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MUS81-EME2 Promotes Replication Fork Restart

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SUMMARY

Replication forks frequently stall at regions of the genome that are difficult to replicate or contain lesions that cause replication blockage. An important mechanism for the restart of a stalled fork involves endonucleolytic cleavage that can lead to fork restoration and replication progression. Here, we show that the structure-selective endonuclease MUS81-EME2 is responsible for fork cleavage and restart in human cells. The MUS81-EME2 protein, whose actions are restricted to S phase, is also responsible for telomere maintenance in telomerase-negative ALT (Alternative Lengthening of Telomeres) cells. In contrast, the G2/M functions of MUS81, such as the cleavage of recombination intermediates and fragile site expression, are promoted by MUS81-EME1. These results define distinct and temporal roles for MUS81-EME1 and MUS81-EME2 in the maintenance of genome stability.

INTRODUCTION

The stability of our genome is dependent upon the efficient and faithful replication of DNA. However, replication forks frequently encounter obstacles to their passage, including DNA base lesions, DNA interstrand crosslinks, difficult-to-replicate sequences, transcription bubbles, or tightly bound proteins (Branzei and Foiani, 2010). It is therefore imperative that replication forks that stall at these structures become reestablished to ensure the transmission of equal copies of the genome to daughter cells. One mechanism for the restart of a stalled replication fork involves nucleolytic cleavage mediated by the MUS81 endonuclease (Hanada et al., 2007; Regairaz et al., 2011). By acting upon the stalled fork, MUS81 generates a DNA double-strand break (DSB) that can be repaired by homologous recombination, leading to the restoration of an active fork. Such break-induced replication processes play critical roles in maintaining genome stability in organisms ranging from bacteria to yeasts and humans (Cox et al., 2000; Llorente et al., 2008).

MUS81 is a member of the XPF/MUS81 family of structure-selective endonucleases (Ciccia et al., 2008). All enzymes belonging to the family form heterodimeric complexes composed of a catalytic and a noncatalytic subunit, and include XPF-ERCC1, FANCM-FAAP24, MUS81-EME1, and MUS81-EME2 (catalytic subunits are indicated first). Although MUS81 has two partners in human cells, EME1 and EME2 (Ciccia et al., 2003, 2007; Oğrünç and Sancar, 2003), the *S. cerevisiae* ortholog of MUS81 associates with a single partner, known as Mms4, to form the Mus81-Mms4 endonuclease (Interthal and Heyer, 2000; Kaliraman et al., 2001; Mullen et al., 2001). Mus81-Mms4 plays an important role in the resolution of homologous recombination intermediates in both mitotic and meiotic cells (Boddy et al., 2001; Matos et al., 2011; Szakal and Branzei, 2013). The Mms4 subunit is the target of cell-cycle regulation, as Cdk/Cdc5-mediated phosphorylation events activate Mus81-Mms4 at the G2/M transition in order to ensure that joint molecule resolution occurs prior to chromosome segregation (Gallo-Fernández et al., 2012; Matos et al., 2011, 2013; Schwartz et al., 2012; Szakal and Branzei, 2013). Importantly, the late cell-cycle activation of Mus81-Mms4 limits inappropriate actions of this nuclease on replication forks in S phase, as premature Cdk/Cdc5 activation has been shown to promote aberrant replication and elevated levels of crossover formation (Matos et al., 2013; Szakal and Branzei, 2013).

In human cells, the functions of MUS81 are more diverse and include replication fork restart (Fugger et al., 2013; Hanada et al., 2007), common fragile site (CFS) expression (Naim et al., 2013; Ying et al., 2013), the resolution of recombination intermediates (Castor et al., 2013; Chen et al., 2001; Wyatt et al., 2013), and telomere maintenance in telomerase-negative cells (Zeng et al., 2009). In this work, we explored the cellular role of the little-studied MUS81-EME2 protein because, until now, it has been assumed that all cellular functions of MUS81 are a consequence of the actions of MUS81-EME1. Biochemical studies indicate that MUS81-EME1 and MUS81-EME2 exhibit related biochemical properties, with both nucleases exhibiting a preference for 3′-flap/fork DNA structures (Pepe and West, 2014). We therefore set out to determine whether the cellular functions of MUS81 are dependent upon EME1 or EME2, whether the two proteins might act at different stages of the cell cycle, and the consequences of loss of MUS81-EME1 or MUS81-EME2 functionality.

RESULTS

Cell-Cycle-Dependent Association of MUS81 with EME2

First, we determined whether the association of MUS81 with EME1 or EME2 was cell-cycle dependent. GFP-FLAG-tagged MUS81 (Wyatt et al., 2013) was expressed at endogenous levels from is own promoter in synchronized HeLa cells carrying a bacterial artificial chromosome (BAC-MUS81$_{FLAP}$; Figure 1A). Cell-stage synchronization was achieved using a double thymidine

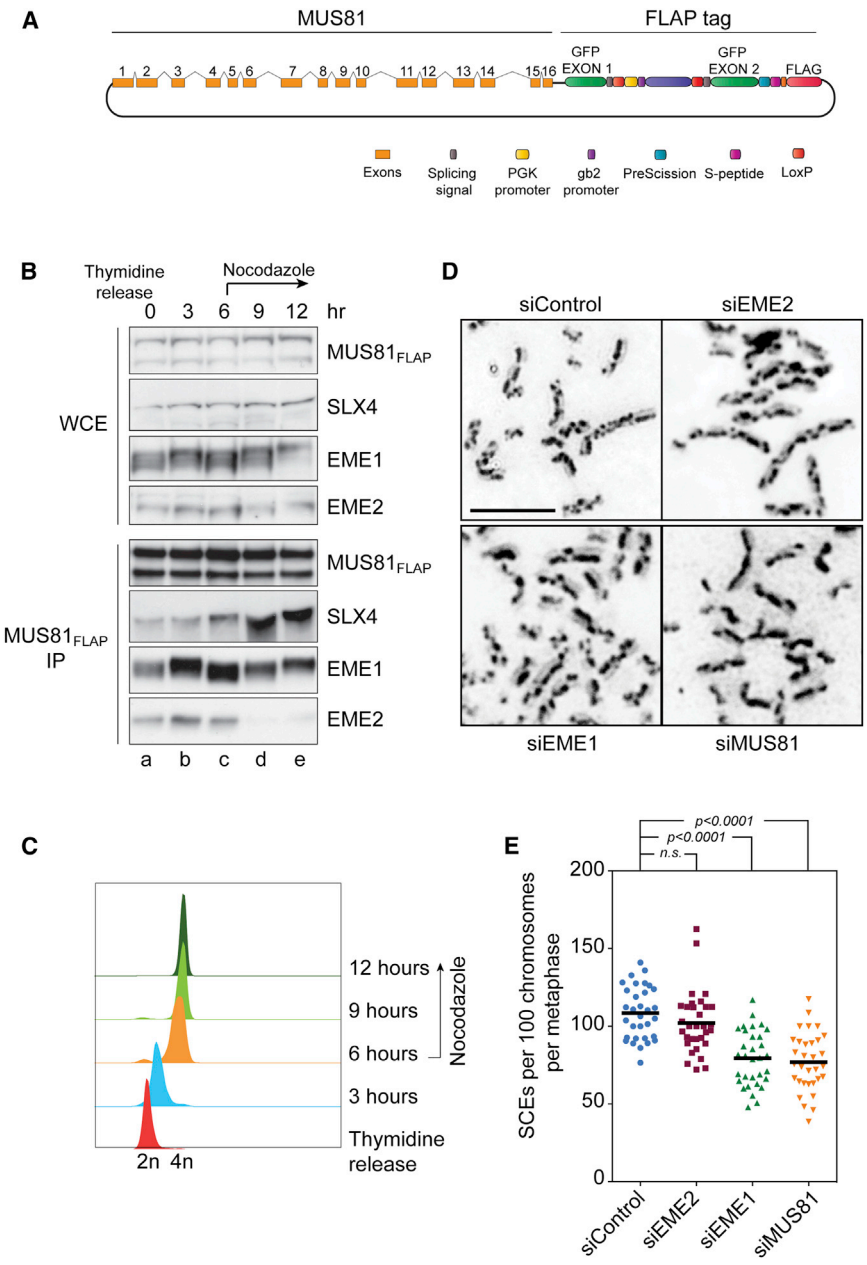

Figure 1. S Phase MUS81-EME2 Is Not Required for SCE Formation

(A) Schematic representation of BAC-MUS81$_{FLAP}$.

(B) HeLa Kyoto cells expressing MUS81$_{FLAP}$ were synchronized at G1/S using a double thymidine block. Six hours after release, the cycling cells were supplemented with nocodazole to promote G2/M phase arrest. Samples were collected at 0, 3, 6, 9, and 12 hr as indicated, and cell-free extracts were prepared. MUS81$_{FLAP}$ was immunoprecipitated from the extracts and the presence of each indicated protein was determined by western blotting.

(C) Cell-cycle progression of the cells used in (B) as determined by FACS analysis.

(D) Representative images of metaphase spreads prepared from BLM-deficient cells (GM08505) transfected with siRNAs against Luciferase (siControl), EME2, EME1, or MUS81. Scale bar, 10 μm.

(E) Quantification of SCE formation in cells treated with the indicated siRNAs, as shown in (D). For each condition, 32 metaphases were analyzed and each data point represents the number of SCEs per 100 chromosomes per metaphase spread; p values were determined using the two-tailed unpaired t test with Welch correction.

block, release into fresh media, and subsequent addition of nocodazole (Figure 1B). Analysis of cellular DNA content by fluorescence-activated cell sorting (FACS) showed that the majority of the cells were efficiently synchronized at G1/S by the thymidine block and proceeded through the cell cycle until prometaphase (Figure 1C). Samples were taken at 3 hr intervals, and MUS81$_{FLAP}$ was immunoprecipitated using anti-GFP beads and analyzed for its association with either EME1 or EME2. MUS81 associated with EME1 throughout the cell cycle (Figure 1B, lanes a–e) and, as reported previously (Wyatt et al., 2013), showed enhanced interaction with a second structure-selective endonuclease, SLX1-SLX4, at prometaphase (lanes d and e). This CDK/PLK1-driven M phase interaction of MUS81-EME1 with SLX1-SLX4 is necessary for formation of the SLX-MUS complex, which is required for Holliday junction resolution (Wyatt et al., 2013). Surprisingly, the association of MUS81 with EME2 was also cell-cycle-stage dependent, except that EME2 was specifically pulled down by MUS81 in S phase (Figure 1B, lanes b and c). EME2 was also detected in the MUS81 pull-down from cells arrested in G1/S (lane a), indicating that the MUS81-EME2 heterodimer begins to form prior to the initiation of DNA replication.

MUS81-EME2 Is Not Required for Holliday Junction Processing

The lack of association of MUS81 with EME2 late in the cell cycle indicates that MUS81-EME2, in contrast to MUS81-EME1, is unlikely to be involved in the resolution of recombination intermediates. To determine if this was the case, we analyzed the effect of small interfering RNA (siRNA)-mediated depletion of MUS81, EME1, or EME2 on the frequency of sister chromatid exchanges (SCEs) in cell lines derived from an individual with Bloom's syndrome (the high SCE frequency in these cells is due to elevated crossover formation arising from MUS81-mediated Holliday junction resolution) (Wechsler et al., 2011; Wyatt et al., 2013). Depletion of MUS81 or EME1, but not EME2, led to a significant reduction in the number of SCEs (Figures 1D and 1E). These results show that Holliday junction resolution, which occurs late in the

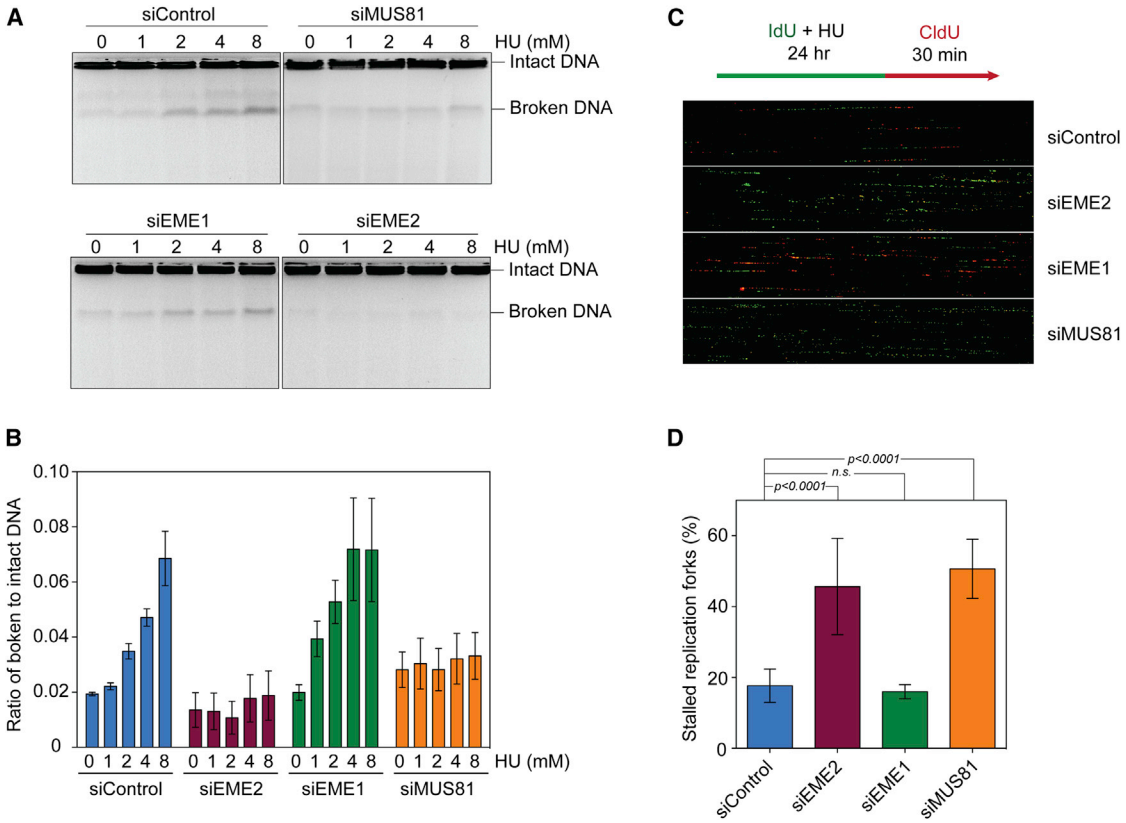

Figure 2. MUS81-EME2 Promotes the Breakage and Restart of Stalled Replication Forks

(A) HeLa cells depleted for MUS81, EME1, or EME2 were treated with the indicated concentrations of HU for 24 hr and the DNA was analyzed for breaks by PFGE.

(B) Quantification of HU-induced DSB formation, as determined in (A), expressed as the ratio of broken to intact DNA. Data are presented as a mean of three experiments (± SEM).

(C) HeLa cells transfected with the indicated siRNAs for 48 hr before addition of HU (2 mM for 24 hr) were analyzed by fiber analyses for replication fork progression. Representative images of iododeoxyuridine (IdU)-labeled (green) and chlorodeoxyuridine (CldU)-labeled (red) DNA fibers are shown.

(D) Quantification of the fiber analyses indicated in (C) ± SEM. Statistical significance was calculated using Fisher's exact test; 300 fibers were scored for each condition.

See also Figure S1.

cell cycle, is dependent upon MUS81-EME1 but does not require MUS81-EME2.

MUS81-EME2 Promotes Replication Fork Restart and Genome Stability

Previously, it was shown that MUS81 is required for DSB formation at stalled replication forks (Fugger et al., 2013; Hanada et al., 2007). The contribution of EME1 and EME2 to fork restart, however, has not been investigated and it has been assumed without supporting evidence that MUS81-EME1 promotes this critical cellular function. Using hydroxyurea (HU), which depletes the cellular pool of deoxynucleotide triphosphates (dNTPs) and causes replication fork stalling and subsequent MUS81-dependent DSB formation, we determined whether DSB formation was dependent on MUS81-EME1 or MUS81-EME2. Forty-eight hours after transfection of HeLa cells with siRNAs against MUS81, EME1, or EME2, the cells were treated with increasing concentrations of HU, and DSB formation was analyzed by pulsed-field gel electrophoresis (PFGE). Consistent with previous observations (Fugger et al., 2013; Hanada et al., 2007), depletion of MUS81 resulted in a significant decrease in DSB formation following treatment with HU (Figures 2A and 2B). However, DSB formation in EME1-depleted cells was comparable to that in control siRNA-transfected cells, whereas depletion of EME2 caused a decrease in DSB formation comparable to that obtained in MUS81-depleted cells. Similar results were obtained using a second, nonoverlapping EME2 siRNA (siEME2 #2; Figure S1A), or when replication forks were stalled using the interstrand crosslinking agent cisplatin (Figures S1B–S1D). Thus, MUS81-EME2, rather than MUS81-EME1, is required for the collapse of stalled RFs after prolonged treatment with either HU or cisplatin.

Next, we compared the ability of MUS81-, EME1-, or EME2-depleted cells to restart stalled replication forks using DNA combing techniques. Remarkably, we found that depletion of MUS81 or EME2, but not EME1, blocked replication fork restart after HU treatment (Figures 2C and 2D). Taken together, these results indicate that MUS81-EME2, rather than MUS81-EME1,

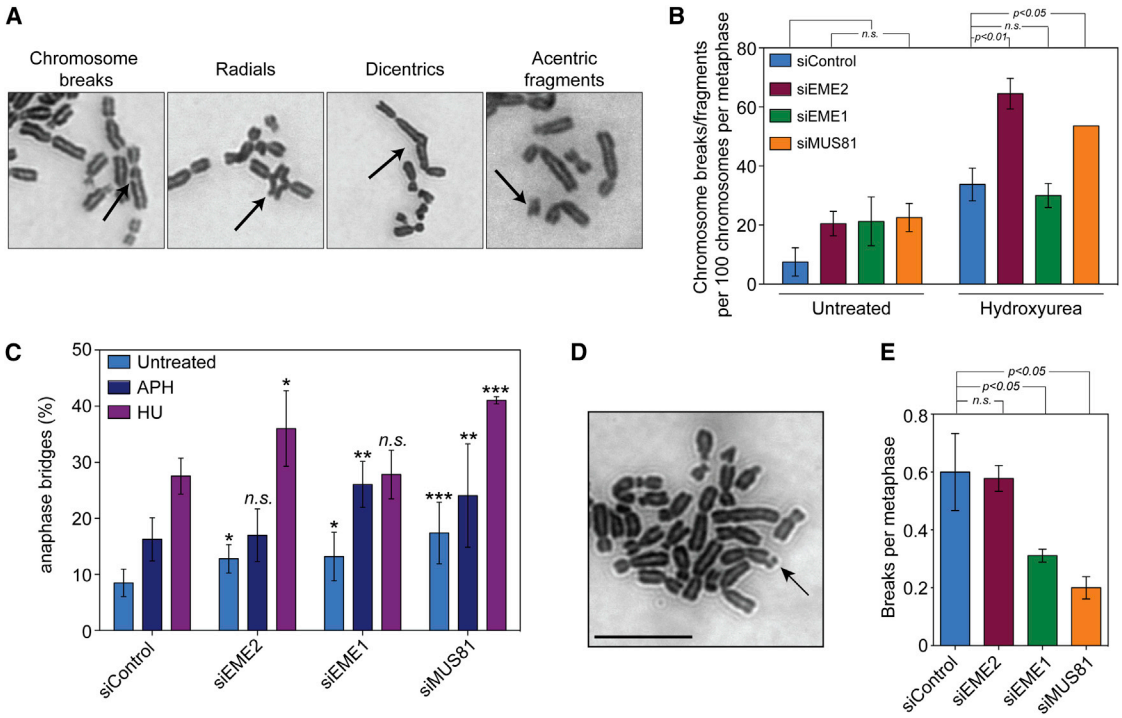

Figure 3. MUS81-EME2 Is Required for Genome Stability

(A) Representative images of chromosomal aberrations observed in metaphase spreads prepared from MUS81- or EME2-depleted RPE-1 hTERT cells following HU treatment. Images were categorized as breaks, radials, dicentrics, or acentrics, and quantified as described in (B) and Table S1.

(B) Quantification of the frequency of chromosomal breaks and fragments in siRNA-depleted RPE-1 hTERT cells with or without HU (1 mM, 24 hr) treatment. Data are presented as the mean of three experiments (± SEM). Statistical significance was calculated using Student's t test. 50 metaphase spreads were scored for each condition.

(C) Quantification of anaphase bridge formation in siRNA-depleted treated cells following treatment with either APH or HU (± SEM). Cells were transfected with the indicated siRNAs for 48 hr before treatment with APH (150 nM for 16 hr) or HU (1 mM for 24 hr). HU-treated cells were incubated in fresh media for 24 hr before staining. Statistical significance was calculated using Fisher's exact test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$); 300 cells were scored for each condition.

(D) Representative image of a metaphase spread of RPE1-hTERT cells transfected with control siRNA and treated with 150 nM APH for 16 hr. Scale bar, 10 μm. The arrow indicates a typical break as quantified in (E).

(E) Quantification of breaks in metaphase spreads in the indicated siRNA-depleted cells following treatment with 150 nM APH for 16 hr (± SEM). Statistical significance was calculated using Student's t test; 50 metaphases were scored for each condition.

is required for the processing and restart of stalled replication forks, consistent with the S phase specificity of MUS81-EME2 complex formation.

Defects in replication fork restart lead to an accumulation of underreplicated DNA that will affect chromosome segregation at mitosis, resulting in increased levels of chromosomal aberrations, such as breaks and fragments, and an increased level of DAPI-positive anaphase bridges. To determine the frequency of chromosomal aberrations after MUS81, EME1, or EME2 depletion, we prepared metaphase spreads from untransformed RPE-1 hTERT cells that were either treated with HU for 24 hr or left untreated. We found that untreated cells depleted for any of the three proteins showed a mild increase in chromosomal aberrations compared with the control (Figures 3A and 3B; Table S1). Treatment with HU, however, further increased the frequency of chromosome aberrations, and cells depleted for either MUS81 or EME2 showed a 2-fold increase in the number of aberrant chromosomes compared with control or EME1-depleted cells (Figure 3B; Table S1).

Next, we analyzed anaphase bridge formation following MUS81, EME1, or EME2 depletion, and found that loss of MUS81 or EME2 resulted in an increase in the number of cells that displayed segregation defects after HU treatment (Figure 3C). These results support the proposal that MUS81-EME2 is important for the maintenance of genome stability following replication fork stalling.

Nucleolytic Cleavage for CFS Expression

Recent studies have shown that MUS81 (and by inference MUS81-EME1) is important for CFS expression, a process by which difficult to replicate regions of the chromosome are broken in order to permit cell division (Naim et al., 2013; Ying et al., 2013). These regions are present on all human chromosomes and have been linked with neurological diseases and cancer predisposition (Durkin and Glover, 2007). To determine whether MUS81-EME1 is indeed responsible for CFS expression, which occurs in long genes that continue replication through the G2 phase of the cell cycle, we treated cells depleted for MUS81,

EME1, or EME2 with a low dose of aphidicolin (APH), which induces mild replication stress, and monitored the number of metaphase breaks. We found that metaphase spreads from MUS81- or EME1-depleted cells, but not those depleted for EME2, exhibited a significant reduction in the frequency of chromosomal breaks when compared with control cells (Figures 3D and 3E). We also determined the frequency of anaphase bridge formation in the APH-treated cells and observed that segregation defects were observed in the MUS81- and EME1-depleted cells, but not in those lacking EME2. These results indicate that MUS81-EME1, rather than MUS81-EME2, is required for CFS expression, consistent with the notion that late replication intermediates are processed at a cell-cycle stage during which the MUS81-EME2 complex fails to exist.

MUS81-EME2 Promotes Telomere Maintenance in ALT Cells

Finally, we determined whether MUS81-EME1 or MUS81-EME2 promotes telomere maintenance in telomerase-negative ALT (Alternative Lengthening of Telomeres) cells. Previously, it was shown that MUS81 localizes to APBs and that its depletion results in increased telomere loss and reduced telomere recombination (Zeng et al., 2009; Zeng and Yang, 2009). Since both EME1 and EME2 localize to APBs in ALT cells (data not shown), we determined the levels of telomere loss by quantitative fluorescent in situ hybridization (Q-FISH) analysis, and the frequency of telomere recombination by chromosome orientation FISH (CO-FISH), after depletion of MUS81, EME1, or EME2. Using ALT-positive U2OS cells, we found that depletion of MUS81, and to a greater extent EME2, resulted in an increased frequency of telomere-free chromosome ends as measured by Q-FISH (Figures 4A, 4B, and S2A). In contrast, the number of telomere-free ends in the EME1-depleted cells was comparable to the control depletion. Increased numbers of telomere-free ends were also observed in codepletions of MUS81 + EME2 or EME1 + EME2 (Figure 4B). However, when we measured telomere length by flow-FISH, we did not observe a significant difference between the control and MUS81-, EME1-, or EME2-depleted cells, indicating that loss of MUS81-EME2 functionality promotes telomere loss rather than gradual telomere shortening (Figure S2B). In contrast to the results observed with ALT-positive cells, which showed a significant increase in the number of telomere-free ends, Q-FISH analysis on metaphase spreads from telomerase-positive HT1080 cells transfected with control, MUS81, EME1, or EME2 siRNAs revealed no significant differences (Figure S3). Consistent with this difference between telomerase-positive and -negative cells, EME2 depletion caused a delay in the cell-cycle progression of ALT, but not telomerase-positive, cells (Figure S4).

We also investigated whether the loss of EME2 function affected the rate of telomeric SCEs (T-SCEs), a measure of telomeric recombination, in ALT cells. We found that depletion of MUS81 or EME2 from U2OS cells led to a significant decrease in the frequency of T-SCEs compared with control-transfected or EME1-depleted cells as measured by CO-FISH (Figures 4C and 4D). Taken together, these results define a role for MUS81-EME2, but not MUS81-EME1, in telomere maintenance in ALT-positive cells.

DISCUSSION

The work presented here shows that MUS81-EME1 and MUS81-EME2 promote distinct cellular functions, and that their actions are dependent on the cell-cycle stage. We find that MUS81-EME2 directs the S phase-specific processing of stalled replication forks to promote fork restart, and that its function is required for telomere maintenance in ALT cells. Depletion of MUS81 or EME2 led to an increased frequency of telomere-free ends in U2OS cells, whereas overall telomere length remained unchanged. These results indicate that a defect in MUS81-EME2 does not lead to gradual telomere loss; rather, the problem lies in telomere replication. Depletion of proteins involved in the repair of stalled replication forks, such as WRN, FEN1, FANCD2, and MUS81, leads to an elevated rate of telomere loss that is specifically observed in ALT cells (Crabbe et al., 2004; Fan et al., 2009; Saharia et al., 2010; Zeng and Yang, 2009). It is therefore possible that ALT cells are sensitive to defects in the repair of stalled forks and, because telomere replication is unidirectional and starts from the subtelomeric region, that the unrepaired forks would cause the loss of distal telomeric sequences.

Conversely, CFS expression and the resolution of recombination intermediates, two events that occur at prometaphase, require MUS81-EME1, but not MUS81-EME2. Most likely, this reflects the cell-cycle-stage-dependent activities of the two MUS81 complexes. Indeed, whereas EME1 interacts with MUS81 throughout the cell cycle, the interaction between MUS81 and EME2 occurs predominantly during S phase. How the formation of the MUS81-EME2 complex is prevented during G2 and M phase is presently unknown and will be the subject of future study. Interestingly, although the MUS81-EME1 complex exists throughout the cell cycle, the interaction with SLX1-SLX4, which is required for Holliday junction resolution and chromosome segregation, occurs predominantly during G2/M (Castor et al., 2013; Garner et al., 2013; Wyatt et al., 2013). Whether formation of the SLX-MUS complex is also required for CFS expression remains to be determined.

In simple organisms such as yeast, Mus81 has Mms4 as a single partner protein, and, in contrast to mammalian cells, there is currently no evidence of interactions between Mus81-Mms4 and Slx1-Slx4. The existence of two MUS81 complexes in mammalian cells presumably reflects the higher level of complexity that is required to coordinate and control the diverse range of cellular events mediated by MUS81. Our work defines distinct MUS81-EME2-specific events that occur in S phase, in contrast to MUS81-EME1's G2/M-specific functions, which occur following SLX1-SLX4 interaction or activation (Wyatt et al., 2013). This distinction holds promise for the development of pharmaceutical agents that can specifically target replication-specific functions of this critical protein.

EXPERIMENTAL PROCEDURES

All experimental procedures are described in detail in Supplemental Experimental Procedures.

Cell Lines and Analyses

The following cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum: telomerase-positive HeLa

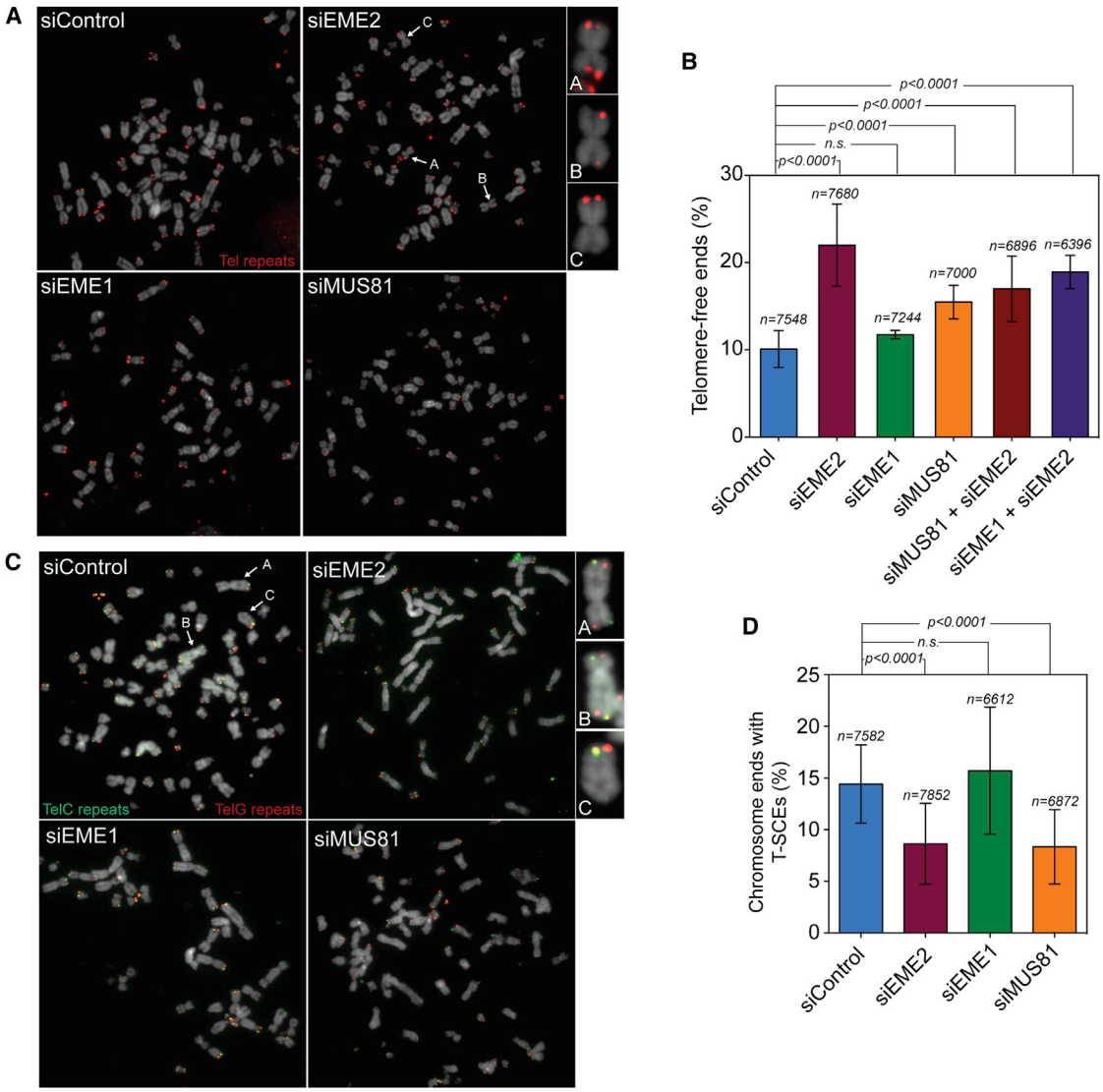

Figure 4. MUS81-EME2 Is Required for Telomere Maintenance in ALT Cells

(A) Representative Q-FISH images of metaphase spreads from U2OS cells depleted for the indicated proteins. Telomeric repeats are indicated in red. The arrows point to a normal chromosome (A) or to chromosomes with telomere-free ends (B and C).

(B) Quantification of telomere-free chromosome ends in the depleted cells (± SEM), as in (A).

(C) Representative CO-FISH images of metaphase spreads from U2OS cells depleted for the indicated proteins. T-SCEs were detected using telomeric G-strand (red) and C-strand (green) PNA probes. Arrows point to chromosomes without T-SCEs (A) and to chromosomes with telomeric exchange signals (yellow, B and C).

(D) Quantification of chromosome ends displaying T-SCEs in the depleted U2OS cells (± SEM), as in (C).

In (B) and (D), statistical significance was calculated using Pearson's chi-square test; n indicates the number of chromosome ends analyzed. See also Figures S2–S4.

Kyoto and HT1080, ALT-positive U2OS and GM847, the SV40-transformed Bloom's syndrome skin fibroblast line GM08505, and the TERT-immortalized retinal pigment epithelial cell line RPE1-hTERT. All cultures were grown at 37°C in a 10% CO_2 humidified incubator.

Proteins were depleted using siRNAs. One day before transfection, the cells were seeded in culture plates and transfected with EME1 (40 nM), EME2 (80 nM), EME2 #2 (80 nM), or MUS81 (60 nM) siRNAs using Lipofectamine RNAiMAX. Cells were transfected with MUS81 siRNA two times within 24 hr, whereas all other transfections were performed once, and cells were collected 72 hr after the first transfection. The control siRNA was Luciferase GL2.

The efficiency of protein depletion was monitored by western blotting (Figures S2C and S2D). Depletion of MUS81 led to a reduction in the levels of MUS81, EME1, and EME2, indicating that the stability of EME1 and EME2 is dependent on interaction with MUS81. Depletion of EME1 also partially depleted MUS81. Depletion of EME2 had no effect on the levels of either MUS81 or EME1.

PFGE and DNA combing were performed essentially as described previously (Hanada et al., 2007; Michalet et al., 1997). SCE analyses were carried out essentially as described previously (Bayani and Squire, 2005). Telomere loss was analyzed by Q-FISH (Blasco et al., 1997; Zijlmans et al., 1997) and

the frequency of telomere recombination was determined by CO-FISH after depletion of MUS81, EME1, or EME2 as described in Supplemental Experimental Procedures.

Immunoprecipitation

HeLa Kyoto cells expressing endogenous levels of $MUS81_{FLAP}$ (Matos et al., 2011) were synchronized by treatment with thymidine (2.5 mM thymidine) and nocodazole (100 ng/ml). MUS81 was immunoprecipitated from extracts using the GFP-tag and the pull-downs were analyzed by western blotting.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.04.007.

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