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Mms1-Mms22 Complex Protects Genomic Integrity in *Schizosaccharomyces pombe*

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Abstract

Mms1 and Mms22 are subunits of an Rtt101-based E3 ubiquitin ligase required for replication of damaged DNA templates in *Saccharomyces cerevisiae*. The function and evolutionary conservation of this DNA repair module are unknown. Here we report the characterization of an Mms1 ortholog in *Schizosaccharomyces pombe*. Fission yeast Mms1 was discovered through its physical association with *S. pombe* Mms22 (also known as Mus7). Loss of *S. pombe* Mms1 results in the accumulation of spontaneous DNA damage, mitotic delay, and hypersensitivity to genotoxins such as camptothecin that perturb replisome progression. Homologous recombination repair proteins Rhp51 and Rad22 (Rad51 and Rad52 orthologs, respectively) are critical for survival in the absence of Mms1; however, there is no such requirement for Mus81-Eme1 Holliday junction resolvase that is essential for recovery from broken replication forks. Mms1 and Mms22 mutants share similar phenotypes and are genetically epistatic under unperturbed growth conditions and following exposure to genotoxins. From these data we conclude that an evolutionary conserved Mms1-Mms22 complex is required for replication of damaged DNA in fission yeast.

Keywords

Mms1; Mms22; Rtt101; DNA repair; genomic instability

1. Introduction

Eukaryotic genomes are endlessly threatened with damage from both normal cellular metabolism and exogenous genotoxic agents. To maintain genome integrity, multiple mechanisms have evolved to sense DNA damage, delay cell cycle progression through checkpoints, and carry out DNA repair. Many of the DNA repair mechanisms are relatively

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Conflict of Interest

The authors declare that there are no conflicts of interest.

well characterized, including the processes of homologous recombination (HR), non-homologous end joining (NHEJ), and nucleotide excision repair (NER). Other DNA repair mechanisms are much more poorly understood, an example being the Mms22-dependent process first identified in the budding yeast *Saccharomyces cerevisiae*. In both budding yeast and the fission yeast *Schizosaccharomyces pombe*, loss of Mms22 results in hypersensitivities to agents that result in DNA damage predominantly in S-phase [1-4]. This, coupled with synthetic genetic interactions with thermosensitive alleles of DNA replication factors, suggests that Mms22p functions to resolve replication intermediates or to prevent damage caused by blocked replication forks [2,4].

In *S. cerevisiae*, Mms22p has been linked, based on genetic and protein–protein interaction analyses, with a set of genes termed the Mms22 module [5,6]. This genetic cluster is postulated to function in the recovery from DNA replication-associated DNA damage and includes, as well as Mms22p, the histone H3 lysine-56 acetyltransferase Rtt109p, the histone chaperone Asf1p, Rtt107p^{Esc4p}, Rtt101p^{Cul8p} and Mms1p.

Rtt107p is a 6-BRCT-repeat protein involved in the resumption of DNA replication after damage [7], which associates with Mms22p [8,9]. Rtt107p interacts by yeast two-hybrid with a number of proteins involved in DNA repair and replication, including HR mediators and Tof1, a subunit of the replication-pausing complex [9]. Rtt107p also physically interacts with Slx4p, a component of the Slx1-Slx4 structure-specific nuclease, and Slx4-dependent phosphorylation of Rtt107p by Mec1p has been proposed to be critical for replication restart following alkylation damage [10]. Rtt107p binds chromatin at stalled replication forks, and binding of Rtt107p to chromatin in response to DNA damage is dependent on both Rtt109p and Rtt101p [11], suggesting it may act as a protein scaffold directly at stalled replication forks.

Rtt101p is a cullin protein that forms part of an E3 ubiquitin ligase complex, and is required for replication fork progression through DNA lesions and naturally occurring pause sites [12]. Following DNA damage, Rtt101p is recruited to chromatin, in a process that also requires the histone acetyltransferase Rtt109p and Rtt107p [11]. Mms22p interacts with Rtt101p [8], an association that is thought to be bridged by the DNA repair protein Mms1p in response to DNA damage [13]. Relatively little is known about Mms1p. Mutants of Mms1 are hypersensitive to genotoxic agents that perturb DNA replication [14,2] and display synthetic interactions with thermosensitive alleles of the DNA replication initiation protein Mcm10p [2]. Genetic epistasis between Mms22p and Mms1p suggests that the two proteins function together [2], and recently Mms1p was identified as a homologue of mammalian DDB1 [13], an adaptor subunit of a Cul4-RING E3 ubiquitin ligase complex, which is involved in the regulation of a number of cellular processes [15].

Together, Rtt101p and Mms1p are thought to be core components of an E3 ubiquitin ligase complex, which recruits specific adaptor proteins to perform different functions [13,16]. Current models predict that Mms22p is one such adaptor that following DNA damage is recruited to ubiquitinate a currently unidentified substrate, presumably involved in the DNA repair process.

Whilst high-throughput protein–protein interaction data suggest that both Rtt101p and Rtt107p interact with Mms22p [8], mutants of *RTT101* and *RTT107* display a synthetic genetic interaction [5], and cells lacking either *RTT101* or *MMS22*, but not *RTT107*, are defective in MMS-induced HR [17], suggesting that Rtt101p and Rtt107p may function in separate Mms22p-containing subcomplexes or that the function of the Rtt101p-ubiquitin ligase complex following replication fork stalling does not involve Rtt107p [5,13].

Whilst the importance of the Mms22 module in the maintenance of genome integrity is well established in budding yeast, relatively little is known in other organisms. Previous studies

identified and characterized mutants of fission yeast Mms22 [4,18], yet whether the protein functions with other Mms22-module members in *S. pombe* is unknown. To further define the function of *S. pombe* Mms22, we undertook a large-scale purification of the protein to attempt to uncover interacting proteins. Here we report the identification and initial characterization of the putative ortholog of budding yeast Mms1. Our studies show that *S. pombe* Mms1 functions with Mms22 to preserve genome integrity and cell viability in the presence of DNA replication-associated DNA damage.

2. Materials and Methods

2.1 Strains and genetic methods

Standard procedures and media for *S. pombe* genetics were used as previously described [19]. The entire open reading frame of *mms1*⁺ was replaced with the hygromycin B (HphMx6) or kanamycin (KanMx6) resistance markers as described [20]. TAP-tagged or 5FLAG-tagged Mms22 protein was generated via a PCR-based method, to introduce an AAGGG linker, the TAP or 5FLAG epitope and a KanMx6 marker gene at the C-terminus of the protein [20]. The same method was used to generate Mms1-TAP, without use of the AAGGG linker [20]. HA-Mms22 was expressed from the *pREP41-N-3HA* plasmid [21]. Strains are listed in Table 1.

2.2 Identification of Mms1 as an Mms22 Interacting Protein

Briefly, a 15L mid-log culture of cells expressing Mms22-TAP at the genomic locus were pelleted and resuspended into 30ml in IP-P-150 buffer (10 mM Tris pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, Complete Protease Inhibitor Cocktail Tablet) and snap frozen in liquid nitrogen. The frozen cells were lysed using a motorized mortar and pestle (Retsch, Newtown, PA) and resuspended into 50ml of Lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, 1 mM NaF, Complete Protease Inhibitors and 1 mM PMSF). Mms22-TAP was purified from the clarified lysate by incubation with 800 l (50% slurry) IgG sepharose beads (GE Healthcare) overnight. After washing, beads were resuspended in 1.5ml TEV cleavage buffer (10mM Tris-HCl pH 8.0, 150mM NaCl, 0.5mM EDTA) containing 15µl 0.1M DTT and 15µl TEV protease. Eluates were trichloroacetic acid (TCA)-precipitated at -20°C overnight, washed with acetone and air-dried for MudPIT analysis.

2.3 Multidimensional Protein Identification Technology (MudPIT) Analysis

Proteins associating with Mms22-TAP were identified by MudPIT analysis as previously described [22,23]. Briefly, the TCA-precipitated sample was resuspended in 8 M urea, reduced, alkylated, diluted to 2 M urea and digested with trypsin (Promega). The digested sample was loaded onto a biphasic reverse phase (Phenomenex), strong cation exchange (Whatman) column and connected to an analytical RP column (Phenomenex). MS analysis was performed on a linear trap quadrupole mass spectrometer (ThermoFisher Scientific) using a four-step MudPIT method. Spectra were searched with Sequest [24] against a *Schizosaccharomyces pombe* protein database and search results were filtered with DTASelect [25].

2.4 Immunoblotting and Immunoprecipitation

Whole-cell extracts were prepared from 50 ml cultures of exponentially growing cells. Briefly, cell pellets were lysed in IP-150 buffer (50 mM Tris pH 8, 150 mM NaCl, 2.5 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, 50 mM NaF, 2 mM PMSF, Complete Protease Inhibitors), in a FASTPREP® 24 (MP Biomedicals), according to manufacturers instructions. For TAP-tag protein precipitation, 5-10 mg of protein extracts were incubated with immunoglobulin G-Sepharose beads (GE Healthcare) for 3 hr at 4°C. Sepharose beads were collected and washed twice in IP-150 buffer, and once in IP-300 buffer (50 mM Tris pH 8, 300 mM NaCl, 2.5mM

EDTA, 10% glycerol, 0.5% Nonidet P-40, Complete Protease Inhibitors). Proteins were separated on a 10% SDS-PAGE gel, transferred to Immobilon membrane, and immunoblotted according to standard techniques. HA-tagged Mms22 was detected with anti-HA antibody (12CA5 at 1:2000; Covance), Mms22-5FLAG with the anti-FLAG M2 reagent (1:2000; Sigma-Aldrich), and Mms1-TAP was detected with Peroxidase-antiperoxidase (PAP) reagent (1:2000; Sigma-Aldrich).

2.5 Microscopy

All microscopy was conducted on live, mid-log phase cells. Cells were photographed using a Nikon Eclipse E800 microscope equipped with a Photometrics Quantix CCD camera. Rad22-YFP-expressing strains were cultured in yeast extract with supplements (YES) for at least 16 hr before foci quantification. At least 250 nuclei were scored in three independent experiments.

2.6 Survival assays

For chronic exposures, mid-log phase cultures were resuspended to 1×10^7 cells/ml and serially diluted fivefold. Dilutions were spotted onto YES agar plates or YES agar containing the indicated amounts of MMS, CPT, or HU. For acute exposures to IR, 1000 cells were plated onto triplicate YES agar plates and immediately irradiated with the indicated dose. Alternatively, YES plates were spotted with serial dilutions of cells, as for chronic drug exposure, and irradiated with the indicated dose of IR or UV. For survival of acute exposure to CPT, mid-log phase cells were cultured in YES media containing 30 μ M CPT for a maximum of 3 hours. Immediately at time 0 min, a volume containing approximately 1000 cells was removed, CPT was washed out, and the cells were plated onto YES agar plates in triplicate. At each subsequent time point, the same volume of culture was removed, and the cells washed and plated. For all survival assays, recovery was for 3-4 days at 30° unless otherwise stated.

3. Results

3.1. Identification of *S. pombe* Mms1

In an attempt to further define the function of *S. pombe* Mms22 in the maintenance of genomic stability, we used multidimensional protein identification technology (MudPIT) to identify proteins that co-precipitate with Mms22. MudPIT combines liquid chromatography, tandem mass spectrometry, and advanced database searching to identify proteins in complex mixtures [23]. Mms22 was TAP-tagged at the C-terminus of the endogenous protein, with an AAGGG linker sequence separating the Mms22 sequence and the TAP tag, to preserve functionality of the protein (Fig. S1). This MudPIT analysis yielded 19.3% coverage (46 peptides) of Mms22, and after subtracting proteins that were present in control samples, the most abundant candidate that specifically co-precipitated with Mms22-TAP was SPAC3H8.05c (19.3% coverage, 20 peptides) (Table 2). *SPAC3H8.05c*⁺ encodes a 1073 amino acid (M_w 120.6kDa) protein of unknown function that is conserved in fungi. It was recently identified as one of 229 mutants from a fission yeast gene deletion library that are sensitive to one or more DNA damaging agents [26]. We proceeded to further characterize this protein, which henceforth we term Mms1 based on a recent study that identified SPAC3H8.05c by bioinformatics analysis as a potential ortholog of *S. cerevisiae* Mms1p [13].

3.2. Mms1 interacts with Mms22 during an unperturbed cell cycle and following DNA damage

Mms1 was identified as a binding partner of TAP-tagged Mms22 by MudPIT analysis (Table 2). To confirm that *S. pombe* Mms22 and Mms1 interact *in vivo*, we performed the reciprocal experiment in cells which expressed Mms1 TAP-tagged at its endogenous locus, as well as ectopically expressed HA-Mms22. Mms22 was overexpressed from the pREP41-N-3HA plasmid to assist in visualization of the protein by immunoblotting, as we found endogenous

Mms22 to be expressed at very low levels (Fig. 1C). Both Mms1-TAP and the exogenous HA-Mms22 were deemed to be functional as the alleles rescued the slow-growth phenotypes and DNA damage sensitivities of *mms1Δ* and *mms22Δ* mutants respectively (Fig. S1). HA-Mms22 co-immunoprecipitated with Mms1-TAP in lysates prepared from asynchronously growing cultures (Fig. 1). To see if this interaction was maintained following DNA damage, cultures were exposed to the Topoisomerase-I (TopI)-inhibitor camptothecin (CPT), an agent that stabilizes TopI-DNA cleavage complexes, which can result in DSB formation when the replication fork collapses on encountering TopI-mediated nicks in the template DNA [27]. The interaction between Mms1-TAP and HA-Mms22 was preserved but not enhanced following exposure to CPT (Fig. 1A). A similar result was obtained following exposure to the alkylating agent MMS (Fig. 1B), and also between Mms1 and Mms22-5FLAG expressed from the endogenous promoter (Fig. 1C), suggesting the two proteins physically interact irrespective of the presence of DNA damage.

3.3. Mms1 mutants experience elevated spontaneous DNA damage

Deletion of *mms1*⁺ produced viable colonies, which in the absence of any genotoxic stress were relatively small in size when compared to isogenic wild type colonies. The *mms1Δ* mutants displayed a heterogeneous cell length, with many cells exhibiting an elongated phenotype (Fig. 2A), indicative of defects in either DNA replication or the repair of DNA breaks that arise spontaneously during DNA replication. To determine whether *mms1Δ* mutants display an elongated phenotype due to checkpoint activation and consequent cell cycle arrest, we deleted *mms1*⁺ in backgrounds defective for the Cds1 or Chk1 checkpoint kinases. Cds1 is the effector kinase of the fission yeast DNA replication checkpoint, which is activated by the stalling of DNA replication forks, whereas Chk1 enforces the G₂-M checkpoint that is activated by damaged DNA [28]. The *mms1Δchk1Δ*, *mms1Δcds1Δ*, and *mms1Δchk1Δcds1Δ* mutants were viable suggesting that absence of Mms1 does not require intact checkpoint pathways for survival (Fig. 2B). Whilst there were no obvious genetic interactions between *mms1Δ* and *chk1Δ*, the double *mms1Δcds1Δ* showed an enhanced sensitivity to chronic low doses of HU (Fig. 2B). Moreover, loss of Mms1 was detrimental to the growth of cells defective in both Cds1 and Chk1 in response to UV, IR and CPT (Fig. 2B), suggesting that Mms1 has checkpoint-independent functions, at least in the tolerance of these DNA-damaging agents.

Deletion of *cds1*⁺ had no effect on the morphology of *mms1Δ* mutants, whereas *chk1*⁺ disruption significantly reduced the length of *mms1Δ* cells (Fig. 2A), indicating that the elongated phenotype of *mms1Δ* mutants is due to accumulation of the cells in G₂ via activation of the Chk1-dependent DNA damage checkpoint. This result is consistent with previous studies of *mms22Δ* mutants [4]. The relative slow growth of *mms1Δ* mutants was also attributed to their lowered viability, in which they displayed a reduced plating efficiency of 57.2±7.2%, compared to 92.1±4.1% for wild type.

To confirm that the activated DNA-damage checkpoint and reduced viability of *mms1Δ* mutants arose due to the occurrence of elevated DNA damage, we scored the number of nuclei exhibiting spontaneous Rad22^{Rad52} foci, as a marker of DNA breaks [29]. Approximately 45% of *mms1Δ* nuclei contained one or more spontaneous Rad22-YFP foci, compared to only 10% in wild type strains, a level of spontaneous DNA damage similar to what we had previously observed for *mms22Δ* mutants (Fig. 3, [4]). Moreover, deletion of Mms22 in the absence of Mms1 function did not exacerbate the amount of DNA damage, as judged by Rad22 foci number (Fig. 3).

3.4. *mms1Δ* and *mms22Δ* mutants display identical phenotypes

In budding yeast, both *mms22Δ* and *mms1Δ* mutants are hypersensitive to a variety of S-phase-specific DNA damaging agents, but strikingly are resistant to ionizing radiation (IR) [1,2,3,

14,17,30], a phenotype which is shared by *S. pombe mms22Δ* mutants [4,18]. As mentioned above, a recent screen identified SPAC3H8.05c/Mms1 amongst a large collection of mutants that display sensitivity to agents that impede DNA replication [26]. In agreement with this, we found that *S. pombe mms1Δ* strains were hypersensitive to CPT, as well as ribonucleotide reductase inhibitor hydroxyurea (HU), which arrests replisome progression by depleting the cellular pool of dNTPs, and methyl methanesulfonate (MMS), which stalls replication fork progression by alkylating DNA template bases (Fig. 4A).

To address whether *mms1Δ* strains were also sensitive to acute exposure of radiation, we assessed the survival of the mutants following irradiation with UV and IR. UV irradiation induces the formation of cyclobutane–pyrimidine dimers (CPDs), which can result in DNA transcription and DNA replication arrest [31]. We observed sensitivity of *mms1Δ* mutants to high doses of UV (Fig. 4A). Whilst IR generates a number of lesions, including single-stranded DNA breaks, double-stranded DNA breaks (DSBs), base damage and base modifications, its toxicity arises mainly from DSBs [32]. We found that, in contrast to HR mutants that are profoundly sensitive to IR, such as those disrupted for *rhp51^{rad51}*, *mms1Δ* mutants were not hypersensitive to IR (Fig. 4A). Furthermore, deletion of *mms22⁺* in the *mms1Δ* background did not confer sensitivity to IR (Fig. 4A) and the levels of sensitivity of *mms1Δ* mutants were comparable to *S. pombe mms22Δ* mutants [4]. These shared phenotypes could indicate that *S. pombe* Mms1 and Mms22 function together within a common pathway. To address this question genetically, we constructed double *mms1Δmms22Δ* deletion strains and compared the phenotype of these strains to the parental *mms1Δ* and *mms22Δ* mutants. In terms of both unperturbed growth and also in response to all DNA damaging agents tested, the double mutants shared the same phenotype as the single parental strains (Fig. 4B). Moreover, the single *mms1Δ* and *mms22Δ* were very similar in terms of their growth and DNA-damage sensitivity, suggesting that the two proteins function together within a common pathway.

3.5 Mms1 and Mms22 mutants share genetic interactions

The elevated numbers of Rad22 foci in *mms22Δ* mutants, coupled with the severe genetic interactions observed between Mms22 and most HR mutants, led us previously to propose that in the absence of Mms22, cells experience an elevated occurrence of spontaneous replication-associated DNA damage that is repaired by HR [4]. In order to further determine whether loss of Mms1 creates a requirement for HR repair to maintain viability, we performed genetic crosses between *mms1Δ* mutants and HR mutants defective for Rad22^{Rad52} or Rhp51^{Rad51}. Similarly, any role for Mms1 in DNA replication fork recapture and replication restart was investigated by disruption of the DNA-processing enzymes Mus81 or Rqh1 in an *mms1Δ* background.

As we observed for Mms22, *mms1Δrad22* or *mms1Δrhp51Δ* double mutants showed a severe synergistic phenotype, with a low number of double mutant colonies arising in tetrad dissections (Fig. 5A). Likewise, deletion of the RecQ helicase Rqh1 in the absence of Mms1 function resulted in a severe synthetic growth defect (Fig. 5A). Deletion of *mms1* in the *mus81Δ* background did not result in a strong synthetic interaction, which was similar to our observations made for Mms22 and Mus81 (Fig. 5B). Moreover, loss of Mms22 function in the *mus81 mms1* background did not further enhance the slow growth and DNA damage sensitivity of the double mutant, suggesting that Mus81 is not essential for viability or DNA damage tolerance in the absence of *mms22* and *mms1* function (Fig. 5B). Together, these data suggest that in the absence of either Mms1 or Mms22, cell viability strongly depends on HR-mediated DNA repair and the DNA helicase Rqh1.

3.6 Mms1 is required for DNA repair following exposure to CPT and MMS

S. pombe mms22Δ mutants are defective at repairing MMS-induced DNA damage [18]. To determine whether the repair of DNA damage is impaired in *mms1Δ* mutants, we monitored the time-dependent disappearance of CPT-induced Rad22-YFP foci (Fig. 6). Following CPT-treatment, the number of wild-type nuclei containing Rad22 foci increased on average from 11.7 ± 2.1 %, to 69.6 ± 1.0 % (Fig. 6B). 6 hours following removal of CPT, the numbers had fallen to 45.6 ± 6.9 %, suggesting that repair of the damaged DNA had occurred in approximately 35% of the cells. In asynchronous cultures, *mms1Δ* cells already exhibited an elevated number of nuclei containing Rad22-YFP foci (Fig. 6). Despite this, *mms1Δ* cells also showed an increase in the number of nuclei with Rad22 foci from 48.4% to 82.1% after CPT treatment, indicating that Mms1 is not required for the initial sensing of DNA damage or the recruitment of the HR protein Rad22. However, even by the later timepoint of 8 hours after CPT removal, the number of nuclei containing at least one Rad22 focus had diminished only slightly, with 76.2% of nuclei still exhibiting foci, suggestive of only 7% of cells having repaired the CPT-induced damage. Similarly, *mms22Δ* cultures showed impaired repair of DNA lesions, as assessed by the persistence of CPT-induced Rad22 foci in that background (Fig. 6B). In line with this, following acute exposure to CPT, *mms1Δ* and *mms22Δ* show markedly reduced viability, with only 17% and 19% of cells respectively surviving a 3 hour exposure to CPT (Fig. 7), suggesting that Mms1 and Mms22 are required for the recovery from CPT-induced DNA damage. At later time points following removal of CPT, the number of Rad22 foci in wild-type cultures continued to decrease, reaching near-basal levels by 12 hours, however the low numbers of viable cells in the *mms1Δ* and *mms22Δ* cultures at this time point made accurate quantification difficult (data not shown). A similar result was observed following treatment with the alkylating agent MMS (a representative data set is shown in Fig. S2). 4 hours following release from the drug, the percent of nuclei displaying Rad22-YFP foci peaked for *wt*, *mms1Δ* and *mms22Δ* cultures (93.4%, 91.8% and 91.1% respectively). By 12 hours, the percent of *wt* nuclei with at least one Rad22 had decreased to 51.1%, unlike the *mms1Δ* and *mms22Δ* cultures, which remained at 81.5% and 84.7%. By 24 hours, *wt* numbers had further decreased to 29.3% whereas, similar to the situation following CPT treatment, the low numbers of viable cells in the *mms1Δ* and *mms22Δ* cultures at this later time point made quantification difficult (data not shown).

Taken together, our data identify fission yeast Mms1 as a protein which functions with Mms22 in a pathway important for the repair of DNA damage which arises during S-phase.

4. Discussion

In this study, we uncovered SPAC3H8.05c as a binding partner of the DNA repair protein Mms22 in *S. pombe*. SPAC3H8.05c was previously identified by bioinformatics analysis as a potential ortholog of budding yeast Mms1p [13], henceforth we termed the *SPAC3H8.05c*⁺ gene product Mms1. We showed that *mms1Δ* mutants are hypersensitive to agents that stall or impede replication fork progression, but not to IR. This suggests that Mms1 likely functions in a DNA repair pathway important for the recovery from S-phase-specific DNA damage that arises from replication fork stalling and collapse, but that Mms1 is not required for the repair DNA DSBs and other lesions induced by IR. Absence of Mms1 protein results in elevated spontaneous DNA damage, cell cycle arrest in G2, and reduced viability, presumably due to defects in DNA repair. Genetic analysis demonstrates that that loss of Mms1 function requires an intact HR pathway for full viability, but not Mus81-Eme1 Holliday junction resolvase [33-35].

In the absence of exogenous DNA damaging agents, *mms1Δ* and *mms22Δ* mutants exhibit a mitotic delay, a phenotype which is dependent on the DNA-damage checkpoint kinase Chk1. Whilst deletion of Chk1 reduced the overall cell length of both *mms1Δ* and *mms22Δ* mutants,

loss of this Chk1-dependent cell cycle delay did not negatively impact on the growth of either strain (Fig. 2A-B, [4]). This suggests that regardless of whether the activation of the Chk1-dependent DNA damage checkpoint occurs, the mutants are unable to recover from the spontaneous DNA damage that arises in the absence of either protein, supporting an important role for Mms1 and Mms22 in DNA repair. In further agreement with this, we show that loss of Mms1 or Mms22 function renders cells unable to repair DNA-damage induced by either the alkylating agent MMS or the Top1-inhibitor CPT, resulting in markedly reduced viability of the mutants when compared to wild type strains, even following relatively short exposures to the drug in the case of CPT (Fig. 7).

We have shown that loss of Mms22, and now also Mms1, results in increased spontaneous Rad22 foci, and leads to the requirement of an intact HR pathway for viability, suggesting that these mutants experience an elevated occurrence of spontaneous DNA damage that must be processed by the HR machinery for repair. This leads to two possible scenarios, one in which Mms1 and Mms22 act in a DNA repair pathway which functions in parallel to HR, and the other in which Mms1 and Mms22, whilst having non-HR functions, are also involved in HR-mediated repair processes. Indeed, Mms22 and Mms1 appear to promote S-phase-specific sister chromatid recombination in budding yeast [17], and the rate of spontaneous Rhp51-dependent conversion-type recombination is reduced in *S. pombe mms22Δ* mutants [18]. It may be that the Mms1-Mms22 complex regulates a specific recombination factor to permit HRR, or alternatively that a sequential recruitment of Mms1-Mms22 regulates some other protein to create a permissive environment for HR-mediated processing and repair, and subsequent restart of the fork.

We identified *S. pombe* Mms1 in a purification of Mms22-TAP, and confirmed this association by small-scale co-immunoprecipitation (Fig. 1). At this point we cannot be certain whether this interaction is direct or bridged by another protein; however, because we achieved substantial and comparable peptide coverage of Mms1 and Mms22 in the Mms22-TAP MudPIT experiment, it is very likely that the two proteins co-exist in a stable complex. This conclusion is consistent with current models for the functions of the two proteins in budding yeast.

S. cerevisiae Mms22p has been proposed to be a substrate-specific adaptor of a DNA repair-specific Rtt101-Mms1 cullin complex, analogous to a CUL4-based ubiquitin ligase, that is involved in the processing of stalled replication forks [13]. Budding yeast Mms1p has been shown to co-precipitate with the cullin Rtt101p, in a DNA damage and Mms22p-independent manner, suggesting that Mms22p is not required for Mms1p-Rtt101p complex assembly [13]. Indeed, functions for the Mms1p-Rtt101p cullin complex have been identified which do not involve the Mms22p protein [13,16]. However, in the presence of MMS-induced DNA damage, Mms22p interacts with the cullin Rtt101p, in an Mms1-dependent manner, suggesting a model in which the Mms1p-Rtt101p complex recruits specific substrate-specific adaptors according to the biological process at hand, with Mms22p being the adaptor involved in DNA-repair [13,16].

Thus, whilst to date a physical interaction between *S. cerevisiae* Mms1p and Mms22p has not been demonstrated, one would predict any association to be dependent on DNA damage. However, we observed an interaction between Mms1 and Mms22 in both the absence and presence of DNA damage (Fig. 1). This difference may be indicative of variations between the two organisms being studied, and could indicate that in *S. pombe*, in response to DNA damage, it is not just Mms22 alone that is recruited to cullin-Mms1, but rather a pre-existing complex of Mms1-Mms22 which relocates to the cullin. Interestingly, we did not recover any cullins in our Mms22-TAP purification, nor in subsequent purifications of Mms1-TAP, though we did recover Mms22 (data not shown).

In budding yeast, Rtt101p, Mms1p and Mms22p have been clustered genetically with Rtt109p, the histone H3 Lysine-56 acetyltransferase [6]. Moreover, recruitment of Rtt101p to chromatin in response to DNA damage depends on Rtt109p [11]. It will be of interest to determine the functional relationship between Rtt109, Mms1 and Mms22 and whether the H3-K56 acetylation machinery is required for the activity of a ubiquitin ligase complex containing Mms1 and Mms22 in fission yeast. Indeed, disruption of *rtt109*⁺ does not further exacerbate the phenotypes of *mms1Δ* or *mms22Δ* mutants (C.L.D and P.R, data not shown), suggesting that the proteins may function within the same pathway. Future studies in both budding and fission yeast will be of importance in uncovering the mechanism and function of the Mms1-Mms22 complex in the recovery from replication-associated DNA damage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Mms1 interacts with Mms22 in the presence or absence of DNA damage

A-B. Co-immunoprecipitation of exogenous 3HA-Mms22 with endogenous Mms1-TAP from lysates prepared from exponentially growing cultures or cultures treated with A. 30 M CPT or B. 0.03% MMS for 3 hours.

C. Co-immunoprecipitation of endogenous Mms22-5FLAG with Mms1-TAP using IgG sepharose beads, from lysates prepared from exponentially growing cultures or cultures treated with 0.03% MMS for 3 hours.

A

B

Figure 2. Spontaneous DNA damage occurs in the absence of Mms1

A. Deletion of Chk1 reduces the elongated phenotype of *mms1* mutants. Cells were cultured to mid-log phase in YES medium and bright-field images taken to assess the cellular length and morphology.

B. Genetic interactions between Mms1 and checkpoint kinase mutants. Five-fold serial dilutions of cells were exposed to the indicated DNA damaging agent and incubated at 30°C for 3 days.

Figure 3. Spontaneous DNA damage occurs in the absence of Mms1

A. Elevated Rad22-YFP foci arise in an *mms1*Δ background. Cells were cultured to midlog phase in YES medium, and imaged live.

B. The numbers of foci in at least 250 nuclei were scored in three independent experiments, and mean values were plotted with error bars representing the standard deviation about the mean.

Figure 4. *mms1Δ* mutants are sensitive to S-phase DNA damaging agents

A. Chronic exposure of *mms1Δ* mutants to DNA damaging agents: five-fold serial dilutions of cells were plated on YES agar containing the indicated DNA damaging agent and incubated at 30°C for 3 days.

Acute exposure to DNA damaging agents: survival curves of *mms1Δ* and *mms1Δmms22Δ* mutants exposed to increasing doses of IR. 500-1000 cells were plated on YES agar in triplicate and immediately exposed to the indicated dose of IR or UV irradiation. Colony numbers were counted following incubation at 30°C for 3 days and the mean colony number for each dose represented graphically (with untreated normalized to 100% survival). The sensitivity of an *rhp51Δ* mutant was analyzed as positive control.

B. Genetic epistasis between Mms1 and Mms22. Five-fold serial dilutions of cells were exposed to the indicated DNA damaging agent and incubated at 30°C for 3 days.

A

B

Figure 5. Importance of HR proteins for survival in the absence of Mms1 function

A. Tetrad dissection of genetic crosses of *mms1Δ* and HR mutants *rad22Δ*, *rhp51Δ* and the RecQ helicase *rqh1Δ*. Representative spores from four asci are shown for each cross.

B. Genetic relationship between Mms1, Mms22 and Mus81. Five-fold serial dilutions of cells were exposed to the indicated DNA damaging agent and incubated at 30°C 3 days.

Figure 6. Mms1 is required for repair of CPT-induced damage

A. Representative images of *wt*, *mms1*Δ and *mms22*Δ mutants displaying Rad22-YFP foci in response to CPT treatment. Images of mid-log cultures were taken prior to addition of 30μM CPT (untreated). CPT treatment was for 3 hours at 30°C. At time point 0 hours, CPT was removed and the cultures released into fresh YES medium. Images were taken at this time point 0 hours, and at 2, 4, 6, 8 and 12 hours following release.

B. Quantification of the percent of nuclei containing one or more Rad22-YFP focus from at least 250 cells, from three independent experiments.

Figure 7. Survival curve of *mms1Δ* and *mms22Δ* following acute exposure to CPT

Exponentially growing cultures were cultured in YES media containing 30μM CPT for a maximum of 3 hours. Immediately at time 0 min, a volume containing approximately 1000 cells was removed, CPT was washed out, and the cells were plated onto YES agar plates in triplicate. At each time point, the same volume of culture was removed, and the cells washed and plated. The mean survival from three independent experiments is represented graphically (with time point 0 min normalized to 100% survival).

Table 1

strains used in this study

Strain	Genotype (all are <i>leu1-32, ura4-D18</i>)	Source
PR109	<i>h-</i>	Lab stock
PR110	<i>h+</i>	Lab stock
VM3725	<i>h- rad22-YFP:KanMx</i>	[36]
CD4091	<i>h- mms22::HphMx, rad22-YFP:KanMx</i>	[4]
EN3095	<i>h- cds1::KanMx</i>	Lab stock
OL4027	<i>h+ chk1::KanMx</i>	Lab stock
CD4101	<i>h- rad22-D2::LEU2</i>	[4]
PS2383	<i>h- smt0 rhp51::ura4⁺</i>	Lab stock
SC3250	<i>h- rqh1::ura4⁺</i>	Lab stock
CD4089	<i>h+ mms22::HphMx</i>	[4]
CD4090	<i>h- mms22::HphMx</i>	[4]
CD4677	<i>h+ mms22::KanMx</i>	This study
CD4678	<i>h- mms22::KanMx</i>	This study
CD4679	<i>h- mms1::KanMx</i>	This study
CD4680	<i>h+ mms1::KanMx</i>	This study
CD4681	<i>h- mms1::HphMx</i>	This study
CD4682	<i>h+ mms1::HphMx</i>	This study
CD4683	<i>h- mms22::HphMx, pREP41-N-3HA-mms22+</i>	This study
CD4684	<i>h+ mms1-TAP:KanMx, pREP41-N-3HA-mms22+</i>	This study
CD4685	<i>h- mms1-TAP:KanMx</i>	This study
CD4686	<i>h- mms1::HphMx, rad22-YFP:KanMx</i>	This study
CD4687	<i>h- mms1::HphMx, mms22::KanMx, rad22-YFP:KanMx</i>	This study
CD4688	<i>h+ mms1::HphMx, chk1::KanMx</i>	This study
CD4689	<i>h+ cds1::KanMx, chk1::KanMx</i>	This study
CD4690	<i>h+ mms1::HphMx, cds1::KanMx</i>	This study
CD4691	<i>h+ mms1::HphMx, cds1::KanMx, chk1::KanMx</i>	This study
CD4692	<i>h+ mus81::NatMx</i>	This study
CD4693	<i>h- smt0 rhp51::ura4⁺, mms1::HphMx</i>	This study
CD4694	<i>h- rqh1::ura4⁺, mms1::HphMx</i>	This study
CD4695	<i>h- mus81::NatMx, mms1::HphMx</i>	This study
CD4696	<i>h- mus81::NatMx, mms22::KanMx</i>	This study
CD4697	<i>h- mms1::KanMx, mms22::HphMx</i>	This study
CD4698	<i>h- mus81::NatMx, mms1::HphMx, mms22::KanMx</i>	This study
CD4699	<i>h+ mms22-TAP:KanMx</i>	This study
CD4801	<i>h+ mms22-5FLAG:KanMx</i>	This study
CD4802	<i>h+ mms1-TAP:KanMx, mms22-5FLAG:KanMx</i>	This study

Table 2
Identification of SPAC3H8.05c as an Mms22-interacting protein

SPAC3H8.05c co-immunoprecipitated with Mms22-TAP from cell lysates. The coverage of both Mms22 and SPAC3H8.05c (which we termed Mms1) and peptides identified by MudPIT analysis are listed.

Protein	Coverage	Peptides
Mms22	19.3%	IGLVPITVPHGFSSDR (x3) YVEVEKLPDLILESYGK (x1) KYFSFDKESDR (x1) QVIDQVLSDWYSGK (x1) HSVNTNAK (x2) TAANNGLSHLQNFSEELLK (x2) TQGLNSILQLDIVTHPPK (x2) VSWLASDLISK (x1) LLSAGQSGLLECYR (x1) YALWEQVNSFFDLQK (x1) FNDLSSEISEDTPDFDFVK (x1) IRDIVIVDNSHLK (x3) EGTDYELAQGMEWFNSILK (x1) KFLQYQSNTPEPK (x1) FLQYQSNTPEPK (x1) KYEVAQILR (x3) TFVSPFLYQTISYLVGNDEDKENYIR (x2) IEEISINYFK (x1) VVGDIQYCSQFANDR (x1) YFMDSTK (x1) LEYTLAR (x1) TLTNASQNALFSFLK (x1) QTETSNLLR (x1) TSGLPSSLICNLQNFLAR (x8) TIQQHCSADWYDNVTNIVHK (x4) FLNVICSYAPDMSGPLTK (x1)
SPAC3H8.05c	19.3%	SVALLSSHFLQSESGR (x4) HSIANILIATENGK (x1) CYLLQLVK (x1) ICLLSVPQIECGDLK (x1) HNTLLDEDINCNTVYATAGIGK (x1) LHLDGVVEDVTESLFLDDIK (x1) DLMNFKSQLLTLK (x1) LNTLSNGTLELQSLPDSFDLHDVPTCITSFSLE (x1) LFPVNWPSMDIK (x2) DHDSNAIELR (x2) HGLCYATGR (x1) SPIVSMSTYK (x1) EQMLQPILTTVSQTK (x1) TNALTNHLK (x2)