assessed whether Eme1 undergoes post-translational modifications in cells lacking Rqh1 or in response to treatment with camptothecin (CPT), which is a prototypical DNA topoisomerase I inhibitor with antitumor activity. Mus81-Eme1 is essential for viability in both situations^{13,19}. A strong phosphatase-sensitive mobility-shift of Eme1 was observed by SDS-polyacrylamide gel electrophoresis (PAGE) following CPT treatment of both WT and *rqh1*Δ cells (Fig. 1a, b). This phosphorylation of Eme1 was notable because CPT treatment triggers a checkpoint arrest in G2 phase prior to activation of Plo1^{PLK1} kinase, and thus the CPT-induced phosphorylation of Eme1 likely involves other protein kinases. A pool of Eme1 was also hyper-phosphorylated in non-treated *rqh1*Δ cells, probably due to the high rate of spontaneous DNA damage that results from loss of Rqh1. This DNA damage-induced hyper-phosphorylation of Eme1 significantly increased the *in vitro* HJ resolvase activity of the partially affinity-purified Mus81-Eme1 complex, as well as its ability to process other secondary DNA structures (Figure 1c, d and Supplementary Fig. 1a, b). Use of a nuclease-dead *mus81*^{DD} strain confirmed that all cuts were Mus81-Eme1 dependent (Supplementary Fig. 1c). These data suggest that DNA damage activates Mus81-Eme1 by a mechanism that might involve Eme1 hyper-phosphorylation.

CPT causes replication fork regression²⁰ and collapse²¹, leading to activation of the checkpoint kinase Chk1 by the ATM-related (ATR) checkpoint kinase ortholog Rad3²². While hyper-phosphorylation of Eme1 was observed in absence of the replication checkpoint kinase Cds1, deletion of the Chk1 DNA damage checkpoint kinase strongly diminished CPT-induced hyper-phosphorylation of Eme1 (Fig. 2a). Elimination of Chk1 also reduced the constitutive hyper-phosphorylation of Eme1 observed in non-treated *rqh1*Δ cells, confirming that accumulation of DNA damage in absence of Rqh1 leads to Chk1-dependent phosphorylation of Eme1 (Fig. 2b). DNA damage induced hyper-phosphorylation of Eme1 was also abolished when the Chk1 activation factors Rad3^{ATR} and Crb2 were deleted (Fig. 2a). The phosphorylation pattern of Eme1 was analyzed by tandem mass-spectrometry to identify phosphorylated sites. Six serine residues were identified within an N-terminal domain of Eme1 (Supplementary Fig. 1d), which is essential for its *in vivo*

functions but dispensable for the basal catalytic activity of the complex *in vitro* (unpublished, PMD, SS, PHLG). Mutating all six residues to alanine abolished CPT-induced phosphorylation of the resulting Eme1^{6SA} mutant protein and prevented stimulation of HJ resolution by Mus81-Eme1 (Fig. 2c and Supplementary Fig. 1e). Stimulation of Mus81-Eme1 following CPT treatment was also abolished in a *rad3Δ* mutant (Supplementary Fig. 1e), confirming that Mus81-Eme1 is stimulated in response to DNA damage through phosphorylation of Eme1. Five of the six serines were in CDK consensus sites (Supplementary Fig. 1c), suggesting that Eme1 phosphorylation might also be cell-cycle regulated in a CDK-dependent manner. DNA damage-independent phosphorylation of Eme1 was further suggested by the increased mobility of Eme1 from untreated cells following incubation with λ phosphatase (Fig. 1b).

CDK-mediated phosphorylation of Eme1 in G2

Using “block and release” of a temperature-sensitive *cdc25-22* strain to synchronize cells in late G2 and follow Eme1 phosphorylation throughout the cell cycle, we observed that fission yeast Eme1 undergoes cell-cycle dependent phosphorylation with hyper-phosphorylation in G2 and M, followed by a dip during S phase (Fig. 3a). Importantly, cell-cycle dependent phosphorylation of Eme1 was not abolished in a *cdc25-22 rad3Δ* mutant, indicating that Eme1 undergoes cell-cycle regulated phosphorylation independently of the DNA damage induced Rad3^{ATR}-mediated phosphorylation described above (Fig. 3b). Using a chromatin fractionation assay²³ we found that both Eme1 and Eme1^{6SA} were tightly associated with chromatin throughout the cell-cycle, indicating that cell-cycle controlled phosphorylation of Eme1 does not modulate its bulk chromatin association (Supplementary Fig. 2).

To confirm that Eme1 undergoes Cdc2^{CDK1}-dependent phosphorylation, we compared the phosphorylation pattern of Eme1 in a *cdc2^{as}* (Cdc2 analogue sensitive) mutant strain in presence or absence of the ATP analog NmPP1²⁴. When cells were synchronized in early S-phase with 12mM HU for 4hrs and released in fresh media in presence or absence of NmPP1, the increase of Eme1 phosphorylation observed upon entry into G2 was abolished in presence of NmPP1 (Fig. 3c), confirming that Eme1 undergoes Cdc2^{CDK1}-dependent phosphorylation during G2 when Cdc2^{CDK1} has a moderate level of activity {Coudreuse: 2010cr}. This cell-cycle regulated phosphorylation of Eme1 in G2 did not require the Cdc5^{PLK1} ortholog Plo1, in contrast to Mms4^{Eme1} phosphorylation in *S. cerevisiae*^{17,18} (Supplementary Fig. 3).

CDK primes Eme1 for phosphorylation after DNA damage

To discriminate whether DNA damage-induced phosphorylation of Eme1 mediated by Rad3^{ATR} may operate in a Cdc2^{CDK1}-dependent way as described for the checkpoint mediator Crb2^{25,26}, we first generated an *eme1^{4SA}* mutant strain where only CDK consensus target sites are mutated (Supplementary Fig. 1d). Eme1^{4SA} was no longer hyper-phosphorylated in response to CPT and the HJ resolvase activity of the resulting Mus81-Eme1^{4SA} complex was no longer stimulated (Fig. 4a). These data suggest that Cdc2^{CDK1}-dependent phosphorylation of Eme1 is a prerequisite for the DNA damage-induced activation of Mus81-Eme1 HJ resolvase. The possibility remained that CPT-induced hyper-phosphorylation of Eme1 resulted from DNA damage checkpoint-dependent cell-cycle arrest in late G2, where Eme1 is hyper-phosphorylated. To rule this out, we tested whether activation of the DNA damage checkpoint in cells already arrested in G2 induced further phosphorylation of CDK-phosphorylated Eme1. Activation of the DNA damage checkpoint following CPT treatment of fission yeast requires ongoing DNA replication²². Therefore, to activate the DNA damage checkpoint of G2 arrested cells and assess whether Eme1 underwent additional DNA damage-induced phosphorylation, *cdc25-22* cells synchronized in late G2 at 36°C were treated with the radiomimetic agent bleomycin. Importantly,

bleomycin treatment induced a strong Chk1-dependent mobility shift of Eme1 that was abolished in *eme1^{6SA}* and *eme1^{4SA}* mutant cells, confirming that DNA damage induced phosphorylation of Eme1 is not limited to topoisomerase inhibition (Fig. 4b–d). Interestingly, bleomycin induced an additional mobility shift of Eme1 in *cdc25-22* cells that had been first arrested in G2 at 36°C (Fig. 4d). Taken together our results show that Eme1 undergoes two phosphorylation events, with a first CDK-dependent phosphorylation that is needed for secondary DNA damage checkpoint-mediated phosphorylation following DNA damage.

Dual control of Mus81-Eme1 necessary in absence of Rqh1^{BLM}

Considering that cells lacking Rqh1^{BLM} rely on the Mus81-Eme1 HJ resolvase for survival¹, we anticipated that Cdc2^{CDK1}- and Rad3^{ATR}-dependent phosphorylation of Eme1 would be crucial in these cells. This prediction was confirmed by the synergistic growth defect caused by introducing the *eme1^{6SA}* and *eme1^{4SA}* mutations in the *rqh1Δ* background (Fig. 5a–c). This relationship contrasts with elimination of Cdc5^{PLK1} sites in *S. cerevisiae* Mms4, which in the absence of exogenous DNA damaging agents has no impact on the viability of cells lacking Sgs1^{Rqh1} DNA helicase or Yen1 HJ resolvase¹⁸. Interestingly, the *eme1^{6SA}* and *eme1^{4SA}* mutants were insensitive to CPT and bleomycin, contrasting with the acute hypersensitivity of the *eme1Δ* mutant (Fig. 5c and Supplementary Fig. 4a). To our knowledge these are the first Mus81-Eme1 separation of function mutants that lack functions essential only in absence of Rqh1. The acute CPT and bleomycin sensitivity of the *eme1^{6SA} rqh1Δ* double mutant compared to an *rqh1Δ* mutant (Figure 5c and Supplementary Fig. 4a), suggests that stimulation of Mus81-Eme1 is also required to process HJs which accumulate in absence of Rqh1 following exogenously induced DNA damage. To test this assumption, we assessed whether the sensitivity to CPT of the *eme1^{6SA} rqh1Δ* double mutant could be rescued by over-producing the bacterial RusA resolvase. Wild type RusA, but not a catalytically inactive mutant that lacks HJ resolvase activity, substantially rescued the CPT sensitivity of an *eme1^{6SA} rqh1Δ* mutant (Fig. 5d). This genetic suppression contrasts with the inability of RusA to rescue the CPT-sensitivity of an *rqh1Δ* single mutant¹⁹. In fact, high expression of active RusA slows growth in an *rqh1Δ* mutant (Supplementary Figure 4b). This result supports the proposal that HJ resolvases must be properly regulated to ensure robust growth.

Gross chromosomal rearrangements in *eme1^{6SA} rqh1Δ* mutants

BLM-related helicases are required to maintain the integrity of specific genomic loci such as the ribosomal DNA (rDNA) tandem repeats^{6,27}. These rDNA repeats are located at both ends of chromosome III in fission yeast. To assess whether Cdc2^{CDK1}- and Rad3^{ATR}-mediated activation of Mus81-Eme1 is required for stability of the rDNA loci in absence of Rqh1, we used pulse-field gel electrophoresis (PFGE) to examine the chromosome structure in *eme1^{6SA} rqh1Δ* double-mutants (Fig. 6a). Whereas chromosomes I and II appeared normal, a striking plasticity of chromosome III (ChrIII) was observed in the *eme1^{6SA} rqh1Δ* clones we tested, with some clones containing two ChrIII of distinct sizes (Fig. 6b and Supplementary Fig. 5). An rDNA probe confirmed that the sizes of the rDNA loci were highly variable in *eme1^{6SA} rqh1Δ* cells (Supplementary Fig. 5). We also noticed that in strains containing two ChrIII, the smallest chromosome contained in some cases as much rDNA as the larger ChrIII, indicating that it was missing non-rDNA regions compared to the larger chromosome. To confirm this we used comparative genomic hybridization (CGH) to compare the genome of an *eme1^{6SA} rqh1Δ* clone with two ChrIII to that of a WT strain. This analysis established that the *eme1^{6SA} rqh1Δ* clone tested is disomic for ChrIII and showed that the smallest chromosome is lacking a large portion of its right arm (Fig. 6b). CGH analysis of other disomic *eme1^{6SA} rqh1Δ* clones indicated that the break-point was different from clone to clone. Taken together, our results confirm that Cdc2^{CDK1}- and DNA damage

checkpoint-dependent phosphorylation of Eme1 is essential for chromosome stability in absence of the BLM-related helicase Rqh1.

DISCUSSION

Structure-specific DNA endonucleases are crucial for the repair of many types of DNA lesions, but it has remained unclear whether their activities are regulated in response to DNA damage. Here, we have found that Mus81-Eme1 activity significantly increases in cells treated with CPT, a drug that causes replication fork collapse. Unexpectedly, this regulation relies on both cell cycle driven phosphorylation of Eme1 by Cdc2^{CDK1} and Rad3^{ATR}-dependent DNA damage-induced phosphorylation. Cdc2^{CDK1}-dependent phosphorylation of Eme1 in G2 licenses Mus81-Eme1 for Rad3^{ATR}-dependent catalytic stimulation in response to DNA damage. We propose that this mechanism ensures rapid activation of Mus81-Eme1 in response to DNA damage specifically in G2, thereby guaranteeing that Holliday junctions that have eluded processing by Rqh1^{BLM} are resolved prior to the onset of mitosis. This DNA damaged-induced activation of Mus81-Eme1 differs from the Cdc5^{PLK1}-mediated activation of Mus81-Mms4^{Eme1} that occurs during the merged G2/M phase in budding yeast^{17,18}. Unlike budding yeast, fission yeast relies on the Cdc25-Wee1 network to regulate Cdc2^{CDK1} and thereby control transition from G2 to M phase. The moderate level of Cdc2^{CDK1} activity during G2 primes Mus81-Eme1 for activation in response to high levels of DNA damage (Fig. 6c). We further propose that this mechanism insures that Mus81-Eme1 activity is maintained at a low basal level except when its function is critical, thereby minimizing the possibility that it will create genome destabilizing lesions when its activity is not needed. The potential for genome destabilizing DNA cleavages by Mus81-Eme1 have been highlighted in several studies^{28–31}.

Our phospho-mutant studies indicate that activation of Mus81-Eme1 is crucial in absence of the Rqh1^{BLM} helicase. Phosphorylation of Eme1 in *rqh1Δ* cells is required to prevent gross chromosomal rearrangements even in absence of any exogenously induced DNA damage (Fig. 6). In contrast, loss of the Cdc5^{PLK1}-mediated phosphorylation of Mms4 has no impact on the viability of *sgs1Δ* and *sgs1Δ yen1Δ* mutants unless they are also treated with DNA damaging agents¹⁸. To our knowledge, this is the first report of a separation of function for Mus81-Eme1 where the essential functions that it fulfills in absence of Rqh1 have been dissociated from those that it fulfills in response to exogenous genotoxic stress in wild type cells.

The severe chromosome instability observed when Eme1 can no longer be phosphorylated in response to the endogenous DNA damage that accumulates in absence of Rqh1^{BLM} underscores the importance of the adaptive control mechanism of Mus81-Eme1 unraveled in this study. Our PFGE and CGH analyses revealed chromosome rearrangements primarily on chromosome III with, in some cases, the appearance of disomic cells with two copies of ChrIII. In fission yeast, only ChrIII aneuploidy is viable. Therefore, it is likely that the severe growth defect phenotype of the *eme1^{6SA} rqh1Δ* and *eme1^{4SA} rqh1Δ* mutants results from unviable chromosomal rearrangements that also affect the other two chromosomes. We observed no phenotype associated with preventing DNA damaged induced phosphorylation of Eme1 in cells that have functional Rqh1. This contrasts with the growth defect of *eme1Δ* null mutants that have lost Mus81-Eme1 functions and suggests that the Cdc2^{CDK1} and Rad3^{ATR} mediated control of Mus81-Eme1 in response to DNA damage acts as a fail-safe mechanism. Recombination structures in wild type cells are resolved by non-endonucleolytic activity of Rqh1^{BLM} helicase (with its associated factors Top III and Rmi1) and the basal endonucleolytic processing activity of Mus81-Eme1. In absence of Rqh1^{BLM}, the basal nuclease activity of Mus81-Eme1 is insufficient to cope with the extra burden of unprocessed recombination intermediates and DNA damage-induced hyper-activation of

Mus81-Eme1 becomes necessary to limit chromosome instability. However, we found that over-expression of constitutively active bacterial RusA resolvase impaired the growth of *rqh1*Δ cells, indicating the importance of limiting resolvase activity even when it is essential for cell viability. The 2 to 3 fold activation of Mus81-Eme1 measured in our assays may be sufficient to ensure effective HJ resolution while still minimizing the risk of Mus81-Eme1 creating new DNA lesions by cleaving transient joint DNA molecules. Restricting Mus81-Eme1 activation to G2 phase may increase the probability that double HJs or regressed replication forks are dissolved or reversed by BLM-related helicases, respectively. There is a growing body of evidence that Mus81-Eme1 is subjected to a complex network of regulatory processes. Clearly, further investigations are needed to determine the precise underlying molecular mechanisms and how these processes are coordinated to maintain genome stability.

Online Methods

Yeast strains

Fission yeast strain genotypes are listed in Table S1. Standard fission yeast methods were used as described previously^{32,33}.

The *eme1*^{6SA} and *eme1*^{4SA} phosphorylation mutants were generated as follows. The *EME1* genomic locus from strain PH81 (h+, leu1-32 ura4-D18 TAP:Eme1 Mus81:13Myc-KanMX6) that produces N-terminally TAP-tagged Eme1, was sub-cloned into a TopoTA vector (Invitrogen). Point mutations were introduced on that *TopoTA-EME1* vector using a Multiprime site directed mutagenesis kit (Stratagene). Mutations were confirmed by DNA sequencing. The mutated *EME1* genomic locus from the *TopoTA-EME1* vector was used to transform strain PH419, in which the entire *EME1* gene is replaced by a URA4 cassette. 5'-FOA resistant clones were selected and confirmed as TAP:Eme1^{6SA} or TAP:Eme1^{4SA} producing strains by genomic DNA sequencing.

For synchronization of cells using *cdc25-22* block and release, cells containing the temperature sensitive *cdc25-22* allele were grown to exponential phase at permissive temperature (25°C), shifted at restrictive temperature (36°C) for 3.5 hours to arrest the cell cycle in G2³². Upon release to permissive temperature (25°C), the cells synchronously enter the cell cycle. Cells were collected and processed every 20 minutes. Progression into S-phase was monitored microscopically by counting cells that contained septa, the appearance of which correlates with S-phase³².

For synchronization of cells in Figure 3c, cells containing the *cdc2*^{as} allele were grown to exponential phase at 25°C before addition of 12mM of HU (Sigma-Aldrich) for 4 hrs to arrest the cells in early S-phase. Following extensive washes, cells were released in fresh medium at 25°C and collected every 20 min. FACS analysis confirmed HU-induced early S-phase arrest and allowed monitoring of cell cycle progression following release. Cdc2^{as} was inhibited by addition of 1 μM NmPP1 (Carbosynth).

RusA complementation assays were performed by transforming *eme1*Δ and *rqh1*Δ *eme1*^{6SA} strains with pREP1-RusA or pREP1-RusA^{D70N} plasmids for over-expression of RusA or the catalytically inactive RusA^{D70N} mutant¹.

Native and TCA protein extraction, immunoblotting

Cellular lysates were prepared from exponentially growing cell cultures treated or not with camptothecin (Sigma-Aldrich) or bleomycin (Calbiochem). Cells were lysed in lysis buffer¹ using a Retsch mechanical grinder in presence of liquid nitrogen. The cellular lysate was clarified and stored at -80°C. Protein concentration of each sample was determined with a

Nanodrop (Thermo-Scientific) and protein amounts were normalized between the different samples. Denatured cell lysates were prepared by TCA precipitation. Cells were resuspended in 20% TCA and vortexed. Following centrifugation, the TCA precipitate was resuspended in SDS-PAGE loading buffer (Invitrogen) containing Tris-Base.

Protein extracts were resolved on Tris-Acetate 3–8% polyacrylamide NUPAGE gels (Invitrogen). Proteins were transferred to a nitrocellulose Hybond-C membrane (Invitrogen). The membrane was blocked in PBS-T milk 5% and probed using anti-Flag (Sigma-Aldrich) antibody (1/5000 dilution).

Nuclease assays

TAP-Eme1 was affinity purified and used in nuclease assays on various DNA substrates as previously described for TAP:Mus81^{1,2}. ³²P-labelled DNA substrates were used in all nuclease assays except in Fig S1.c where fluorescent Cy5-labelled substrates were used. The HJ substrate used in this study was the X12 mobile HJ.

Pulse Field Gel Electrophoresis

PFGE of whole chromosomes was performed as previously described⁶. $5 \cdot 10^8$ cells were spheroplasted with 0.6 mg ml^{-1} Zymolase-100T (Seikagru) at 37 °C for 2h, resuspended in 1% low melting point agarose (SIGMA) at a concentration of 10^8 cells per plug. Agarose plugs were loaded onto 0.8% agarose gels. PFGE was performed on a BioRad CHEF DR-III system in $1 \times$ TAE (40 mM Tris-acetate buffer, 2 mM Na₂EDTA pH 8.3) at 14 °C using the following program: 48 h at 2 V cm^{-1} , 106° angle, 30 min switch time. After electrophoresis, DNA was visualized by ethidium bromide staining, and gels were processed for Southern blotting, which was performed using an rDNA probe and a Wee1 probe (used as a probe to visualize Chr III).

Comparative Genomic Hybridization

Agilent *S. pombe* ChIP on CHIP 4x44K G8410A Genome Microarrays were used to perform comparative genomic hybridizations. Genomic DNA from strains PH463 and PH466 was prepared using a Qiagen genomic kit and DNA was directly labeled using the Agilent Genomic DNA Enzymatic labeling Kit (5190-0453). Hybridizations were performed according to Agilent recommendations.

Proteomic identification of Eme1 phosphorylation sites

TAP-Eme1 was affinity purified from WT and *cds1Δ* + HU 10mM cells. Western blot analysis showed that Eme1 is hyper-phosphorylated to the same extent in CPT-treated and HU-treated *cds1Δ* cells (data not shown).

TCA precipitates were proteolytically digested by Lys-C and trypsin analyzed by MudPIT on a LTQ mass spectrometer (ThermoFisher) as previously described³⁴. Data was analyzed with the SEQUEST and DTASelect algorithms using a differential modification search that considered +80 on serine, threonine, and tyrosine residues³⁵. Sites found with high confidence as constitutively phosphorylated or phosphorylated in response to DNA damage were further validated by manual inspection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank M. Grenon, N. Rhind, C. Chahwan, N. Boddy, J.H. Guervilly and G. Almouzni for critical reading of the manuscript and stimulating discussions throughout the course of this study and L. Brossy for technical help. Many thanks to V. Simanis (ISREC, Lausanne, Switzerland) for kindly providing the *cdc2^{as}* strain and to Y. Denis for technical help with the CGH analysis. PHLG acknowledges financial support from Fondation Arc pour la Recherche sur le Cancer (SF1052), Centre National de la Recherche Scientifique (ATIP program) and Agence Nationale de la Recherche (ANR-10-BLAN-1512-01). BL was supported by a grant from Agence Nationale de la Recherche (ANR-10-BLAN-1606-03). Work in the laboratory of PR was supported by the US National Institutes of Health (RO1 GM59447).

References

1. Boddy MN, et al. Mus81-Eme1 are essential components of a Holliday junction resolvase. *Cell*. 2001; 107:537–548. [PubMed: 11719193]
2. Gaillard PHL, Noguchi E, Shanahan P, Russell P. The endogenous Mus81-Eme1 complex resolves Holliday junctions by a nick and counternick mechanism. *Mol Cell*. 2003; 12:747–759. [PubMed: 14527419]
3. Whitby MC, Osman F, Dixon J. Cleavage of model replication forks by fission yeast Mus81-Eme1 and budding yeast Mus81-Mms4. *J Biol Chem*. 2003; 278:6928–6935. [PubMed: 12473680]
4. Rass U, et al. Mechanism of Holliday junction resolution by the human GEN1 protein. *Genes Dev*. 2010; 24:1559–1569. [PubMed: 20634321]
5. Fricke WM, Brill SJ. Slx1-Slx4 is a second structure-specific endonuclease functionally redundant with Sgs1-Top3. *Genes Dev*. 2003; 17:1768–1778. [PubMed: 12832395]
6. Coulon S, et al. Slx1-Slx4 are subunits of a structure-specific endonuclease that maintains ribosomal DNA in fission yeast. *Mol Biol Cell*. 2004; 15:71–80. [PubMed: 14528010]
7. Ehmsen KT, Heyer WD. A junction branch point adjacent to a DNA backbone nick directs substrate cleavage by *Saccharomyces cerevisiae* Mus81-Mms4. *Nucleic Acids Res*. 2009; 37:2026–2036. [PubMed: 19211663]
8. Muñoz-Galván S, et al. Distinct roles of Mus81, Yen1, Slx1-Slx4, and Rad1 nucleases in the repair of replication-born double-strand breaks by sister chromatid exchange. *Mol Cell Biol*. 2012; 32:1592–1603. [PubMed: 22354996]
9. Ho CK, Mazón G, Lam AF, Symington LS. Mus81 and Yen1 promote reciprocal exchange during mitotic recombination to maintain genome integrity in budding yeast. *Mol Cell*. 2010; 40:988–1000. [PubMed: 21172663]
10. Ashton TM, Mankouri HW, Heidenblut A, McHugh PJ, Hickson ID. Pathways for Holliday junction processing during homologous recombination in *Saccharomyces cerevisiae*. *Mol Cell Biol*. 2011; 31:1921–1933. [PubMed: 21343337]
11. Wechsler T, Newman S, West SC. Aberrant chromosome morphology in human cells defective for Holliday junction resolution. *Nature*. 2011; 471:642–646. [PubMed: 21399624]
12. Wu L, Hickson ID. The Bloom's syndrome helicase suppresses crossing over during homologous recombination. *Nature*. 2003; 426:870–874. [PubMed: 14685245]
13. Boddy MN, et al. Damage tolerance protein Mus81 associates with the FHA1 domain of checkpoint kinase Cds1. *Mol Cell Biol*. 2000; 20:8758–8766. [PubMed: 11073977]
14. Kaliraman V, Mullen JR, Fricke WM, Bastin-Shanower SA, Brill SJ. Functional overlap between Sgs1-Top3 and the Mms4-Mus81 endonuclease. *Genes Dev*. 2001; 15:2730–2740. [PubMed: 11641278]
15. Andersen SL, Kuo HK, Savukoski D, Brodsky MH, Sekelsky J. Three structure-selective endonucleases are essential in the absence of BLM helicase in *Drosophila*. *PLoS Genet*. 2011; 7:e1002315. [PubMed: 22022278]
16. Mullen JR, Kaliraman V, Ibrahim SS, Brill SJ. Requirement for three novel protein complexes in the absence of the Sgs1 DNA helicase in *Saccharomyces cerevisiae*. *Genetics*. 2001; 157:103–118. [PubMed: 11139495]

17. Matos J, Blanco MG, Maslen S, Skehel JM, West SC. Regulatory control of the resolution of DNA recombination intermediates during meiosis and mitosis. *Cell*. 2011; 147:158–172. [PubMed: 21962513]
18. Gallo-Fernandez M, Saugar I, Ortiz-Bazan MA, Vazquez MV, Tercero JA. Cell cycle-dependent regulation of the nuclease activity of Mus81-Eme1/Mms4. *Nucleic Acids Res*. 2012;10.1093/nar/gks599
19. Doe CL, Ahn JS, Dixon J, Whitby MC. Mus81-Eme1 and Rqh1 involvement in processing stalled and collapsed replication forks. *J Biol Chem*. 2002; 277:32753–32759. [PubMed: 12084712]
20. Ray Chaudhuri A, et al. Topoisomerase I poisoning results in PARP-mediated replication fork reversal. *Nat Struct Mol Biol*. 2012; 19:417–423. [PubMed: 22388737]
21. Regairaz M, et al. Mus81-mediated DNA cleavage resolves replication forks stalled by topoisomerase I-DNA complexes. *J Cell Biol*. 2011; 195:739–749. [PubMed: 22123861]
22. Wan S, Capasso H, Walworth NC. The topoisomerase I poison camptothecin generates a Chk1-dependent DNA damage checkpoint signal in fission yeast. *Yeast*. 1999; 15:821–828. [PubMed: 10407262]
23. Goshima G, Iwasaki O, Obuse C, Yanagida M. The role of Ppe1/PP6 phosphatase for equal chromosome segregation in fission yeast kinetochore. *EMBO J*. 2003; 22:2752–2763. [PubMed: 12773390]
24. Dischinger S, Krapp A, Xie L, Paulson JR, Simanis V. Chemical genetic analysis of the regulatory role of Cdc2p in the *S. pombe* septation initiation network. *J Cell Sci*. 2008; 121:843–853. [PubMed: 18303049]
25. Esashi F, Yanagida M. Cdc2 phosphorylation of Crb2 is required for reestablishing cell cycle progression after the damage checkpoint. *Mol Cell*. 1999; 4:167–174. [PubMed: 10488332]
26. Caspari T, Murray JM, Carr AM. Cdc2-cyclin B kinase activity links Crb2 and Rqh1-topoisomerase III. *Genes Dev*. 2002; 16:1195–1208. [PubMed: 12023299]
27. Kaliraman V, Brill SJ. Role of SGS1 and SLX4 in maintaining rDNA structure in *Saccharomyces cerevisiae*. *Curr Genet*. 2002; 41:389–400. [PubMed: 12228808]
28. Forment JV, Blasius M, Guerini I, Jackson SP. Structure-specific DNA endonuclease Mus81/Eme1 generates DNA damage caused by Chk1 inactivation. *PLoS ONE*. 2011; 6:e23517. [PubMed: 21858151]
29. Froget B, Blaisonneau J, Lambert S, Baldacci G. Cleavage of stalled forks by fission yeast Mus81/Eme1 in absence of DNA replication checkpoint. *Mol Biol Cell*. 2008; 19:445–456. [PubMed: 18032583]
30. Beck H, et al. Cyclin-dependent kinase suppression by WEE1 kinase protects the genome through control of replication initiation and nucleotide consumption. *Mol Cell Biol*. 2012; 32:4226–4236. [PubMed: 22907750]
31. Domínguez-Kelly R, et al. Wee1 controls genomic stability during replication by regulating the Mus81-Eme1 endonuclease. *J Cell Biol*. 2011; 194:567–579. [PubMed: 21859861]
32. Forsburg SL. Growth and manipulation of *S. pombe*. *Curr Protoc Mol Biol*. 2003; Chapter 13(Unit 13.16)
33. Bähler J, et al. Heterologous modules for efficient and versatile PCR-based gene targeting in *Schizosaccharomyces pombe*. *Yeast*. 1998; 14:943–951. [PubMed: 9717240]
34. Maccoss MJ, et al. Shotgun identification of protein modifications from protein complexes and lens tissue. *Proc Natl Acad Sci USA*. 2002; 99:7900–7905. [PubMed: 12060738]
35. Tabb DL, McDonald WH, Yates JR. DTASelect and Contrast: tools for assembling and comparing protein identifications from shotgun proteomics. *J Proteome Res*. 2002; 1:21–26. [PubMed: 12643522]

Figure 1. Phosphorylation of Eme1 and hyper-activation of Mus81-Eme1 in response to DNA damage

(a) Western blot assay on total lysates of wild-type (WT) or *rqh1Δ* cells producing TAP-tagged Eme1 (See Supplementary Figure 1 for structure of the TAP-tag). Cells were either non-treated or exposed to 40 μM camptothecin (CPT). Wild-type (WT) (PH423), *rqh1Δ* (PH454). **(b)** Western blot assay on TAP-Eme1 affinity purified from untreated or 40 μM CPT-treated WT cells preincubated with active λ phosphatase (PPase) or heat inactivated λ phosphatase (PPase 95°C). **(c)** A ³²P-labelled (red dot) Holliday Junction (HJ) was incubated with identical amounts of the Mus81-Eme1 samples purified from untreated or 40 μM CPT-treated cells shown in **b**. The reaction products were separated by neutral PAGE. **(d)** Average (+/-SEM) stimulation fold of HJ resolution by Mus81-Eme1 following CPT treatment calculated from n=8 independent experiments. The histogram shows the ratio of the HJ resolvase activity of the Mus81-Eme1 affinity-purified from CPT-treated cells over that of Mus81-Eme1 affinity purified from untreated cells.

Figure 2. Eme1 is phosphorylated by the DNA damage checkpoint

(a) Western blot assay on total lysates derived from the indicated untreated or 40 μ M CPT-treated strains producing TAP-tagged Eme1; WT: strain PH466, *cds1Δ* (PH113), *chk1Δ* (PH120), *rad3Δ* (PH132), *crb2Δ* (PH189). (b) Western blot assay on total lysates from untreated or 40 μ M CPT-treated WT, *rqh1Δ* and *chk1Δ rqh1Δ* strains. (c), Upper panel: Western blot assay on affinity-purified Mus81-Eme1 from untreated or 40 μ M CPT-treated WT (PH423) and *eme1^{6SA}* (PH481) cells, Lower panel: stimulation fold of HJ resolution. The histogram shows the ratio of the HJ resolvase activity of the Mus81-Eme1 affinity-purified from CPT-treated cells over that of Mus81-Eme1 affinity purified from untreated cells. The samples used in the *in vitro* nuclease assay are those shown on upper panel.

Figure 3. Eme1 is phosphorylated in a Cdc2^{CDK1}-dependent manner

(a) *cdc25-22* cells producing TAP-tagged Eme1 (PH171) or Eme1^{6SA} (PH491) were blocked at 36°C for 4 hrs before release at 25°C. Top: Septation index measured for each strain. Middle: Western blot detection of TAP-tagged Eme1. Bottom: Western blot detection of TAP-tagged Eme1^{6SA}. (b) Western blot detection of TAP-Eme1 from block and released *cdc25-22 rad3Δ* (PH538) mutant cells. (c) *cdc2^{as}* mutant cells (PH602) were incubated with 12 mM HU for 4 hrs to arrest cell cycle progression in early S-phase before release in fresh media with or without NmPP1. Top: FACS analysis of asynchronous (As), blocked and released cells. Bottom: Western blot detection of TAP-Eme1 from blocked and released (+ or – NmPP1) cells.

Figure 4. Cdc2^{CDK1}-dependent phosphorylation prime Eme1 for DNA damage dependent hyper-activation of Mus81-Eme1

(a) Upper panel: Western blot detection of TAP-Eme1 from untreated or 40μM CPT-treated WT (PH466) and *eme1^{4SA}* (PH546) strains. Lower panel: Hyper-activation of HJ resolvase activity of Mus81-Eme1 after 40 μM CPT-treatment of WT and *eme1^{4SA}* mutant cells. Mus81-Eme1 HJ resolvase activation was calculated by dividing the activity of Mus81-Eme1 affinity purified from CPT-treated cells by the activity of Mus81-Eme1 affinity purified from untreated cells. (b) Western blot detection of TAP-Eme1 in total lysates derived of the indicated untreated, 40 μM CPT or 5 mg/ml bleomycin-treated strains producing TAP-tagged Eme1; WT (PH466), *cds1Δ* (PH113), *chk1Δ* (PH120). (c) as in b, *eme1^{6SA}* (PH491), *eme1^{4SA}* (PH546), WT (PH466). (d) Western blot assay on the total lysates from the indicated strains grown asynchronously (As) at 25°C or synchronized and kept in late G2 at 36°C, without or with bleomycin. *cdc25^{ts}* (PH171), *cdc25^{ts} rad3Δ* (PH538).

Figure 5. Hyper-activation of Mus81-Eme1 is critical in absence of Rqh1^{BLM}
(a) Western blot detection of TAP-Eme1 in cell lysates of the indicated untreated strains. **(b)** Left pannel: Tetrad analysis of a *eme1^{4SA}* x *rqh1Δ* mating germinated at 30°C. Right pannel: representative image of *eme1^{6SA} rqh1Δ* (PH462) colonies 16 hrs after plating on YES rich media. **(c)** 5-fold dilutions of cells with the indicated genotype were plated on medium supplemented or not with the indicated concentrations of CPT followed by incubation at 30°C. *TAP-Eme1* (PH466); *eme1Δ* (PH419); *rqh1Δ* (SC3250); *rqh1Δ TAP-Eme1* (PH454); *rqh1Δ eme1^{6SA}* (PH462). **(d)** 5-fold dilutions of *eme1Δ* (PH466) and *eme1^{6SA} rqh1Δ* (PH454) cells over-producing the bacterial wild-type RusA HJ resolvase or a nuclease dead RusAND version. Cells were plated on medium supplemented or not with the indicated concentrations of CPT followed by incubation at 30°C.

Figure 6. DNA damage induced phosphorylation of Eme1 prevents gross chromosomal rearrangements in absence of Rqh1^{BLM}

(a) Left: Schematic representation of *S. pombe* three chromosomes (Chr) and distribution of rDNA at both ends of ChrIII. Right: Genomic DNA samples of WT and the indicated mutant strains were subjected to pulse-field gel electrophoresis. The gel was stained with ethidium bromide. A dashed line indicates the position of WT ChrIII. WT (PH466); *eme1^{6SA}* (PH481); *rqh1Δ* (PH454); *rqh1Δ eme1^{6SA}* (PH462, PH463, PH464, PH465). (b) Comparative Genome Hybridization of the ChrIII disomic *eme1^{6SA} rqh1Δ* clone (PH463) indicated by “*” in panel a. (c) Model; see text for details.