Mus81-Eme1 and Rqh1 involvement in processing stalled and collapsed replication forks

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Running title: Processing DNA junctions by Mus81-Eme1 and Rqh1
SUMMARY

The processing of stalled replication forks and repair of collapsed replication forks are essential functions in all organisms. In fission yeast DNA junctions at stalled replication forks appear to be processed by either the Rqh1 DNA helicase or Mus81-Eme1 endonuclease. Accordingly we show that the hypersensitivity to agents that cause replication fork stalling of mus81, eme1 and rqh1 mutants is suppressed by a Holliday junction resolvase (RusA), as is the synthetic lethality of a mus81 rqh1 double mutant. Recombinant Mus81-Eme1, purified from Escherichia coli, readily cleaves replication fork structures but cleaves synthetic Holliday junctions relatively poorly in vitro. From these data we propose that Mus81-Eme1 can process stalled replication forks before they have regressed to form a Holliday junction. We also implicate Mus81-Eme1 and Rqh1 in the repair of collapsed replication forks. Here Mus81-Eme1 and Rqh1 seem to function on different substrates since RusA can substitute for Mus81-Eme1 but not Rqh1.
INTRODUCTION

A key intermediate of homologous recombination is the four-way DNA (Holliday) junction (HJ), which is formed by the reciprocal exchange of strands between homologous DNA molecules in a reaction catalysed by the RecA/Rad51 family of proteins. HJs can also form from the regression of stalled replication forks (1-3). Once formed HJs have to be removed from the DNA to enable chromosome segregation.

In *Escherichia coli* RuvABC processes HJs by the combined action of a branch migration enzyme (RuvAB) and a junction-targeted endonuclease (RuvC). In the absence of RuvABC the presence of an alternative branch migration enzyme (RecG) together with mutations that activate the expression of an alternative resolvase (RusA) means that recombination and DNA repair can proceed at near wild-type levels (reviewed in 4). A key role for these enzymes is to facilitate the re-start of replication when replication forks stall at blockages in the DNA template (reviewed in 5). One strategy involves the regression of the fork by RecG, which forms an HJ when the two nascent DNA strands anneal. Cleavage of this HJ by RuvABC collapses the replication fork and the resulting free double-strand end is processed to generate a 3'-OH terminated single-strand tail that, in a RecA-mediated reaction, invades the intact chromosome to form a displacement (D-) loop. PriA promotes assembly of the replisome at the 3' invading strand end of the D-
loop. At the opposite end of the D-loop an HJ is formed that is cleaved by RuvABC to complete the process.

To identify genes involved in processing HJs, in the fission yeast *Schizosaccharomyces pombe*, we have systematically screened recombination/repair mutants for suppression of their phenotypes by RusA. We have reported previously that RusA partially suppresses aberrant chromosome segregation in *rqh1* mutants (6). *rqh1* encodes a member of the RecQ family of DNA helicases that also includes the Bloom’s, Werner’s, and Rothmund-Thomson’s Syndrome helicases in humans (7). RecQ helicases are implicated in controlling recombination at stalled replication forks by reversing the regression of the fork and thereby preventing its collapse through cleavage of the HJ that is formed (6,8,9). It appears that RusA provides an alternative way of removing HJs that would otherwise remain unprocessed in a *rqh1* mutant and impede sister chromatid segregation (6).

Mus81-Eme1 is a heterodimeric endonuclease that is required for spore viability, tolerance of ultraviolet light (UV) and hydroxyurea (HU), and viability in the absence of Rqh1 (10). Mus81-Eme1 is also an essential component of an activity, purified from *S. pombe* cells, which resolves synthetic HJs *in vitro* (a similar activity has been detected with human Mus81 purified from HeLa cells) (11,12). Furthermore, RusA suppresses the meiotic defects of a *mus81* mutant (11). Based on these data Russell and co-workers have
proposed that Mus81-Eme1 resolves HJs during meiotic recombination and, in vegetative cells, provides an alternative to Rqh1 for processing HJs formed at regressed replication forks (11). The *Saccharomyces cerevisiae* Mus81 forms a heteromeric complex with Mms4, which shares sequence similarity to Eme1 (13,14). ScMus81-Mms4, like SpMus81-Eme1, is required for the tolerance of agents that cause replication fork stalling and for viability in the absence of Sgs1 (a homologue of Rqh1) (14). However, unlike SpMus81-Eme1, spore viability is not dramatically reduced in the absence of ScMus81-Mms4 (15). Furthermore, recombinant ScMus81-Mms4 purified from *E. coli* cleaves synthetic HJs very poorly, whereas, replication fork substrates are cleaved well (13). These data have lead to the proposal that ScMus81-Mms4, rather than resolving HJs, cleaves stalled replication forks to facilitate replication restart and in meiosis removes 3' DNA flaps formed during the repair of double strand breaks (DSBs) by a pathway of “strand displacement and annealing” (13,15).

Here we provide additional evidence that Mus81-Eme1 and Rqh1 provide overlapping activities for processing DNA junctions at stalled replication forks. However, based on the *in vitro* activity of recombinant protein, we propose that Mus81-Eme1 cleaves replication forks before they have regressed to form an HJ. We also present evidence that Mus81-Eme1 and Rqh1 promote the repair of collapsed replication forks, although here their functions appear not to overlap.
EXPERIMENTAL PROCEDURES

General techniques - Procedures for S. pombe genetics are described by Moreno et al (16). Spot assays and media are described by Doe et al (6).

Strains - Strains used in this study are ura4-D18 and leu1-32 unless otherwise indicated: MCW448, h+ ade6-704; MCW449, h+ ade6-704; MCW682, h+ mus81::kanMx6 ade6-704; MCW744, h+ mus81::kanMx6 ade6-704; MCW793, h+ eme1::ura4+ ade6-704; MCW825, h+ rqh1::kanMx6 ade6-704; MCW827, h+ mus81::kanMx6 eme1::ura4+ ade6-704; MCW149, h+ rqh1::ura4+ ade6-704; MCW296, h+ rad50::kanMx6 his3-D1; MCW3, h+ rhp51::ura4+ his3-D1; MCW642, h+ rad22A::ura4+ ade6-469; MCW121, rhp54::ura4+, his3-D1; MCW248, h+ rhp55::ura4+ ade7-152; MCW708, h+ leu1::Pnmt-NLS-rusA-GFP-KanMx6; MCW788, h+ rqh1::ura4+ leu1::Pnmt-NLS-rusA-GFP-KanMx6; MCW790, h+ rqh1::ura4+ leu1::Pnmt-NLS-rusAD70N-GFP-KanMx6; MCW836, h+ mus81::kanMx6 leu1::Pnmt-NLS-rusA-GFP-KanMx6; MCW838, h+ rqh1::ura4+ mus81::kanMx6 leu1::Pnmt-NLS-rusA-GFP-KanMx6; MCW869, h+ top1::LEU2+ ura4+; MCW907, h+ rqh1::kanMx6 top1::LEU2+; MCW910, h+ mus81::kanMx6 top1::LEU2+. The mus81::kanMx6 and rqh1::kanMx6 deletion mutants, and leu1::Pnmt-NLS-rusA-GFP-KanMx6 strains were made by PCR-based gene targeting.
The eme1::ura\textsuperscript{4+} insertion mutant was made by inserting the ura\textsuperscript{4+} gene into a cloned copy of eme1 at an SphI site 860 bp downstream from the start codon. The eme1::ura\textsuperscript{4+} fragment was then liberated from its plasmid, transformed into MCW448 and stable Ura\textsuperscript{+} transformants selected. Genuine mus81::kan\textsuperscript{t} and eme1::ura\textsuperscript{4+} strains were identified by genomic Southern analysis.

**Plasmids** - pREP-rus plasmids are described by Doe et al (6). pMW439 expresses NLS-RusA-GFP from the T7 phage \(\Theta10\) promoter in pT7-7 (18). mus81 was cloned as a PCR-amplified fragment from genomic DNA with NdeI and BamHI flanking restriction sites to enable cloning downstream of the nmt promoter in pREP41 (pMW524) and the T7 phage \(\Theta10\) promoter in pT7-7 (pMW525) and pET14b (Novagen) (pMW510). eme1 cDNA was cloned by RT-PCR using the Access RT-PCR System (Promega). The nucleotide sequence of the cloned eme1 cDNA revealed that the first of its two putative introns is larger than predicted in the current \(S.\) pombe gene database by 9 bp (\(S.\) pombe GeneDB). A gtttgt sequence 5' of the predicted gtattt splice donor site appears to be the real donor site (data not shown). The eme1 cDNA was cloned into pET14b to make pMW560, which expresses Eme1 with a hexa-histidine tag at its N-terminus. pMW562 was made by cloning mus81 with the T7 phage \(\Theta10\) promoter from pMW525 into
pMW560 to make a plasmid that expresses both His-tagged Eme1 and untagged Mus81 from independent T7 phage Ø10 promoters.

**Purification of NLS-RusA-GFP** - 1-Liter batches of *E. coli* BL21(DE3) pLysS (18) containing pMW439 were grown with aeration at 25°C in Luria-Bertani broth containing 125 µg/ml carbenicillin and 50 µg/ml chloramphenicol. At a cell density corresponding to an A<sub>600</sub> of 0.5, NLS-RusA-GFP was induced by adding isopropyl-1-thio-ß-D-galactopyranoside to a final concentration of 1 mM, following which the cells were incubated for a further 2 h. The cells were then harvested by centrifugation, resuspended in lysis buffer (100 mM Tris-HCl, pH 8.0, 2 mM EDTA, 5% glycerol), and frozen at -80°C until required. All subsequent steps were at 4°C. Defrosted cells were mixed with 0.5 M NaCl, 0.1% Triton X-100, 1 mM DTT and protease inhibitors before passage through a French pressure cell at 30,000 p.s.i.. Cell debris was then removed by centrifugation at 43,700 g for 30 min and the supernatant was dialysed against Buffer A (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 10% glycerol) containing 0.2 M NaCl. The dialysed sample was loaded onto a 10 ml phosphocellulose column, which was washed with 50 ml Buffer A + 0.2 M NaCl before eluting protein with a 160 ml gradient from 0.2 to 1.0 M NaCl. NLS-RusA-GFP eluting between 0.7 to 0.8 M NaCl was pooled and dialysed against Buffer A + 0.2 M NaCl. The dialysed sample was
centrifuged at 27,200 g for 15 min and pelleted material, consisting mainly of NLS-
RusA-GFP, was re-dissolved in Buffer A + 1.0 M NaCl before aliquoting and storage at
–20°C.

**Purification of Mus81-Eme1 -** *E. coli* BL21-RIL cells (Stratagene) containing
pMW562 were grown and induced as for NLS-RusA-GFP above. Harvested cells were
resuspended in Buffer H (50 mM potassium phosphate, pH 8.0, 10 mM ß-
mercaptoethanol, 0.3 M NaCl, 10% glycerol) with protease inhibitors and 1% Triton X-
100, and lysed by passage through a French pressure cell at 30,000 p.s.i.. After
centrifugation at 43,700 g for 50 min the supernatant was loaded directly onto a 2 ml Ni-
NTA superflow column (Qiagen), which was washed with 60 ml Buffer H + 25 mM
imidazole before eluting bound Mus81-Eme1 with Buffer H + 200 mM imidazole into 2
ml fractions. The second 2ml fraction contained the peak of Mus81-Eme1 and was loaded
directly onto a HiLoad 16/60 Superdex 200 gel filtration column (Amersham Pharmacia),
which was then developed with 120 ml Buffer A + 150 mM NaCl. Fractions containing
Mus81-Eme1 were detected by both SDS-PAGE and screening fractions for fork and X-
12 cleavage activity, the peak fractions were then pooled, dialysed against storage buffer
(50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 50% glycerol, 150 mM NaCl) and
stored as aliquots at –80°C. Amounts of protein were estimated using a Biorad protein assay kit and BSA as the standard.

**DNA substrates and nuclease assay** - Oligonucleotides 1 – 7 and 11 used to make the DNA substrates have been described, as has X-12 (19). The fork substrate was made from oligonucleotides 2, 5, 11 and 14 (oligo 14 = 5’-ATCACTGGC ACTGGT AATTCGGC-3’), and regressed forks 1 and 2 were made from oligonucleotides 2, 5, 7 and 14, and 2, 5, 6 and 11 respectively. The details of DNA substrate preparation have been described (19). Nuclease reaction mixtures (20 µl) contained 0.5 nM labelled substrate DNA in 25 mM Tris-HCl (pH 8.0), 1 mM DTT, 100 µg/ml bovine serum albumin, 6% glycerol, 10 mM MgCl₂, and protein as indicated. Reactions were incubated at 30°C for 30 min, stopped by the addition of 5 µl of stop mix (2.5% SDS, 200 mM EDTA, 10 mg/ml proteinase K) followed by a further 15 min at 30°C, and analysed by electrophoresis through a 10% native polyacrylamide gel with phosphorimaging on a Fuji FLA3000.

**RESULTS**

*mus81, eme1, and rqh1 mutants are hypersensitive to camptothecin* - *mus81, eme1 and rqh1 mutants are hypersensitive to agents such as UV light, HU and methyl
methane sulfonate (MMS) that cause replication forks to stall (10,11,20,21). To see if they are also hypersensitive to replication fork collapse mus81, eme1 and rqh1 single mutant strains and a mus81 eme1 double mutant strain were tested for their sensitivity to the topoisomerase I (Top1) poison camptothecin (CPT) (Fig. 1). CPT stabilises Top1-DNA covalent intermediates by inhibiting the re-ligation step and in so doing leads to the accumulation of strand-breaks in the DNA (22). The toxic effect of CPT appears to be due to the collapse of replication forks at these strand-breaks (23-28). mus81 and eme1 mutant strains are extremely sensitive to CPT, whereas, rqh1- cells are sensitive but less so (Fig. 1 A). S. cerevisiae mus81/mms4 top1 and sgs1 top1 double mutants each grow slower than their respective single mutants (14) and therefore at least part of the CPT hypersensitivity of mus81/eme1 and rqh1 mutants might be attributable to an overlap in function with Top1. To investigate this possibility we constructed mus81- top1- and rqh1- top1- double mutant strains and compared them to their respective single mutant strains for viability in the presence and absence of CPT (Fig. 1 C). In the absence of CPT the mus81- top1- double mutant strain grew about as well as the mus81- single mutant strain, and the rqh1- top1- double mutant grew only slightly slower than the rqh1- single mutant strain. These data show that the hypersensitivity of mus81- and rqh1- to CPT is not due to an overlap in function with Top1. Instead the rescue of mus81- and rqh1- hypersensitivity to CPT by deleting top1 (Fig. 1 C) shows that Top1 is required to mediate the toxic effect
of CPT. These data indicate that Mus81-Eme1 and Rqh1 promote the repair of collapsed replication forks.

In *S. cerevisiae* the repair of collapsed replication forks is dependent on the *RAD52* epistasis group of proteins (29). A comparison of the *mus81* mutant with strains mutated for several *S. pombe* homologues of the *RAD52* epistasis group shows that it is more sensitive to CPT than *rad50, rhp51, rhp55* and *rad22A* mutant strains (Fig. 1 B). Interestingly, the only member of this group that is more sensitive than *mus81* is *rhp54*. These data emphasise the importance of Mus81 and Eme1 for the repair of collapsed replication forks.

*RusA does not suppress rqh1* hypersensitivity to CPT or ionizing radiation* - The hypersensitivity of an *rqh1* mutant to UV and HU can be partially suppressed by the *E. coli* RusA resolvase that is highly specific for cleaving HJs (6). For these experiments RusA was modified to include an N-terminal nuclear localisation sequence (NLS) that efficiently targets it to the nucleus of *S. pombe*, and a C-terminal green fluorescent protein (GFP) tag that provides a marker for expression and localisation. Expression of NLS-RusA-GFP was controlled from the thiamine-repressible *nmt1* promoter on a pREP1 plasmid (pREP1-rus). A pREP41 derivative (pREP41-rus) was also used that expressed the NLS-RusA-GFP from an attenuated *nmt1* promoter. Using these plasmids,
or strains containing the $P_{nmt}$-NLS-rusA-GFP cassette integrated into the chromosome, we tested whether the hypersensitivity of an $rqh1$ mutant to MMS and CPT is suppressed by RusA (Fig. 2 and data not shown). An approximate ten-fold improvement in survival in the presence of MMS is obtained when NLS-RusA-GFP is expressed from the chromosome or from a plasmid in the absence of thiamine similar to the suppression of UV and HU hypersensitivity observed previously (6). The expression of a nuclease-dead D70N mutant of RusA that retains wild-type levels of DNA binding failed to suppress MMS sensitivity confirming that suppression depends on the cleavage of HJs. In contrast, neither NLS-RusA-GFP nor NLS-RusAD70N-GFP suppresses the hypersensitivity of $rqh1$ to CPT (Fig. 2 and data not shown). Furthermore, the hypersensitivity of $rqh1$ to ionizing radiation is also not suppressed by NLS-RusA-GFP (data not shown). From these results we conclude that Rqh1 has a role in promoting the repair of collapsed replication forks and DSBs that cannot be substituted by an HJ resolvase.

**RusA can substitute Mus81-Eme1’s role in DNA repair -**

$emel$ mutant strains were tested for suppression of their hypersensitivities to UV, HU, MMS and CPT by RusA. In the case of the $mus81$ strain expression of NLS-RusA-GFP from either pREP1-rus or pREP41-rus suppresses its sensitivity to UV, HU, MMS and CPT about as well as plasmid-expressed Mus81 (pREP41-mus81) (Fig. 3). Interestingly
pREP1-rus provides better levels of suppression than pREP41-rus for HU, MMS and CPT sensitivities, but not for UV sensitivity. This may be due to a toxic effect of too much HJ resolution in the presence of UV damage. When the expression of NLS-RusA-GFP is repressed in the presence of thiamine the hypersensitivities of mus81- cells transformed with pREP41-rus and pREP41-mus81 are indistinguishable from those transformed with the empty pREP1 vector. However, some beneficial effect of pREP1-rus is still observed due to incomplete repression of the nmt1 promoter. To see if suppression depends on the resolution of HJs the nuclease-dead D70N mutant of RusA was used. Neither pREP1-rusD70N nor pREP41-rusD70N suppresses the DNA damage sensitivity of the mus81- strain consistent with the resolution of HJs being necessary for suppression. In fact a marked negative effect of pREP1-rusD70N was observed possibly due to high-levels of the mutant RusA protein binding to HJs and preventing their processing. Similar results to those shown in figure 3 were obtained with eme1 and mus81 eme1 mutant strains (data not shown). The ability of RusA to substitute for Mus81-Eme1 provides strong evidence that the essential function of these proteins is to resolve HJs or DNA junctions that would otherwise become HJs. Furthermore, the suppression of CPT hypersensitivity by RusA provides an interesting contrast to rqh1- cells where RusA fails to reduce CPT sensitivity. Seemingly Mus81-Eme1’s critical role
in repairing collapsed replication forks is to resolve HJs or DNA junctions that can mature into HJs.

*The inviability of a rqh1 mus81 double mutant is suppressed by RusA* - Mus81 is essential for viability in the absence of Rqh1 (10). We have found that the same is true for Eme1. To see if the essential defect here is an inability to process HJs or DNA junctions that can become HJs we attempted to rescue the inviability of *rqh1* - *mus81* - cells by the expression of RusA. *rqh1* - and *mus81* - strains carrying chromosomally integrated *Pnmt-NLS-rusA-GFP* were crossed in the absence of thiamine. Asci from the cross were then microdissected and the liberated spores grown in the absence of thiamine on nutrient agar plates. Once grown the colonies were replica plated onto media containing thiamine, allowed to grow and then replica plated again onto thiamine-containing media and also onto media with no thiamine. Using this strategy putative *rqh1* *mus81* double mutants that were viable when RusA was expressed but dead when it was repressed were identified (Fig. 4). The double mutant status of these strains was confirmed by the segregation of *rqh1* - and *mus81* - cells after back-crossing to a wild-type strain (data not shown). These data indicate that the essential defect in *rqh1* *mus81* - cells is an inability to process HJs or DNA junctions that can become HJs. The fact that RusA only suppresses *rqh1* - hypersensitivity to agents that cause fork stalling suggests that the lethal DNA
junctions in rqh1 mus81 cells arise at stalled forks as opposed to during the repair of collapsed forks.

**NLS-RusA-GFP is highly specific for cleaving HJs in vitro** - Our interpretation of the preceding data depends on the high specificity of RusA for cleaving HJs. RusA is a homodimer of 14 kDa subunits that binds to a range of DNA junctions, however, its nuclease activity is essentially restricted to HJs where cleavage occurs 5' of CC dinucleotides positioned at or close to the junction crossover point (30,31). This has been reinforced by recent work from Bolt and Lloyd who have shown that optimal nuclease activity by RusA depends on the interaction of both its subunits with symmetrically positioned cleavage sites ensuring that cleavage activity is directed to HJs with high specificity2. To further substantiate the contention that RusA is a valid probe for HJs we have compared the ability of purified NLS-RusA-GFP to cleave a synthetic HJ (X-12), a model replication fork, and two junctions that resemble replication forks that have regressed to expose either a 3' or 5' single-stranded tail (regressed forks 1 and 2 respectively). Each substrate is related by a common radiolabelled strand and contain potential CC dinucleotide cleavage sites for NLS-RusA-GFP (Fig. 5 B). As expected NLS-RusA-GFP cleaves X-12 very efficiently to generate nicked linear duplex products (Fig. 5 C, lanes b and c). In contrast, NLS-RusA-GFP failed to cleave the fork substrate
(lanes g and h) and generated only a very low level of cleavage products with both regressed forks 1 and 2 (lanes l and m, and q and r). These data are consistent with the high specificity of RusA for HJs.

**RusA cannot substitute for Rad2, Rad16, Swi10 or Exo1** - To further investigate the specificity of NLS-RusA-GFP we tested whether it could suppress the DNA damage sensitivity of *rad16, swi10, rad2* and *exo1* mutants. These genes all encode DNA structure-specific nucleases. Rad16, like Mus81, is a member of the XPF family of endonucleases, and forms a complex with Swi10 that makes the 5' incision during nucleotide excision repair and removes 3'-ended single-stranded flaps during the repair of DSBs by single-strand annealing (32,33). Rad2 is a 5'-FLAP endonuclease that functions both in DNA replication and repair (33,34). Exo1 is a member of the same nuclease family as Rad2 and shares some overlap in function with it (33,34). Expression of NLS-RusA-GFP from either pREP1-rus or pREP41-rus failed to suppress the various sensitivities of *rad16, swi10, rad2* and *exo1* mutants to UV, MMS or HU (data not shown). The failure of NLS-RusA-GFP to substitute for any of these nucleases supports the view that its activity *in vivo* is as specific as it is *in vitro*.
Recombinant Mus81-Eme1 cleaves model replication forks well but HJs very poorly in vitro - To analyse the substrate specificity of Mus81-Eme1 in vitro Mus81 and His-tagged Eme1 were co-expressed in E. coli and purified by affinity chromatography on a Ni-NTA resin column followed by gel filtration on a Superdex 200 column. Two bands of protein with the expected sizes of Mus81 and His-tagged Eme1 co-purified in an approximately 1:1 ratio through both columns (Fig. 5 A and data not shown). These were confirmed as Mus81 and Eme1 by western blot analysis and N-terminal amino acid sequencing (data not shown). The majority of the other protein bands detectable in figure 5 A lane b are proteolytic fragments of Mus81 and Eme1. Purified Mus81-Eme1 was tested for its ability to cleave various DNA substrates (Fig. 5 C and Fig. 6). Mus81-Eme1 cleaved the synthetic HJ X-12 very poorly generating low levels of nicked linear duplex product (Fig. 5 C lanes d and e, and Fig. 6 lanes d-f). Altering the concentration of MgCl₂ between 0.5 and 10 mM made no difference to the efficiency of this reaction (data not shown). In contrast to its poor cleavage of X-12 Mus81-Eme1 readily cleaved a model replication fork and regressed forks 1 and 2 to generate nicked linear duplex products (Fig. 5 C lanes i and j, n and o, and s and t, and Fig. 6 lanes h-l). No cleavage products were detected with these substrates using His-tagged Mus81 purified without Eme1 or His-tagged Eme1 purified without Mus81 (data not shown). These data show that
recombinant Mus81-Eme1 cleaves replication fork substrates with much higher efficiency than HJs.

**DISCUSSION**

*Rqh1 and Mus81-Eme1 provide alternative pathways for processing DNA junctions at stalled replication forks* - In both prokaryotes and eukaryotes DNA replication rarely proceeds unhindered. Nucleotide depletion and obstacles such as transcription complexes and lesions in the DNA template can impede the progression of the replisome (35). Stalled replication forks can be targets for recombination enzymes that generate D-loops and HJs between the newly formed sister chromatids. HJs can also form by the regression of the stalled fork where the nascent DNA strands anneal to each other (1-3). Fork reversal has been touted as serving a number of useful purposes, including providing room for the repair of DNA lesions, a mechanism for template switching and a substrate for enabling fork collapse and subsequent recombination-dependent restart of replication (6,35). This latter strategy appears to be a major pathway for restarting replication in *E. coli*, but its importance in eukaryotes is less clear.

Both *rqh1* and *mus81/eme1* cells are hypersensitive to UV light, HU and MMS that are known to cause replication forks to stall. Furthermore, mutations in *rqh1* and
mus81 impair the survival of DNA polymerase mutants (10,20). These data indicate that Rqh1 and Mus81-Eme1 help to promote the processing of stalled replication forks. The ability of a bacterial HJ resolvase to suppress the hypersensitivity of rqh1, mus81 and eme1 to UV, HU and MMS implicates both Rqh1 and Mus81-Eme1 in processing DNA junctions at stalled replication forks. Furthermore, the ability of RusA to rescue the synthetic lethality of a rqh1 mus81 double mutant indicates that Rqh1 and Mus81-Eme1 operate in overlapping pathways for processing these DNA junctions.

Based on the known in vitro properties of RecQ helicases we have proposed previously that Rqh1 catalyses the reverse branch migration of HJs to reset regressed replication forks (6). In doing this it protects the fork from collapse and averts the need for recombinational repair, which can make errors that generate genomic rearrangements. This appears to be a common function of RecQ helicases, e.g. Bloom’s syndrome cells show increased numbers of DSBs during replication and a high frequency of sister chromatid exchange consistent with an increased frequency of fork collapse (36,37).

Based on the detection of HJ resolvase activity in partially purified samples of Mus81-Eme1 from S. pombe cells, Boddy et al have proposed that Mus81-Eme1 cleaves HJs at stalled replication forks and in so doing collapses the fork to stimulate a recombination-dependent restart of replication (11). Our observation that RusA can efficiently substitute for Mus81-Eme1 in vegetative cells supports this idea. However,
recombinant Mus81-Eme1 produced in \textit{E. coli} cleaves HJs very poorly \textit{in vitro}, whereas replication fork substrates are cleaved very well (Fig. 5 C). The same observation has been made for Mus81-Mms4 from \textit{S. cerevisiae} (13). How can we reconcile these data? One possibility is that the hexahistidine N-terminal tag on Eme1 and Mms4, used to facilitate their purification from \textit{E. coli}, affects substrate specificity. However, at least in the case of Eme1 we have found that a similar N-terminal tag does not affect its ability to complement the DNA damage sensitivity of an \textit{eme1} mutant\textsuperscript{3}. A second possibility is that Mus81-Eme1 purified from \textit{E. coli} is missing a cofactor or post-translational modification that enables it to efficiently cleave HJs. Certainly, the Mus81-Eme1 sample used by Boddy \textit{et al} was relatively crude and therefore could contain a protein that activates Mus81-Eme1 HJ cleavage activity (11). In addition Mus81 interacts with the FHA1 domain of Cds1 and undergoes a Cds1-dependent phosphorylation following exposure to HU (10), which may be required to activate Mus81-Eme1 to cleave HJs. The third possibility is that the relative inability of recombinant Mus81-Eme1 to cleave an X-junction versus a fork substrate is a true reflection of its substrate specificity \textit{in vivo}. Currently we favour this idea, at least regarding Mus81-Eme1’s role in processing stalled replication forks. However, if Mus81-Eme1 does preferentially act on replication fork structures, as opposed to HJs, how can a bona fide HJ resolvase like RusA substitute for it \textit{in vivo}? One possibility is that, despite its high specificity for cleaving HJs \textit{in vitro},
RusA can cleave stalled replication forks in vivo. Alternatively, stalled forks that remain uncleaved in the absence of Mus81-Eme1 regress to form an HJ that can be processed by RusA. This would resemble the situation in E. coli where RusA’s ability to suppress the UV sensitivity of ruvABC mutants seems to depend on RecG converting stalled replication forks into HJs (3,38,39).

**Why does RusA suppress rqh1- phenotypes when Mus81-Eme1 is present?** - As pointed out recently by Haber and Heyer the fact that the addition of RusA can ameliorate rqh1- HU, MMS and UV hypersensitivity suggests that there may be a limiting amount of Mus81-Eme1 resolvase activity in these cells (40). However, we have been unable to suppress rqh1- sensitivity to HU, MMS and UV by overexpressing Mus81-Eme13. An alternative explanation is that if Mus81-Eme1 is an inefficient HJ resolvase in vivo then suppression of rqh1- by RusA could reflect processing of stalled replication forks that have regressed beyond the point at which Mus81-Eme1 can act effectively.

**A role for Mus81-Eme1 in the repair of collapsed replication forks** - CPT stabilises the covalent linkage between the active site tyrosine of Top1 and DNA (22). It is generally regarded that the toxic effect of CPT is mediated by the collision of replication forks with these covalent complexes, which causes the replication fork to collapse (23-28). In S. cerevisiae repair of these collapsed replication forks is dependent
on the *RAD52* epistasis group of proteins and Tdp1 that hydrolyses the bond between Top1 and DNA, especially when the Top1-DNA complex is exposed at the end of a DNA molecule (41). In *S. pombe* homologues of the *RAD52* epistasis group appear to play equally important roles in repairing collapsed replication forks judged by the hypersensitivity of their mutants to CPT (Fig. 1 B). The exquisite sensitivity of *mus81* and *eme1* mutants to CPT suggests that Mus81-Eme1 likewise plays an important role in the repair of collapsed replication forks. The ability of RusA to substitute for this role suggests that Mus81-Eme1 promotes the repair of collapsed replication forks by resolving HJs. Alternatively, Mus81-Eme1’s ability to cleave three-stranded junctions *in vitro* (11) indicates that it might be capable of resolving the three-stranded junction that is formed at one end of a D-loop. Such three-stranded junctions if left uncleaved can branch migrate to form HJs, which could explain why RusA can substitute for Mus81-Eme1 during the repair of collapsed replication forks.

**A role for Rqh1 in the repair of collapsed replication forks and DSBs** - The hypersensitivity of a *rqh1* mutant to CPT and γ-rays suggests that Rqh1 promotes the repair of collapsed replication forks and DSBs. However, its function here appears to be different from that at stalled replication forks since RusA has no ameliorating effect on the γ-ray and CPT hypersensitivity of *rqh1* cells. In *E. coli* the 3' → 5' DNA helicase activity of RecQ combined with the 5' → 3' single-strand exonuclease activity of RecJ
can substitute for RecBCD in the processing of a DNA double-strand end to generate a 3’
single-stranded tail onto which RecA can nucleate (42). It is possible that Rqh1 may
serve a similar function in S. pombe to help initiate the recombinational repair of DSBs
and collapsed replication forks. However, the recent observation that the hypersensitivity
of a sgs1' mutant to CPT can be rescued by a helicase-defective Sgs1 suggests an
alternative model (43). Both Sgs1 and Rqh1 interact with the type I topoisomerase Top3
and it appears that this association is critical for resistance to CPT at least in S. cerevisiae
(44, 45). Possibly Rqh1 promotes the repair of DSBs and collapsed replication forks by
targeting and/or activating Top3, which in turn could affect recombinational repair by
controlling DNA supercoiling and/or the interwinding of DNA strands in plectonemic
recombination junctions (46).

**Conclusion** – The identification of Mus81-Eme1 as an essential component of a
nuclear-acting HJ resolvase has provided considerable insight into its potential roles
during vegetative growth and meiosis (11). The data described in this paper establishes
that Mus81-Eme1 and Rqh1 provide alternative ways of processing DNA junctions at
stalled replication forks. In their absence the inability to deal with these junctions has
lethal consequences. The fact that the addition of a bacterial HJ resolvase restores
viability indicates that HJs must form which presumably impede the completion of DNA
replication and/or the segregation of sister chromatids at cell division. However, Mus81-
Eme1 may not wait for an HJ to form at a stalled replication fork before collapsing it as evidenced by the \textit{in vitro} activity of recombinant protein. Indeed, for Mus81-Eme1 to act as an effective HJ resolvase it would appear to require activation by another protein(s) or some kind of post-translational modification. Further studies will be required to establish what this activating factor is.

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**REFERENCES**


FOOTNOTES

1The abbreviations used are: HJ, Holliday junction; UV, ultraviolet light; HU, hydroxyurea; MMS, methylmethane sulfonate; CPT, camptothecin.

2Robert Lloyd (University of Nottingham, UK) personal communication.

3Our unpublished observations.

FIGURE LEGENDS

Fig. 1. A comparison of various strains for sensitivity to CPT. The strains used were (A) MCW149, MCW448, MCW682, MCW793, and MCW827; (B) MCW3, MCW121, MCW248, MCW296, MCW449, MCW642, and MCW744; (C) MCW449, MCW744, MCW825, MCW869, MCW907 and MCW910. Strains were cultured in YES prior to spotting onto YES containing CPT as indicated. Neat spots represent 10^5 cells plated.

Fig. 2. Effect of RusA on the MMS and CPT sensitivity of a rqh1− mutant. The strains used were MCW149, MCW708, MCW788 and MCW790. Strains were cultured in the presence or absence of thiamine, serially diluted and spotted onto appropriately
supplemented EMM containing thiamine, MMS and CPT as indicated. Neat spots represent $10^5$ cells plated.

**Fig. 3.** Effect of RusA on the UV, HU, MMS and CPT sensitivity of a *mus81* mutant strain. The strain used was MCW682 carrying plasmids as indicated. Strains were cultured in the presence or absence of thiamine, serially diluted and spotted onto appropriately supplemented EMM containing thiamine, HU, MMS and CPT as indicated. Neat spots represent $10^5$ cells plated.

**Fig. 4.** RusA rescues the synthetic lethality of a *mus81 rqh1* double mutant. The strains used were MCW708, MCW788, MCW836 and MCW838. The strains were streaked onto EMM, grown at 30°C for 3 days and then replica plated onto EMM containing thiamine. This replica was grown for 2 days at 30°C and then replica plated onto EMM and EMM plus thiamine. These plates were then photographed after 2 days at 30°C.

**Fig. 5.** Purification of recombinant Mus81-Eme1 and comparison of its nuclease activity with NLS-RusA-GFP. (A) SDS-PAGE analysis of purified Mus81-Eme1. Mus81-Eme1 (lane b) (0.3 µg) in storage buffer was mixed with SDS loading buffer and boiled prior to loading onto the gel. The gel was stained with Coomassie blue. (B) Schematic showing the core sequences of the fork, and regressed forks 1 and 2. The bold lines indicate the elements in common between each of the substrates and the asterisk indicates the position
of the radiolabel. The solid arrowheads indicate potential cleavage sites for RusA. Please note that the core sequences of X-12 are not shown. (C) Nuclease assay. Reactions contain DNA substrates, Mus81-Eme1 (‘+’ = 20 ng; ‘++’ = 40 ng) and NLS-RusA-GFP (‘+’ = 100 ng; ‘++’ = 200 ng) as indicated. A schematic of each substrate is shown with an asterisk to indicate the position of its radiolabel at the 5’ end of oligo 2. The shaded box on X-12 indicates the central core of homology in which the junction point is free to branch migrate. Reaction products are indicated on the side of the panel.

Fig. 6. Comparison of Mus81-Eme1 cleavage of X-12 and a fork substrate. Reactions contain DNA substrates and Mus81-Eme1 as indicated. A schematic of each substrate is shown with an asterisk to indicate the position of its radiolabel at the 5’ end of oligo 2. The shaded box on X-12 indicates the central core of homology in which the junction point is free to branch migrate. Reaction products are indicated on the side of the panel.
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