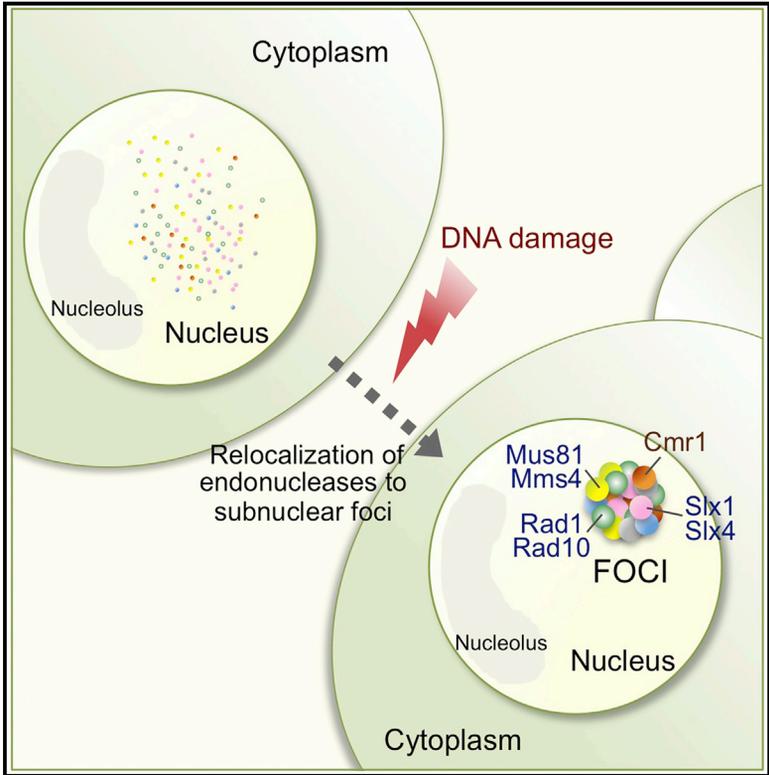


Subnuclear Relocalization of Structure-Specific Endonucleases in Response to DNA Damage

Graphical Abstract



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In Brief

Saugar et al. find that, under conditions of DNA damage, the structure-specific endonuclease Mus81-Mms4/EME1 relocalizes to subnuclear foci, where it colocalizes with the endonucleases Rad1-Rad10 and Slx1-Slx4. Relocalization takes place to a class of stress-induced foci defined by the Cmr1 protein and correlates with the function of the endonuclease.

Highlights

- The endonuclease Mus81-Mms4 relocalizes to subnuclear foci following DNA damage
- Mus81-Mms4 colocalizes to foci with the endonucleases Rad1-Rad10 and Slx1-Slx4
- The endonucleases relocalize to a class of DNA damage response foci defined by Cmr1
- Mus81-Mms4 relocalization to foci correlates with the function of the endonuclease



Subnuclear Relocalization of Structure-Specific Endonucleases in Response to DNA Damage

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SUMMARY

Structure-specific endonucleases contribute to the maintenance of genome integrity by cleaving DNA intermediates that need to be resolved for faithful DNA repair, replication, or recombination. Despite advances in the understanding of their function and regulation, it is less clear how these proteins respond to genotoxic stress. Here, we show that the structure-specific endonuclease Mus81-Mms4/EME1 relocalizes to subnuclear foci following DNA damage and colocalizes with the endonucleases Rad1-Rad10 (XPF-ERCC1) and Slx1-Slx4. Recruitment takes place into a class of stress foci defined by Cmr1/WDR76, a protein involved in preserving genome stability, and depends on the E2-ubiquitin-conjugating enzyme Rad6 and the E3-ubiquitin ligase Bre1. Foci dynamics show that, in the presence of DNA intermediates that need resolution by Mus81-Mms4, Mus81 foci persist until this endonuclease is activated by Mms4 phosphorylation. Our data suggest that subnuclear relocalization is relevant for the function of Mus81-Mms4 and, probably, of the endonucleases that colocalize with it.

INTRODUCTION

Structure-specific endonucleases play a crucial role in the maintenance of genome stability, as they are necessary to resolve a variety of secondary DNA intermediates that are generated during DNA replication, DNA repair, or DNA recombination (Dehé and Gaillard, 2017; Rass, 2013; Schwartz and Heyer, 2011). Their nucleolytic function ensures faithful completion of these processes, thereby contributing importantly to preserving genomic integrity in every cell cycle.

The activity of structure-specific endonucleases is highly regulated to guarantee the efficient resolution of their DNA substrates. At the same time, these proteins need to be tightly controlled to avoid the unscheduled cleavage of DNA intermediates, which could lead to genomic instability due to chromosomal rearrangements, high levels of chromatid exchanges, or faulty replication. These controls are achieved by different regulatory mechanisms that include posttranslational modifications and interactions with other factors that stimulate their activity (Cussiol et al., 2017; Dehé and Gaillard, 2017; Minocherhomji

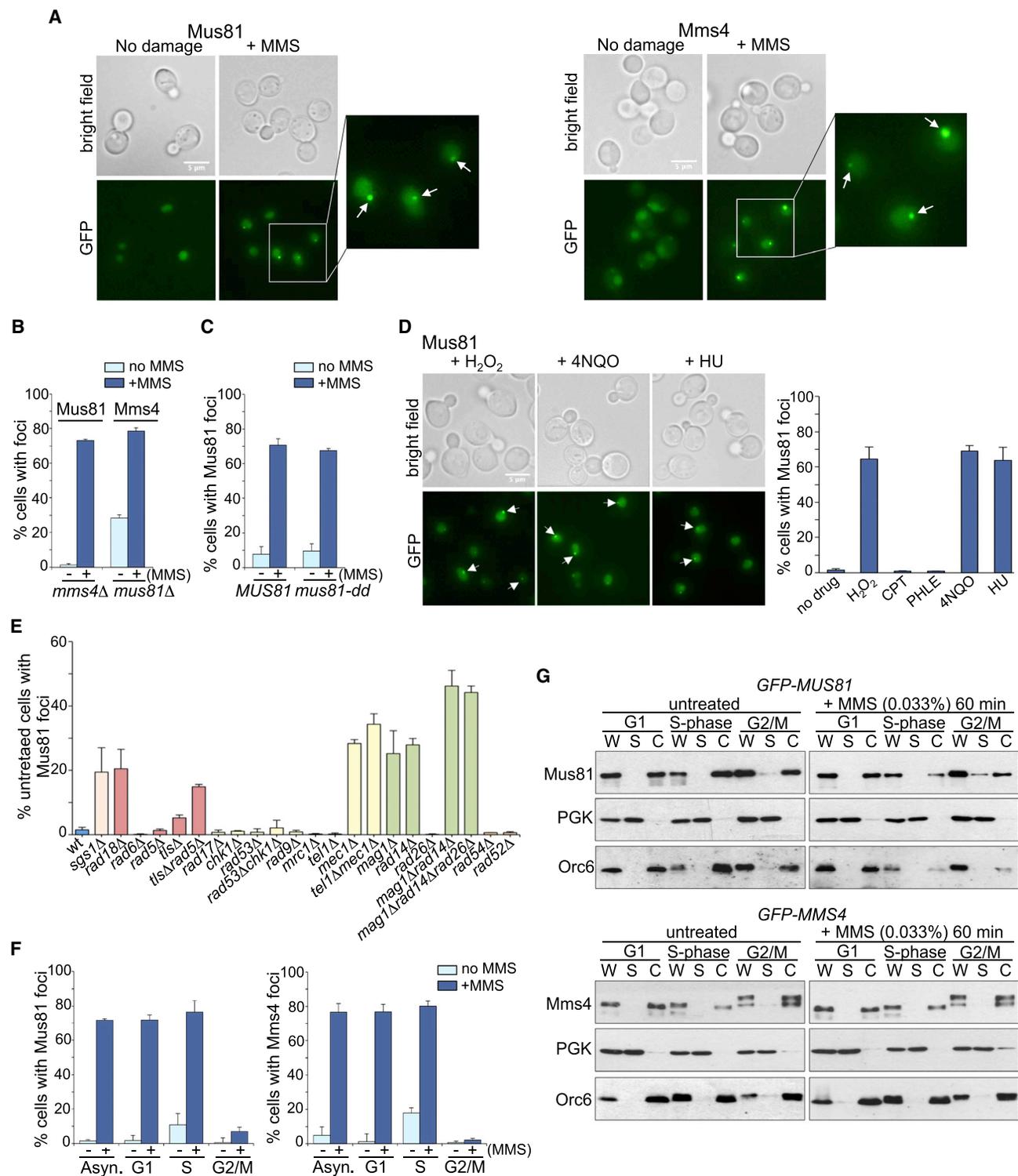
and Hickson, 2014; Sarangi and Zhao, 2015; West et al., 2015). For example, in budding yeast and human cells, the activity of the endonuclease Mus81-Mms4/EME1 is strictly regulated by cyclin-dependent kinase (CDK)- and polo-like kinase (PLK)-dependent phosphorylation of the non-catalytic subunit Mms4/EME1 (Gallo-Fernández et al., 2012; Matos et al., 2011; Saugar et al., 2013; Szakal and Branzei, 2013) and, in fission yeast, by CDK-dependent phosphorylation of Eme1 (Dehé et al., 2013). At least in budding yeast, Mms4 phosphorylation also depends on Cdc7-Dbf4 (Princz et al., 2017). This modification restricts the full activity of this endonuclease to the end of S-phase and mitosis.

The function of structure-specific endonucleases is important for cell survival under DNA damage conditions, which can be caused by endogenous and environmental agents, as well as by cancer therapeutic treatments. However, despite the role of these proteins in coping with DNA lesions, little is known about how they respond to the presence of DNA damage. Protein relocalization is a hallmark of the cellular response to DNA damage or replicative stress (Lisby et al., 2004; Tkach et al., 2012), but the potential regulation of structure-specific endonucleases by this mechanism remains poorly understood. Here, we show that Mus81-Mms4/EME1 and other endonucleases undergo cellular relocalization upon DNA damage. Mus81-Mms4 is recruited into subnuclear foci following genotoxic stress and colocalizes at these sites with the endonucleases Rad1-Rad10 and Slx1-Slx4. This relocalization could be important for the function of these proteins.

RESULTS

Mus81-Mms4 Relocalizes to Subnuclear Foci in Response to DNA Damage

To study the localization of different structure-specific endonucleases, we tagged the proteins with GFP, which yielded *Saccharomyces cerevisiae* strains that behaved similarly to parental controls (Figure S1A). We started analyzing Mus81-Mms4 and found that in untreated cells, the two subunits appeared diffused in the nucleus, whereas they formed subnuclear foci after treatment with the DNA-damaging agent methyl methanesulfonate (MMS) (Figures 1A and 1F). These foci localized to perinuclear regions and usually appeared as one dot per cell (Figure 1A). Mus81 and Mms4 accumulation into foci was not due to an increase in their levels after DNA damage (Figures S1B and S1C), thus reflecting relocalization. Of note, although Mus81 and Mms4 form a complex that is essential for the nuclease activity (Kaliraman et al., 2001), both subunits



(legend continued on next page)

relocalized to foci independently of each other (Figure 1B). This indicates that neither the formation nor the activity of the Mus81-Mms4 complex affect the relocalization of their subunits. Moreover, Mus81 recruitment into foci does not require the nuclease activity of this catalytic subunit, as a Mus81 nuclease-dead mutant relocalizes normally to foci after DNA damage (Figure 1C).

We then studied the formation of Mus81 foci following treatment with other drugs that cause DNA damage or replicative stress. A high percentage of cells showed Mus81 foci after exposure to H₂O₂, 4NQO, or hydroxyurea, but not to camptothecin or phleomycin (Figure 1D). Previously, it had been shown that Mus81 forms zeocin-induced foci (Sebesta et al., 2017). We also analyzed Mus81 foci formation in different DNA repair, DNA damage tolerance, or genome integrity checkpoint mutants. Cells lacking the RecQ helicase Sgs1 (BLM), which has overlapping functions with Mus81-Mms4 (Kaliraman et al., 2001), or the E3-ubiquitin ligase Rad18, involved in DNA damage tolerance, showed a significant increase in the percentage of cells containing Mus81 foci with respect to the wild-type control (Figure 1E). Likewise, a higher number of cells with Mus81 foci were detected in mutants lacking Mec1 (ATR), but not in those deficient in other checkpoint proteins. There was also an increase in the number of cells with Mus81 foci in mutants lacking nucleotide excision repair or base excision repair, but not homologous recombination (Figure 1E). Collectively, these results show that Mus81-Mms4 relocalizes to subnuclear foci in response to DNA damage or replicative stress and also in mutants that may accumulate unrepaired endogenous DNA lesions or unresolved DNA intermediates.

We next analyzed Mus81 and Mms4 foci along the cell cycle. Both proteins formed foci after induced DNA damage in G1- and S-phase cells, but not in G2/M cells (Figures 1F, S2A, and S2B). This was not a consequence of the nocodazole treatment used for G2/M synchronization, as cells blocked in G1 and then released into S-phase in medium containing this drug formed Mus81 and Mms4 foci upon DNA damage (Figure S2C). We also examined the chromatin association of this endonuclease throughout the cell cycle. As shown in Figure 1G, Mus81 and Mms4 were mostly bound to chromatin in G1-phase, S-phase, and G2/M-arrested cells, both under unperturbed conditions and in response to DNA damage. This result indicates that Mus81 and Mms4 are chromatin components and therefore that their relocalization to foci under DNA-damaging conditions occurs within the chromatin.

Structure-Specific Endonucleases Colocalize to Foci following DNA Damage

In metazoans, MUS81-EME1/Mms4 forms a multi-protein complex with other structure-specific endonucleases, such as XPF-ERCC1 (Rad1-Rad10 in *S. cerevisiae*) and SLX1-SLX4 (Fekairi et al., 2009; Muñoz et al., 2009; Svendsen et al., 2009). In budding yeast, Mus81-Mms4 can associate at least with Slx4 (Gritenaite et al., 2014). We therefore asked whether Rad1-Rad10 and Slx1-Slx4 behave similarly to Mus81-Mms4 in the response to DNA damage. The catalytic subunits Rad1 and Slx1 relocalized to subnuclear foci in a high percentage of cells after MMS treatment (Figures 2A and 2B), similar to Mus81. Likewise, Slx4, which is not only a subunit of Slx1-Slx4, but is also a platform for several proteins involved in genome integrity maintenance (Cussiol et al., 2017), formed foci upon exposure to MMS (Figures 2A and 2B), in agreement with previous data (Tkach et al., 2012). As for Mus81-Mms4, the accumulation of Rad1, Slx1, and Slx4 into foci was not due to increased protein levels upon DNA damage (Figures S1D–S1F), thus reflecting relocalization.

The observed foci were similar to those of Mus81-Mms4, and, remarkably, the analyzed endonucleases colocalized at these sites (Figures 2C and 2D). These results strongly suggest that Mus81-Mms4, Rad1-Rad10, and Slx1-Slx4 are recruited into the same foci through a common DNA-damage-induced signal. Nevertheless, despite their colocalization, they formed foci independently of each other, even in the absence of the Slx4 scaffold (Figure 2E).

Endonuclease Relocalization Is Rad6- and Bre1-Dependent and Occurs to a Class of DNA-Damage-Induced Foci Defined by Cmr1

To investigate the potential signal(s) for endonuclease recruitment into foci, we analyzed possible genetic dependencies for foci formation. We examined Mus81 foci after DNA damage in the mutants that did not accumulate this protein when cells were untreated (Figure 1E). We detected Mus81 foci in the absence of homologous recombination or checkpoint proteins after MMS treatment. By contrast, no Mus81 foci were found in cells lacking the E2-ubiquitin-conjugating enzyme Rad6 (human HR6A and HR6B) (Figure 3A). Rad6 participates in several processes in conjunction with different E3-ubiquitin ligases, Rad18, Ubr1, and Bre1 (Game and Chernikova, 2009), and we assessed the contribution of these to Mus81 foci formation (Figure 3B). Cells lacking the DNA damage tolerance protein Rad18, and cells lacking Ubr1, which together with Rad6 is necessary for protein degradation by the N-end rule pathway, showed Mus81

(D) Cycling *GFP-MUS81* (YSG39 strain) cells were treated for 60 min with 0.4 mM H₂O₂, 250 μM camptothecin, 50 μg/mL phleomycin, 0.5 μg/mL 4NQO, or 0.2 M hydroxyurea. The percentage of cells showing Mus81 foci is shown in each case. Arrows indicate selected foci.

(E) Percentage of cycling cells of different mutants containing spontaneous Mus81 foci. The strains are listed in Table S1.

(F) Percentage of cells containing Mus81 (YSG39 strain) or Mms4 (YSG42 strain) foci at different stages of the cell cycle. For G1 analysis, cells were synchronized using α factor and held for an additional 60 min in G1 in medium without or with 0.033% MMS. For S-phase, G1-blocked cells were released in medium either without (30 min) or with 0.033% MMS (60 min). For G2/M, cells were synchronized with nocodazole and held in G2/M for an additional 60 min in medium \pm MMS (0.033%).

(G) Chromatin-binding assays of Mus81 and Mms4. Whole cell extracts (W) from G1-, S-, or G2/M-phase cells were fractionated into a soluble fraction (S) and a chromatin-enriched fraction (C) and analyzed by immunoblot. Synchronization and MMS treatment were as in (F). Strains: *GFP-MUS81* (YSG39); *GFP-MMS4* (YSG42). PGK and Orc6 were used as controls for soluble and chromatin fractions, respectively.

In all cases, the histograms represent the mean \pm SD from three different experiments. See also Figures S1 and S2.

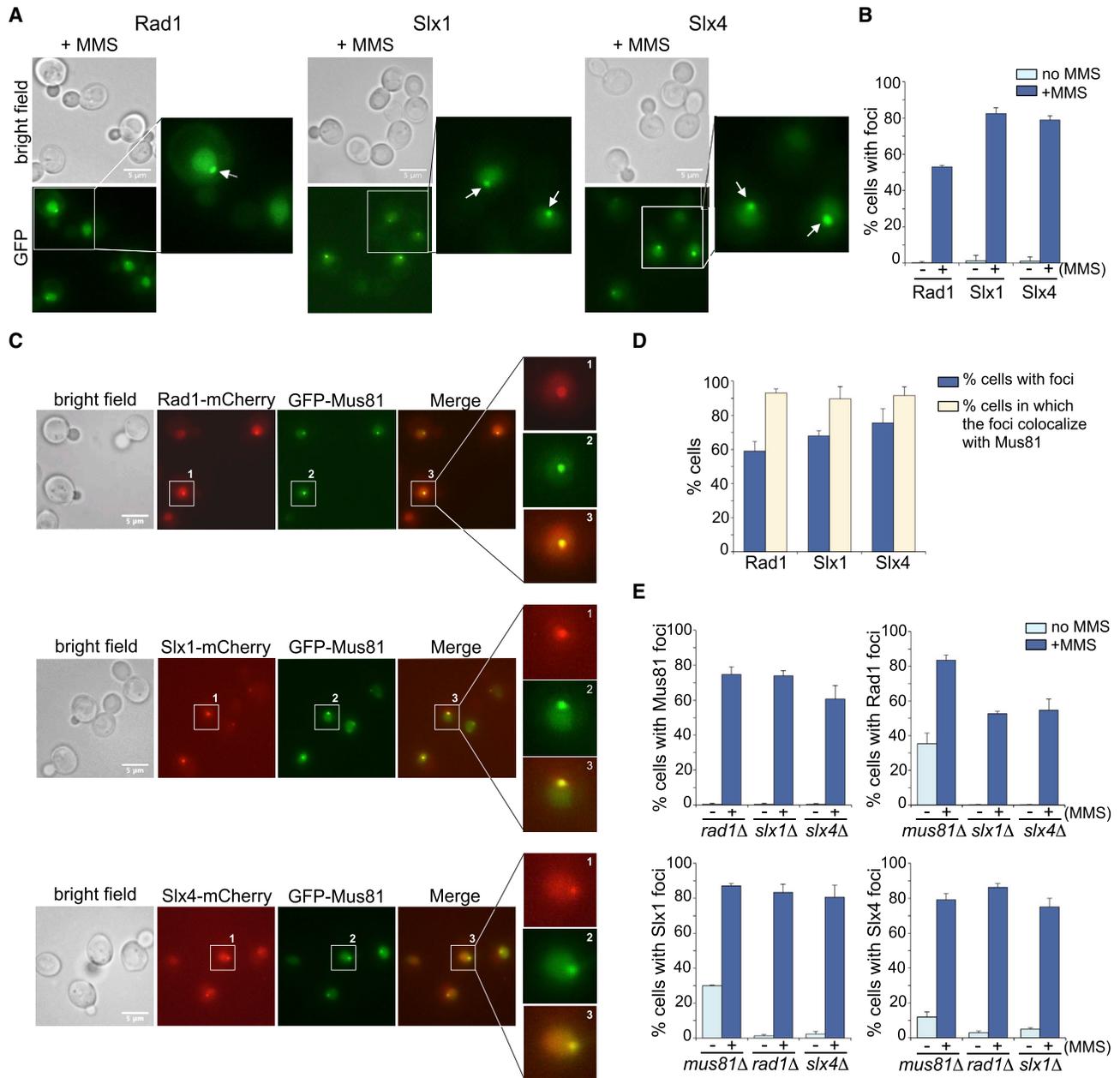


Figure 2. Rad1, Slx1, and Slx4 Relocalize to DNA-Damage-Induced Foci and Colocalize with Mus81

(A) Cycling *GFP-RAD1* (YSG271 strain), *GFP-SLX1* (YSG356 strain), and *GFP-SLX4* (YSG235 strain) cells were examined by fluorescence microscopy before and after treatment with 0.033% MMS for 60 min. For simplicity, only images of MMS-treated cells are shown. Arrows indicate selected foci.

(B) Percentage of cells containing foci of Rad1, Slx1, or Slx4, before and after treatment with 0.033% MMS for 60 min.

(C) Colocalization of the endonucleases at DNA-damage-induced foci. Cycling *RAD1-mChERRY GFP-MUS81* (YSG280 strain) (upper panel), *SLX1-mChERRY GFP-MUS81* (YSG363 strain) (middle panel), and *SLX4-mChERRY GFP-MUS81* (YSG274 strain) (bottom panel) cells were examined by fluorescence microscopy after treatment with 0.033% MMS for 60 min.

(D) Quantification of the data in (C).

(E) Cycling cells were examined by fluorescence microscopy before and after treatment with 0.033% MMS for 60 min. Histograms show the percentage of cells containing foci of every protein in the absence of the others. Strains: *GFP-MUS81 rad1Δ* (YSG299), *GFP-MUS81 slx1Δ* (YSG353), and *GFP-MUS81 slx4Δ* (YSG81) (upper panel, left); *GFP-RAD1 mus81Δ* (YSG307), *GFP-RAD1 slx1Δ* (YSG376), and *GFP-RAD1 slx4Δ* (YSG305) (upper panel, right); *GFP-SLX1 mus81Δ* (YSG372), *GFP-SLX1 rad1Δ* (YSG373), and *GFP-SLX1 slx4Δ* (YSG374) (bottom panel, left); and *GFP-SLX4 mus81Δ* (YSG304), *GFP-SLX4 rad1Δ* (YSG302), and *GFP-SLX4 slx1Δ* (YSG377) (bottom panel, right).

In all cases, the histograms represent the mean \pm SD from three different experiments. See also Figure S1.

foci after exposure to MMS. However, Mus81 foci formation was highly reduced in the absence of Bre1 (human RNF20 and RNF40) (Figure 3B), which in conjunction with Rad6 monoubiquitylates histone H2B (Hwang et al., 2003; Wood et al., 2003). However, Mus81 foci formation was not affected in cells lacking Dot1 or Set1, which function downstream of Bre1 to methylate histone H3 (Feng et al., 2002; Sun and Allis, 2002). The Rad6/Bre1 dependency for foci formation suggests that histone ubiquitylation might be required for Mus81 relocalization. Notably, the formation of Rad1, Slx1, and Slx4 foci was also Rad6 and Bre1 dependent (Figure 3C), reinforcing the idea of a common signal for their colocalization. Interestingly, although this signal for recruitment into foci is triggered by DNA damage and might involve histone modification, it was independent of H2A phosphorylation, as Mus81 relocalizes to foci in the non-phosphorylatable mutant *H2A-S129A* and in null mutants of its readers (*ttt107Δ* or *rad9Δ*) (Figure S3A).

To further investigate the nature of endonuclease foci, we analyzed whether they colocalize with any known nuclear structure. We did not find colocalization of Mus81 foci with DNA recombination/repair (represented by Rad52), DNA replication (Rfa1), nucleolus (Nop1), nuclear pore (Nup84), telomeres (Rap1), or spindle pole body (SPC42) proteins (Figures 3D and 3F). Remarkably, however, Mus81 foci colocalized with Cmr1/WDR76 (Figures 3E and 3F), a protein related to histones that is involved in genome stability maintenance and interacts with DNA repair, chromatin, and replication factors (Gallina et al., 2015; Tkach et al., 2012). Cmr1 has defined a class of DNA damage response foci to where different proteins relocalize, some of which are implicated in mitotic cell cycle, chromosome organization, or spindle organization (Gallina et al., 2015; Tkach et al., 2012). Supporting that these endonucleases are components of this type of foci, their relocalization was dependent on Btn2, a protein that directs Cmr1 to these sites and is likely to be structurally involved in their formation (Gallina et al., 2015) (Figure 3G). Nevertheless, the endonucleases were recruited into these foci independently of Cmr1 (Figure 3H). Importantly, the Rad6/Bre1 dependency for endonuclease relocalization could be a feature for other components of this class of foci, as Cmr1 recruitment into foci is reduced in the absence of these proteins (Figure 3I). Of note, although Cmr1-foci formation is dependent on the histone deacetylase Hos2 (Tkach et al., 2012), Mus81, Rad1, Slx1, and Slx4 formed DNA-damage-induced foci normally in cells lacking this protein (Figure S3B). These results indicate the existence of common and particular signals for protein recruitment into this type of foci.

Foci Dynamics Correlate with Endonuclease Function

We next investigated the biological significance of endonuclease relocalization. Like Cmr1, but different to other proteins that are recruited into these foci (Gallina et al., 2015), the nucleases did not relocalize to foci for degradation or as a consequence of misfolding, as they did not undergo changes in their levels in response to DNA damage and were functional (Figure S1). Moreover, the relocalization was not affected by the presence of the microtubule polymerization inhibitor nocodazole (Figure S2C), which is thought to be a requisite for a protein to be considered as misfolded (Gallina et al., 2015 and references therein).

One possibility was that the recruitment into foci could be related to the regulation of the nuclease activity. To test this idea, we focused on Mus81-Mms4, whose regulation has been extensively studied. This endonuclease is fully activated by phosphorylation of Mms4, which only occurs after bulk genome replication (Gallo-Fernández et al., 2012; Matos et al., 2011; Princz et al., 2017; Saugar et al., 2013; Szakal and Branzei, 2013) and is a mode of regulation that is not altered by DNA damage (Saugar et al., 2013). As we detected foci in G1- and S-phases, but not in G2/M, it was possible that Mus81-Mms4 was recruited into foci to help avoid Mms4 phosphorylation until G2/M. To investigate this hypothesis, we analyzed the phosphorylation of this protein during the cell cycle in *btn2Δ* cells, in which there is no foci formation, in the absence and in the presence of DNA damage. We found that Mms4 phosphorylation was prevented if cells did not reach G2/M, as it happened when *btn2Δ* and *BTN2⁺* control cells were exposed to MMS after release from G1 arrest (Figures S4A and S4B). Therefore, Mus81-Mms4 recruitment into foci following DNA damage is not required to avoid Mms4 phosphorylation until G2/M. Conversely, protein recruitment into foci is not a prerequisite for Mms4 phosphorylation, as *rad53Δbtn2Δ* cells, which do not form Mus81 foci, showed premature Mms4 phosphorylation in the presence of MMS with respect to control cells (Figures S4A and S4C), as a consequence of a defective checkpoint (Szakal and Branzei, 2013).

We then analyzed whether Mus81-Mms4 relocalization to foci could be related to the function of the endonuclease, rather than to the regulation of the complex by phosphorylation. We reasoned that, if this were the case, Mus81 foci should persist until Mus81-Mms4 can carry out its function under conditions in which some DNA structures need to be resolved by this endonuclease. To test this hypothesis, we evaluated the dynamics of Mus81 foci assembly/disassembly after treatment of cells in S-phase with MMS and subsequent removal of the drug (Figures 4A–4C). In control cells, Mus81 foci began to disappear soon after MMS removal (Figure 4B), indicating that the signal for protein recruitment into foci was no longer present. By contrast, in cells lacking the RecQ helicase Sgs1, which after MMS treatment accumulate DNA recombination intermediates that are resolved later by Mus81-Mms4 (Ashton et al., 2011; Szakal and Branzei, 2013), Mus81 foci persist until the complex is fully activated by Mms4 phosphorylation (Figures 4B and 4C). This suggests that, once activated, Mus81-Mms4 resolves the accumulated intermediates and the signaling for protein relocalization disappears. Consistent with these data, in the absence of Mms4, and therefore of nuclease activation, a high percentage of cells did not disassemble Mus81 foci or did so much later under similar experimental conditions (Figures 4D–4F), very likely because some DNA structures that needed resolution by the endonuclease persisted and there was signaling for Mus81 recruitment. In agreement with these results, Mus81 foci gradually disappeared under the same conditions, even before Mus81-Mms4 activation, in mutants such as *sgs1Δctf4Δ* and *sgs1Δrad51Δ* (Figures S4D and S4E), in which the accumulation of DNA recombination intermediates is greatly reduced (Branzei et al., 2008; Fumasoni et al., 2015; Liberi et al., 2005). Finally, supporting these results, Mus81 foci disappeared earlier in a

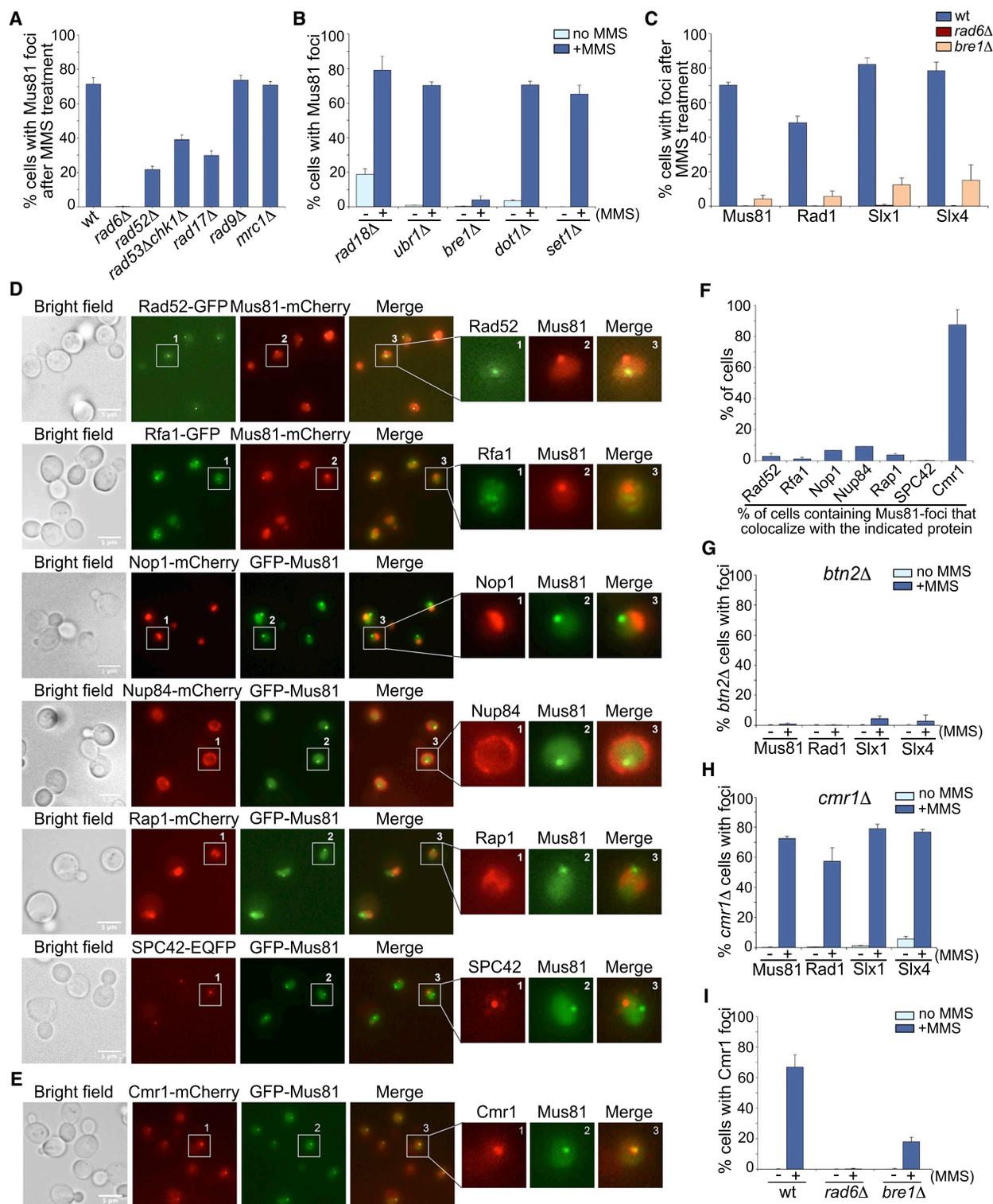


Figure 3. Endonuclease Relocalization Occurs to a Class of Foci Defined by Cmr1 and Depends on Rad6 and Bre1

(A) Percentage of cells containing Mus81 foci in different mutants after treatment of cycling cells with 0.033% MMS for 60 min. Strains: *GFP-MUS81* (wild-type, WT, YSG39), *GFP-MUS81 rad6Δ* (YSG215), *GFP-MUS81 rad52Δ* (YSG51), *GFP-MUS81 rad53Δ chk1Δ* (YSG55), *GFP-MUS81 rad17Δ* (YSG74), *GFP-MUS81 rad9Δ* (YSG54), and *GFP-MUS81 mrc1Δ* (YSG188).

(B) Percentage of cells containing Mus81 foci in different mutants before and after treatment of cycling cells with 0.033% MMS for 60 min. Strains: *GFP-MUS81 rad18Δ* (YSG96), *GFP-MUS81 ubr1Δ* (YSG232), *GFP-MUS81 bre1Δ* (YJT167), *GFP-MUS81 dot1Δ* (YJT168), and *GFP-MUS81 set1Δ* (YSG312).

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significant number of cells when Mms4 was prematurely phosphorylated due to ectopic expression of Cdc5 (PLK1) (Figures 4G–4I), most probably because there was anticipated resolution of accumulated DNA intermediates by this endonuclease, which stopped signaling for protein recruitment into foci. Altogether, our results indicate that Mus81 is recruited into foci following DNA damage, and that these foci persist until Mus81-Mms4 is fully activated and carries out its function in the resolution of accumulated DNA intermediates, which in turn eliminates the signaling for recruitment. These results correlate the localization of the Mus81-Mms4 complex at foci with its function, suggesting that subnuclear relocalization in response to DNA damage is important for the role of this endonuclease.

DISCUSSION

The data presented in this work have shown that the structure-specific endonucleases Mus81-Mms4, Rad1-Rad10, and Slx1-Slx4 relocalize to subnuclear foci in response to DNA damage, where all of them colocalize. Using complementary experiments, we have found a direct correlation between the persistence of Mus81 foci and the existence of DNA intermediates that need to be resolved by Mus81-Mms4. Foci dynamics has indicated that, if these intermediates are present: (1) Mus81 foci persist until Mus81-Mms4 is fully activated by Mms4 phosphorylation and can carry out its nucleolytic function; (2) Mus81 foci do not disappear or do so much later when Mus81-Mms4 is not activated; and (3) Mus81 foci disappear earlier when Mus81-Mms4 is prematurely activated. In addition, Mus81 foci do not remain (after eliminating the DNA-damaging agent) when there is no DNA intermediates accumulation, even in the absence of Mus81-Mms4 activation. These findings correlate the relocalization of Mus81-Mms4 with its function, strongly suggesting that shuttling through the DNA-damage-induced foci could be important for the role of this complex and, by extension, of the other endonucleases.

Our results have shown that recruitment into foci does not prevent the phosphorylation of Mms4 that leads to Mus81-Mms4 activation, and it is also not necessary for the modification of Mms4. We propose that the accumulation or sequestration of the endonucleases into foci following genotoxic stress could serve as a prepping mechanism that readies these proteins for

further action, probably facilitating their subsequent function if substrates needing nucleolytic resolution are formed. It is also reasonable to think that the colocalization of the endonucleases might favor the coordination among them and, in turn, their correct function when this is necessary. Importantly, endonuclease colocalization is likely a universal mechanism, as in human cells MUS81 colocalizes with ERCC1 after aphidicolin treatment (Naim et al., 2013; Ying et al., 2013), and SLX4 colocalizes with XPF-ERCC1 foci even in the absence of genotoxic stress (Wilson et al., 2013). Interestingly, although the endonucleases colocalize, they are recruited into foci independently of each other, suggesting that their interactions, as well as the formation of complexes and macro-complexes, might occur and perhaps be facilitated after relocalization to these sites.

Our results have also shown that, under DNA-damaging conditions, the endonucleases colocalize with Cmr1, which marks a class of DNA damage response foci (Tkach et al., 2012). This protein is involved in the maintenance of genome integrity and has been associated with histones, as well as with DNA repair, DNA replication, and checkpoint proteins (Gallina et al., 2015; Tkach et al., 2012). Recently, it has been proposed that Cmr1 defines a nuclear compartment called INQ that is established in response to genotoxic stress and is composed of a large number of proteins, including some involved in DNA metabolism and cell-cycle control (Gallina et al., 2015). The diversity of proteins found at these foci (Gallina et al., 2015; Tkach et al., 2012) indicates that multiple processes can be regulated by protein recruitment into these compartments. Here, we have identified structure-specific endonucleases as components of these foci and therefore new processes than can be controlled by relocalization to these sites. In turn, this finding opens up the possibility that still unexplored interactions exist between the endonucleases and other components of these foci. These data highlight the relevance of this type of foci as subnuclear sites that are important for diverse biological processes, some of which perhaps remain unidentified.

Notably, endonuclease recruitment into foci depends on Rad6 and Bre1. Due to the role of these proteins in histone modification (Hwang et al., 2003; Wood et al., 2003), these data suggest that the relocalization of the endonucleases after DNA damage may involve at least histone ubiquitylation. This potential modification might remodel the chromatin to allow the access of some proteins to DNA or to provide a permissive environment for these

(C) Percentage of cells containing Mus81-, Rad1-, Slx1-, or Slx4-foci in *rad6Δ* or *bre1Δ* mutants after treatment of cycling cells with 0.033% MMS. Strains: *GFP-MUS81* (WT, YSG39), *GFP-MUS81 rad6Δ* (YSG215), *GFP-MUS81 bre1Δ* (YJT167), *GFP-RAD1* (WT, YSG271), *GFP-RAD1 rad6Δ* (YSG287), *GFP-RAD1 bre1Δ* (YSG315), *GFP-SLX1* (WT, YSG356), *GFP-SLX1 rad6Δ* (YSG368), *GFP-SLX1 bre1Δ* (YSG370), *GFP-SLX4* (WT, YSG235), *GFP-SLX4 rad6Δ* (YSG246), and *GFP-SLX4 bre1Δ* (YSG264).

(D) Analysis of Mus81 foci nuclear localization. Exponentially growing cultures were examined by fluorescence microscopy after treatment with 0.033% MMS for 60 min. Strains: *RAD52-GFP MUS81-mCHERRY* (YSG283), *RFA1-GFP MUS81-mCHERRY* (YSG309), *NOP1-mCHERRY GFP-MUS81* (YSG276), *NUP84-mCHERRY GFP-MUS81* (YSG278), *RAP1-mCHERRY GFP-MUS81* (YSG326), and *SPC42-EQFP GFP-MUS81* (YSG308). Quantification is shown in (F).

(E) Colocalization of Mus81 and Cmr1 foci. Cycling *CMR1-mCHERRY GFP-MUS81* (YSG324 strain) cells were examined by fluorescence microscopy after treatment with 0.033% MMS for 60 min. Quantification is shown in (F).

(F) Quantification of the experiments in (D) and (E).

(G and H) Percentage of cells containing foci of each nuclease in cells lacking Btn2 (G) or Cmr1 (H), before and after treatment of exponentially growing cultures with 0.033% MMS for 60 min. Strains: *GFP-MUS81 btn2Δ* (YSG310), *GFP-RAD1 btn2Δ* (YSG322), and *GFP-SLX1 btn2Δ* (YSG369); *GFP-SLX4 btn2Δ* (YSG320); *GFP-MUS81 cmr1Δ* (YSG338), *GFP-RAD1 cmr1Δ* (YSG341), *GFP-SLX1 cmr1Δ* (YSG375), and *GFP-SLX4 cmr1Δ* (YSG340).

(I) Percentage of cells containing Cmr1 foci in *rad6Δ* and *bre1Δ* mutants after treatment of exponentially growing cultures with 0.033% MMS for 60 min. Strains: *CMR1-GFP* (WT, YSG342), *CMR1-GFP rad6Δ* (YSG344), and *CMR1-GFP bre1Δ* (YSG345).

In all cases, the histograms represent the mean \pm SD from three different experiments. See also Figure S3.

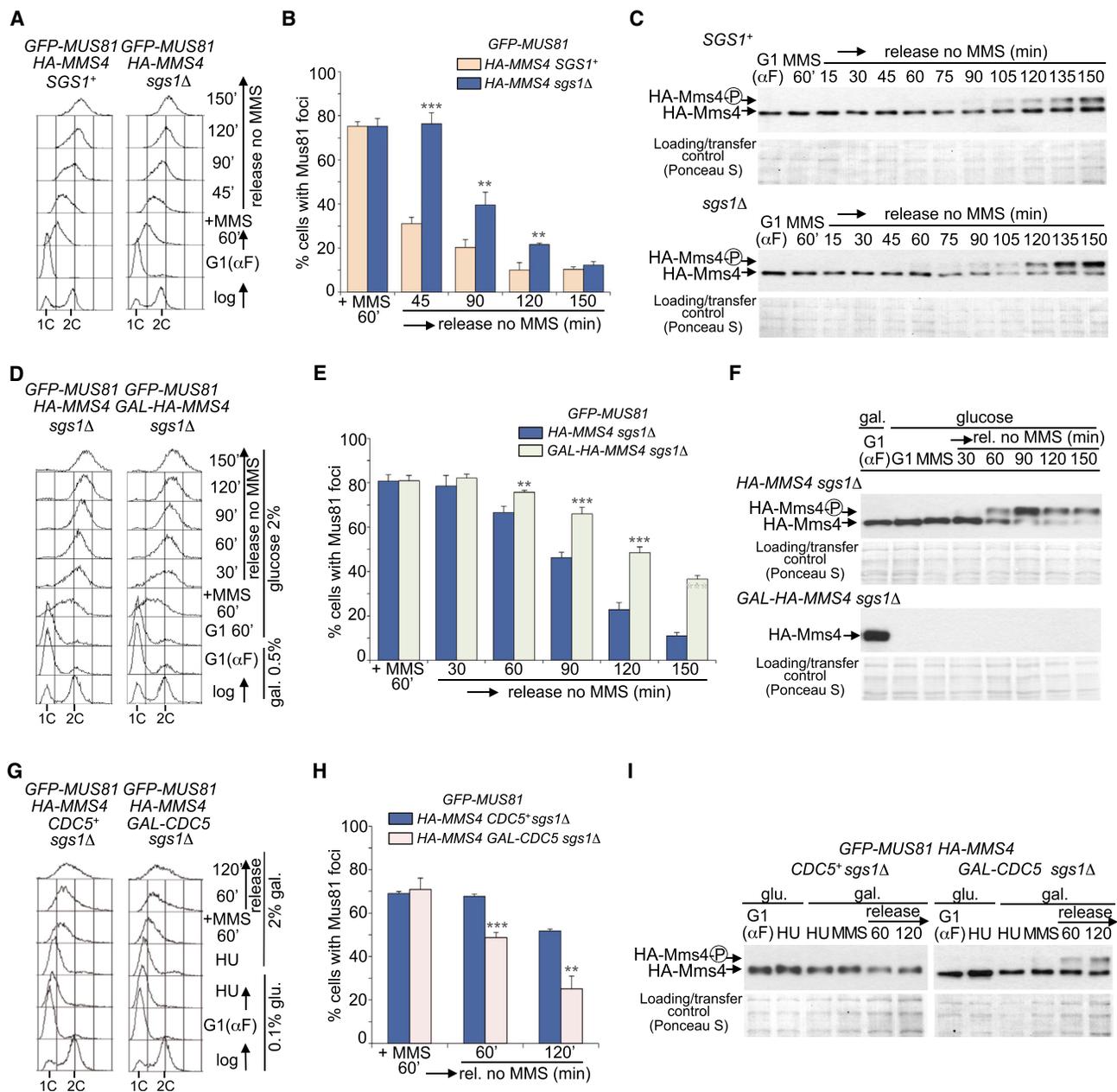


Figure 4. Foci Dynamics Correlate with Mus81-Mms4 Activity

(A–C) Cycling *GFP-MUS81 HA-MMS4 SGS1+* (YSG45 strain) and *GFP-MUS81 HA-MMS4 sgs1Δ* (YSG133 strain) cells were synchronized in G1 and released into S-phase in medium containing 0.033% MMS for 60 min. MMS was then removed to allow cell-cycle progression. (A) Cell-cycle progression was monitored by flow cytometry. (B) Percentage of cells containing Mus81 foci at every time point. (C) Immunoblot analysis of Mms4 during the course of the experiment.

(D–F) *GFP-MUS81 HA-MMS4 sgs1Δ* (YSG133 strain) and *GFP-MUS81 pGAL-HA-MMS4 sgs1Δ* (YSG99 strain) cells were synchronized in G1 in medium with 0.5% galactose. The cells were held in G1 for 60 min in medium with 2% glucose (to switch off the *GAL* promoter to allow Mms4 elimination) and then released in medium containing 0.033% MMS. The MMS was removed after 60 min to allow cell-cycle progression. (D) Cell-cycle progression was monitored by flow cytometry. (E) Percentage of cells containing Mus81 foci at every time point. (F) Immunoblot analysis of Mms4 during the course of the experiment.

(G–I) *GFP-MUS81 HA-MMS4 sgs1Δ CDC5+* (YSG133 strain) and *GFP-MUS81 HA-MMS4 sgs1Δ pGAL-CDC5* (YSG95 strain) cells were grown in medium with 0.1% glucose (*GAL* promoter OFF), synchronized in G1, and released in the presence of 0.2 M hydroxyurea (HU) for 60 min. The cultures were transferred to medium with 2% galactose (*GAL* promoter ON) for 60 min to induce Cdc5 expression. After this arrest in early S-phase, HU was removed and the cells were treated with 0.033% MMS for 60 min, after which, the drug was removed to allow cell-cycle progression.

(legend continued on next page)

proteins to accumulate when cells need to cope with DNA damage. Alternatively, or additionally, histone modification might allow the recruitment of some protein(s) that could be necessary for nuclease relocalization to foci.

Efficient regulation of structure-specific endonucleases is required to ensure resolution of certain DNA intermediates, as well as to prevent the potential genomic instability derived from their incorrect or untimely function. Subnuclear relocalization upon DNA damage should contribute to the important role of these proteins in the maintenance of genome integrity.

EXPERIMENTAL PROCEDURES

Yeast Strains

The yeast strains are listed in Table S1. Yeasts were routinely grown in YP medium with 2% glucose.

Cell-Cycle Synchronization, Flow Cytometry, and Chromatin-Binding Assays

Cells were synchronized in G1 with α factor (5–10 μ g/mL) and in G2/M with nocodazole (2 μ g/mL). Samples for flow cytometry were processed as described (Saugar et al., 2013) and analyzed using a FACSCalibur flow cytometer (BD Biosciences). Chromatin isolation was as described (Donovan et al., 1997).

Fluorescence Microscopy

For fluorescence microscopy analysis, cells were usually grown at 30°C in minimal medium supplemented with adenine (130 μ g/mL) and yeast synthetic drop out. The experiments with *rad6* Δ and *bre1* Δ strains were carried out at 25°C, due to the described temperature sensitivity of these mutants. Live cells were examined using a Zeiss Axiovert 200 fluorescence microscope with a 100 \times oil immersion objective (1.45 NA) and MetaMorph imaging software. For each field of cells captured, five fluorescent images were obtained at 1 μ m intervals along the z axis using a Hamamatsu charge-coupled device (CCD) camera. The images were processed and colored using ImageJ software and prepared for publication using Adobe Photoshop. The percentage of cells containing foci was calculated in each case after analyzing at least three independent experiments. Approximately 300 cells were counted in each experiment.

Protein Analysis

Protein extracts for immunoblot were prepared from trichloroacetic acid-treated cells, as described (Saugar et al., 2013). Haemagglutinin (HA)- and GFP-tagged proteins were detected with 12CA5 (Centro de Biología Molecular Severo Ochoa [CBMSO]) and anti-GFP (Roche) antibodies, respectively. The anti-Orc6 antibody (SB49) was a gift from Cancer Research UK. The anti-PGK antibody was from Invitrogen. The secondary antibody was horseradish peroxidase (HRP)-coupled anti-mouse (Vector Labs).

Statistical Analysis

Statistical analysis was performed using a two-tailed Student's t test. A p value of < 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2017.07.059>.

(G) Cell-cycle progression was monitored by flow cytometry.

(H) Percentage of cells containing Mus81 foci at every time point.

(I) Immunoblot analysis of Mms4 during the course of the experiment.

In all cases, the histograms represent the mean \pm SD from three different experiments. Data were analyzed using Student's t test. **p < 0.01 and ***p < 0.001. See also Figure S4.

AUTHOR CONTRIBUTIONS

I.S. and A.J.-M. performed the experiments. I.S. and J.A.T. designed the experiments and analyzed the data. J.A.T. supervised the project and wrote the manuscript.

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