Schizosaccharomyces pombe MutSα and MutLα Maintain Stability of Tetra-Nucleotide Repeats and Msh3 of Hepta-Nucleotide Repeats

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ABSTRACT Defective mismatch repair (MMR) in humans is associated with colon cancer and instability of microsatellites, that is, DNA sequences with one or several nucleotides repeated. Key factors of eukaryotic MMR are the heterodimers MutSα (Msh2-Msh6), which recognizes base-base mismatches and unpaired nucleotides in DNA, and MutLα (Mlh1-Pms1), which facilitates downstream steps. In addition, MutSβ (Msh2-Msh3) recognizes DNA loops of various sizes, although our previous data and the data presented here suggest that Msh3 of Schizosaccharomyces pombe does not play a role in MMR. To test microsatellite stability in S. pombe and hence DNA loop repair, we have inserted tetra-, penta-, and hepta-nucleotide repeats in the ade6 gene and determined their Ade+ reversion rates and spectra in wild type and various mutants. Our data indicate that loops with four unpaired nucleotides in the nascent and the template strand are the upper limit of MutSα- and MutLα-mediated MMR in S. pombe. Stability of hepta-nucleotide repeats requires Msh3 and Exo1 in MMR-independent processes as well as the DNA repair proteins Rad50, Rad51, and Rad2FEN1. Most strikingly, mutation rates in the double mutants msh3 exo1 and msh3 rad51 were decreased when compared to respective single mutants, indicating that Msh3 prevents error prone processes carried out by Exo1 and Rad51. We conclude that Msh3 has no obvious function in MMR in S. pombe, but contributes to DNA repeat stability in MMR-independent processes.

KEYWORDS mismatch repair microsatellite instability homologous recombination repair repetitive DNA FEN1

Repetitive DNA elements are widespread in genomes. They are located in centromeres, telomeres, rDNA genes, transposons, and intergenic regions (Padeken et al. 2015). One class of repetitive DNA comprises microsatellites, which are short tandem repeats of one to several nucleotides. Their lengths often differ between individuals of a given species, but are identical in cells of an individual organism. During DNA replication, repeat units can slip and misalign with repeats of the opposite strand. Unrecognized slippage in the template strand leads to deletions, and in the nascent strand to insertions after the next round of replication. The major pathway for maintaining repeat tract lengths of microsatellites is mismatch repair (MMR). Eukaryotic MMR is initiated either by Msh2-Msh6 (MutSα) or by Msh2-Msh3 (MutSβ) (Alani 1996; Habraken et al. 1996; Johnson et al. 1996; Marsischky et al. 1996). Depending on the eukaryote, some differences exist in the recognition spectrum of the two complexes (Marti et al. 2002). Generally, MutSα is responsible for repair of base-base mismatches and loops, and MutSβ for repair of loops. After mismatch binding, MutL heterodimers are recruited, with Mlh1-Pms1 (MutLα) (termed Mlh1-Pms2 in humans) being the major factor for MMR (Jiricny 2013). The newly synthesized strand with the erroneous nucleotide(s) is subsequently degraded either by the 5’-exonuclease activity of Exo1 or by the endonuclease activity of MutLα (Kadyrov et al. 2006, 2007; Smith et al. 2013; Goellner et al. 2014, 2015). The homotrimeric processivity factor PCNA binds to MutSα, MutSβ, and MutLα and coordinates correct incision of the newly synthesized strand (Flores-Rozas et al. 2000, Klescikowska et al. 2001; Lee and Alani 2006; Iyer et al. 2010;
Pluciennik et al. (2010). After excision beyond the mismatch, a replicative DNA polymerase fills the resulting gap and the remaining nick is ligated.

A defect in the human MMR genes MSH2 and MLH1 causes microsatellite instability and a predisposition to colon and other types of cancer (Lynch et al. 2009; Da Silva et al. 2016). Mutations in other MMR genes are rarely correlated with cancer, probably due to functional redundancy. In contrast to other types of repetitive elements, microsatellites are often situated in genes, which is a critical factor for tumor development and important for the choice of drugs for treatment of cancer patients. For example, deletions in a T11 repeat in intron 1 of MRE11 originated from transformations of OL2137 (h− ura4-D18), with DNA fragments obtained by fusion PCRs. pFA6a-hphMX (Hentges et al. 2005) was used as template to amplify the hypogymcic resistance cassette. Genomic DNA of strain RO144 (smt-0) was used to amplify either 450 bp of the 3′ UTR of the smh2 locus or 500 bp of the 5′ UTR of the smh6 locus. Primers for the smh2 disruption were msh2_For5 5′-GAGGTTTTTATTTTATTTATTTGAG GACTTAACTGTGGCAAGAAGTTCCTTCTCTGTTTTTATAACCA TTTGCAGGTTGCGGTTTGAAGACAATTCAATCACTGACCTGGCGG GGTATAATTA; msh2_Rev5 5′-TTTCTCGTTTTAGTAAAAATTT TATTTTACAAAAAGGCTTCCAAAACAACTGATCTGTTTT GAATCCCTAATATTGCTGTCGAGTTATTTAACG; msh2_Rev3 5′-GCTAACAAAGATTTAATGGCG for the smh6 disruption were msh6_For5 5′-TATATAGTATATTTTGTCCT CTGTTAGCTTTTACTCATTTAGAGGCCAGCTCTGGTTTTGAAA TACCTAGAATCTGCGCAAAAAACACTGCTTTGCGTGGATCC CGCGGTATATTTA; msh6_Rev5 5′-ATAAGCTAATGATTAAAAATTTAAAAAAGGAACGTTCGGGCTCGTGCTCGTGGATTGCGGAGCTCGTTTCAATTAGTTGTTGAA TTAATAGCTATATATTTGCTGTCGAGTTATTTAACG; msh2_Rev2 5′-CTCATCTTACCTAAAC for the smh6 disruption were msh6_Rev2 5′-GAAAGCGTCTGTTTCAATTAGTTGTTGAA TTAATAGCTATATATTTGCTGTCGAGTTATTTAACG; msh2_Rev3 5′-GCTAACAAAGATTTAATGGCG.

Recombination processes can alter lengths of repetitive DNA either by unequal crossover between repeats or through secondary structures formed between repeats in the same strand. The Mre11-Rad50-Nbs1 (MRN) complex has single-stranded 3′-exonuclease and endonuclease activities as well as structural functions in recombination processes (Cejka et al. 2015). After 5′–3′ resection of DNA double-strand breaks or ends by Exo1 or other 3′-exonucleases, Rad51-dependent homologous recombination (HR) can occur by invasion of the 3′-independent single-stranded end (SSA) into a complementary DNA molecule. Rad51-independent single-strand annealing (SSA) can also occur between two repeats and leads to deletion of the intervening sequence. SSA requires the nucleotide excision repair factors Rad1-Rad10 of Saccharomyces cerevisiae (XPF-ERCC1 in human) and MutSβ (Bhargava et al. 2016). FEN1 is a flap endonuclease with multiple roles in DNA metabolism. FEN1 is involved in processing of Okazaki fragments during replication, in long-patch base excision repair and in other processes (Marti and Fleck 2004). F. cerevisiae, rad27 (a FEN1 homolog) mutants exhibit instability of mono- and dinucleotide repeats and generate duplicates of sequences flanked by repeats (Johnson et al. 1995; Tishkoff et al. 1997; Kirchner et al. 2000). In addition, FEN1 has been implicated in trinucleotide repeat stability (Freudenreich et al. 1998; Liu and Wilson 2012) and repair of large loops with up to 216 unpaired nucleotides (Sommer et al. 2008).

The genome of fission yeast Schizosaccharomyces pombe encodes the MutS homologs Msh1, Msh2, Msh3, and Msh6, the MutL homologs Mlh1 and Pms1, and the exonuclease Exo1. Based on homology with S. cerevisiae Msh1, S. pombe Msh1 likely acts in MMR of mitochondrial DNA. S. pombe Msh2, Msh6, Mlh1, and Pms1 are indispensable for repair of base-base mismatches and small loops with one or two nucleotides (Schär et al. 1997; Rudolph et al. 1999; Mansour et al. 2001; Tornier et al. 2001; Marti et al. 2003). In contrast, Msh3 seems to have no, or a minor and rather MMR-independent, function in repair of base-base mismatches and small loops. Exo1 appears to be involved in MMR of base-base mismatches but has a rather MMR-independent function in repair of small loops (Rudolph et al. 1998; Mansour et al. 2001; Marti et al. 2003).

In the present study, we tested stability of tetra-, penta-, and heptanucleotide repeats in S. pombe. Our aim was to analyze whether stability of such repeats is dependent on MMR, and if so, whether MutSo, MutSβ, or both are involved. In addition, we analyzed msh1, and thus MutLx-deficient strains as well as exo1 mutants.

**MATERIALS AND METHODS**

**General yeast genetic methods, media, and S. pombe strains**

The S. pombe media minimal medium agar (MMA), yeast extract agar (YEA), and yeast extract liquid (YEL), and general genetic methods were used as described (Gutz et al. 1974). S. pombe strains used in this study were derived from Ru39 h− msh2-3his3 his3-D1 (Rudolph et al. 1999); KK83 smt-0 msh3-3loxP:ura4-3loxM:leu1-32 ura4-D18 (D. Villahermosa, K. Knapp, and O. Fleck, unpublished data); OL197 h− msh1-kanMX his3-D1 ura4-D18 (Marti et al. 2003); Ru42 h− exo1::ura4-400 (Schär et al. 2001 and Wilson 2012), and repair of large loops with up to 216 unpaired nucleotides

**Construction of (GACC)_n repeats in the ade6 gene**

In pAN-K, a pUC18 derivative, the kanamycin resistance gene was replaced by a 340-bp DraIII-HindIII fragment of the ade6 gene containing a (GACC)_7 repeat near the DraIII site (underlined in primer ade6-GACC7; see below). The fragment was obtained by PCR with primers ade6-GACC7 5′-GCCACACTTTGATGACAAGCAGAGCCGAGCGGAGCCGAGCTTATGAAGAAAGGGAACGTTCGGGCTCGTGCTCGTGGATTGCGGAGCTCGTTTCAATTAGTTGTTGAA TTAATAGCTATATATTTGCTGTCGAGTTATTTAACG; ade6-GACC7 5′-CTCATCTTACCTAAAC for the ade6-GACC7 5′-GCCACACTTTGATGACAAGCAGAGCCGAGCGGAGCGGAGCGCTTATGAAGAAAGGGAACGTTCGGGCTCGTGCTCGTGGATTGCGGAGCTCGTTTCAATTAGTTGTTGAA TTAATAGCTATATATTTGCTGTCGAGTTATTTAACG; ade6-GACC7 5′-CTCATCTTACCTAAAC for the ade6-GACC7 5′-GCCACACTTTGATGACAAGCAGAGCCGAGCGGAGCGGAGCGCTTATGAAGAAAGGGAACGTTCGGGCTCGTGCTCGTGGATTGCGGAGCTCGTTTCAATTAGTTGTTGAA TTAATAGCTATATATTTGCTGTCGAGTTATTTAACG; ade6-GACC7 5′-CTCATCTTACCTAAAC for the ade6-GACC7 5′-GCCACACTTTGATGACAAGCAGAGCCGAGCGGAGCGGAGCGCTTATGAAGAAAGGGAACGTTCGGGCTCGTGCTCGTGGATTGCGGAGCTCGTTTCAATTAGTTGTTGAA TTAATAGCTATATATTTGCTGTCGAGTTATTTAACG; ade6-GACC7 5′-CTCATCTTACCTAAAC for the ade6-GACC7 5′-GCCACACTTTGATGACAAGCAGAGCCGAGCGGAGCGGAGCGCTTATGAAGAAAGGGAACGTTCGGGCTCGTGCTCGTGGATTGCGGAGCTCGTTTCAATTAGTTGTTGAA TTAATAGCTATATATTTGCTGTCGAGTTATTTAACG; ade6-GACC7 5′-CTCATCTTACCTAAAC for the ade6-GACC7 5′-GCCACACTTTGATGACAAGCAGAGCCGAGCGGAGCGGAGCGCTTATGAAGAAAGGGAACGTTCGGGCTCGTGCTCGTGGATTGCGGAGCTCGTTTCAATTAGTTGTTGAA TTAATAGCTATATATTTGCTGTCGAGTTATTTAACG; ade6-GACC7 5′-CTCATCTTACCTAAAC for the ade6-GACC7 5′-GCCACACTTTGATGACAAGCAGAGCCGAGCGGAGCGGAGCGCTTATGAAGAAAGGGAACGTTCGGGCTCGTGCTCGTGGATTGCGGAGCTCGTTTCAATTAGTTGTTGAA TTAATAGCTATATATTTGCTGTCGAGTTATTTAACG; ade6-GACC7 5′-CTCATCTTACCTAAAC for the ade6-GACC7 5′-GCCACACTTTGATGACAAGCAGAGCCGAGCGGAGCGGAGCGCTTATGAAGAAAGGGAACGTTCGGGCTCGTGCTCGTGGATTGCGGAGCTCGTTTCAATTAGTTGTTGAA TTAATAGCTATATATTTGCTGTCGAGTTATTTAACG; ade6-GACC7 5′-CTCATCTTACCTAAAC for the ade6-GACC7 5′-GCCACACTTTGATGACAAGCAGAGCCGAGCGGAGCGGAGCGCTTATGAAGAAAGGGAACGTTCGGGCTCGTGCTCGTGGATTGCGGAGCTCGTTTCAATTAGTTGTTGAA TTAATAGCTATATATTTGCTGTCGAGTTATTTAACG; ade6-GACC7 5′-CTCATCTTACCTAAAC for the ade6-GACC7 5′-GCCACACTTTGATGACAAGCAGAGC}}
and ade6_d_ura4_R 5'-GAATGGTCTCTAGTGTAGGATAAAC ATAAACATTTTCCGCTCAAAACTCGTACTACCACATCGCAATT TCGATGAGAGAACGGAGTCCATGGAGAAAGGAACGAACTTTTCAACATAAGGACGATG TAAACTTTTCCGTCTAAACTGCGTACTACCATCACTGCAATT flpendent ages and SDs were calculated from averages of at least three inde- pendent tests, each with seven cultures, and up to 20 random revertants (where available) per culture. 

**Construction of penta- and hepta-nucleotide repeats in the ade6 gene**

We subjected 100 pmol of each of two 120-nucleotide-long oligonucleotides, with ~40 nucleotides complementary to each other at their 5’ ends, to primer extension in 50 μl reactions containing 1 U GoTaq polymerase, 2.5 mM MgCl2, 50 μM each dNTP in Colorless GoTaq Flexi buffer (Promega). After initial denaturation for 1 min at 94°C, we applied two cycles with 30 sec at 94°C, 30 sec at 45°C, and 30 sec at 72°C, followed by five cycles with 30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C. Reaction samples were transformed into strain DE1 using the Flexi buffer (Promega). After initial denaturation for 1 min at 94°C, we applied two cycles with 30 sec at 94°C, followed by 10 cycles with 30 sec at 94°C, 10 sec at 65°C, and 30 sec at 72°C.

**Figure 1** Schematic of the position and nature of the various repeats. In the ade6::ura4 disruption strain, 13 nucleotides have been deleted and replaced by the ura4 marker. This strain was transformed with DNA fragments to produce ade6 mutants with the indicated repeats (highlighted by blue arrows). Nucleotides within the repeats that differ from the wild-type sequence are shown in red. Integration of the repeats caused framenshifts with nearby located stop codons. The numbers of net inserted nucleotides and of the major deletion/insertion events that lead to Ade+ reversions are given on the right. (+14) indicates that, although this event restores the open reading frame, it was not found among the revertants sequenced (Table 6). In (GACC)8 & (ATCGTCC)5, as indicated on the right.

**Table 1** Construction of penta- and hepta-nucleotide repeats in the ade6 gene

| Repeat | Net inserted nucleotides | Deletion/insertion events |
|--------|--------------------------|---------------------------|
| ade6+  |                          |                           |
| ade6::ura4 |                          |                           |
| ade6::(GACC)7 | +22 / -4 | +8 |
| ade6::(ATCGTCC)7 | +17 / -8 | +4 |
| ade6::(CTGCC)7 | +19 / -10 | +5 |
| ade6::(ATCGTCC)7 | +25 / -7 (+14) |                           |
| ade6::(ATCGTCC)7 | +26 / -14 / +7 |                           |

**Determination of mutation rates and spectra**

Mutation rates were determined by fluctuation tests as described (Mansour et al. 2001). In brief, seven tubes containing 2 ml YEL were inoculated with a single small colony and incubated at 30°C until cultures were grown to stationary phase. Appropriate dilutions were plated on YEA for selection of Ade+ revertants. Colonies were counted after 5 d of growth at 30°C, except for strains with rad50 or rad51 background, where colonies were counted after 6 d to compensate for their slow growth. Reversion rates were calculated from at least three independent fluctuation tests. Statistical significance was calculated with a two-tailed Student’s t-test.

The nature of mutations was determined by sequencing of PCR products from genomic DNA using primers ade6-F 5’-ATTACACT GATGGCTTTGGC and ade6_R 5’-ACAGAGAACGTTAGCGATC. In the case of ade6-(GACC)7-ΔT, repeat tract changes were also ana- lyzed by inspection of the color of Ade+ revertants (Figure 2A). The color was best determined when revertants were restreaked on YE without supplemented adenine. The proportion of white and pink color was best determined when revertants were restreaked on YEA and visually scored.

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RESULTS

Genetic assay for microsatellite instability in S. pombe

We have previously reported that in-frame nucleotide insertions and codon changes in a region around nucleotide 1397 of the ade6 gene [the ATG start codon is at 875 as defined by Szankasi et al. (1988)] in S. pombe did not disrupt or only slightly disrupted its functionality (Mansour et al. 2001; Marti et al. 2003). Here, we inserted tetra-, penta-, and hepta-nucleotide repeats in this part of ade6 in order to analyze microsatellite instability in wild type and MMR mutants (Figure 1). Insertion of the repeats caused frameshifts, which rendered cells auxotrophic for adenine due to nearby located stop codons. Such ade6 mutants required a supply of adenine for growth and turned red on the medium with a limited amount of adenine due to accumulation of a red pigment. Reversions of such strains to Ade+ can occur by deletions or insertions of repeats, when these events restore the open reading frame. With (GACC)\_\_T, (CTGCC)\_T, and (ATCGTCC)\_T, insertions of one repeat unit and deletions of two repeat units are the major events detectable. The opposite is the case with (GACC)\_8 and (ATCGTCC)\_7, where deletions of one repeat unit and insertions of two repeat units are the principal events that can be detected, although in the case of (ATCGTCC)\_T, we identified exclusive deletions as described below.

In the case of ade6-(GACC)\_T, deletions and insertions can be distinguished by the color of revertants (Figure 2A). Deletions caused white Ade+, while insertions caused pink Ade+. The pink color likely reflects that ade6 is not fully functional and thus that the red pigment is produced in low quantities. Sequencing of 18 Ade+ revertants of the (GACC)\_T repeat in the various genetic backgrounds revealed deletions and sequencing of 23 (GACC)\_T revertants revealed insertions (Table 1). All revertants with deletions were white and all revertants with insertions were pink. Thus, the occurrence of deletions and insertions in the (GACC)\_T repeat can be easily determined from a large number of revertants. Analyzing revertants of the other ade6 repeats did not allow a distinction by color, probably because Ade+ originating from deletions and insertions were both not fully functional. In these cases, PCR products of independent Ade+ revertants were subjected to sequencing to identify the number of repeats.

GACC tetra-nucleotide repeats were unstable in msh2, msh6, and mlh1 mutants

We first analyzed stability of GACC tetra-nucleotide repeats in wild type, and in mutants deleted for either msh2, msh3, msh6, mlh1, or exo1. In wild type, ade6-(GACC)\_8 and ade6-(GACC)\_T reverted to Ade+ with 1.2 \times 10^{-5} and 2.1 \times 10^{-5} reversions per cell division, respectively (Table 2). The ade6-(GACC)\_8 reversion rates increased 39–44-fold in msh2, msh6, and mlh1 mutants and slightly decreased in msh3 and exo1 mutants. Similarly, ade6-(GACC)\_T reversion rates increased 17–22-fold in msh2, msh6, and mlh1 mutants and decreased 3–5-fold in msh3 and exo1 mutants. None of the differences between msh3, exo1, and wild type was statistically significant.

Figure 2 Distribution of deletions and insertions in the ade6-(GACC)\_T repeat. (A) Deletions and insertions within the (GACC)\_T repeat that lead to Ade+ can be distinguished by their color. ade6-(GACC)\_T mutants form red colonies on medium with limited amount of adenine due to a defective ade6 gene. They can revert to Ade+ by deletion of two or five repeat units, producing white Ade+, or by insertion of one or four repeat units, producing pink Ade+. (B) Percentage of deletions in the various strain backgrounds. Wild type, msh3, and exo1 mainly reverted to Ade+ by deletions, while msh2, msh6, and mlh1 mutants mainly reverted by insertions. Significantly different to wild type: ** * p < 0.01; *** p < 0.001. Shown are average values with SDs. Reversion spectra are also presented as pie charts, with the proportion of deletions and insertions indicated in white and pink, respectively.

Data availability

S. pombe strains are available on request. The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.
Table 1 Reversion spectra of tetra-nucleotide repeats

| Relevant Genotype | (GACC)$_2$ | (GACC)$_3$ | (GACC)$_4$ | (GACC)$_5$ | (GACC)$_6$ | (GACC)$_7$ | (GACC)$_8$ |
|------------------|------------|------------|------------|------------|------------|------------|------------|
|                   | −4 bp      | +8 bp      | +20 bp     |            |            |            |            |
| Wild type         | 0          | 10         | 0          | 0          | 0          | 0          | 0          |
| msh2             | 8          | 2          | 0          | 2          | 0          | 0          | 0          |
| msh3             | 4          | 6          | 1          | 7          | 1          | 7          | 1          |
| msh6             | 9          | 6          | 0          | 10         | 10         | 6           | 9          |

Revertants were determined from random independent revertants. WT, wild type.

$\chi^2$ and p-values (in parenthesis) are shown for the distribution of deletions vs. insertions and were calculated with an online program (http://www.socscistatistics.com/tests/chisquare/).
remained about the same in msh3 rad50, msh3 rad2FEN1, and exo1 rad50 but decreased in the msh3 rad51 and exo1 rad51 double mutants when compared to respective single mutants (Table 7). When wild type and the other single mutants, rad50 and rad51 strains mainly reverted to Ade+ by insertion of one repeat unit (Table 6). In the rad2FEN1 mutant, 50% of the reversion events were due to deletion of two repeats, which was not significantly different to wild type. A reduction of reversion rates in the msh3 rad51 and exo1 rad51 double mutants indicates that Exo1 and Rad51 act error prone on the (ATCGTCC)5 repeat when msh3 is mutated.

**DISCUSSION**

**MutSα and MutLα are essential for S. pombe MMR, which is limited to loops with up to four nucleotides**

Eukaryotic MMR is initiated by MutSα for repair of base-base mismatches and loops or by MutSβ for repair of loops (Marti et al. 2002; Jiricny 2013). In *S. cerevisiae*, msh3 and msh6 mutants show little to moderate increases of mutation rates in mono- and dinucleotide repeats (Johnson et al. 1996; Marischky et al. 1996; Greene and Jinks-Robertson 1997; Sia et al. 1997). On the other hand, such repeats are highly unstable in msh3 msh6 double mutants and within the range of the msh2 instability, indicating redundancy of MutSα and MutSβ for small loops in this organism. In humans, MutSα is the major factor for recognition of base-base mismatches and loops, while MutSβ rather serves as a backup (Drummond et al. 1997; Genschel et al. 1998; Marra et al. 1998).

In *S. pombe*, we knew to this date that MMR is able to repair base-base mismatches and loops with up to two nucleotides (Schär et al. 1997; Rudolph et al. 1999; Mansour et al. 2001; Tornier et al. 2001; Marti et al. 2003). This requires MutSα and MutLα but not MutSβ. In the present study, we expanded analysis of loop repair in *S. pombe* to four to seven unpaired nucleotides. Our aim was to determine the contributions of Msh2, Msh3, Msh6, Mlh1, and Exo1, and particularly the relative roles of MutSα and MutSβ in stability of repeats with four or more iterated nucleotides in this model organism. The microsatellites tested were (GACC)7 and (GACC)8 tetra-, (CTGCC)6 with four or more iterated nucleotides in this model organism. The assay with the (GACC)7 repeat allowed distinguishing deletions from insertions by the color of Ade+ revertants (Figure 2A). We found that wild type, msh3, and exo1 mainly reverted by eight-nucleotide deletions, and msh2, msh6, and mlh1 mainly by four-nucleotide insertions (Figure 2B and Table 1). Thus, this assay also revealed that MutSα but not MutSβ initiates MMR of loops with four nucleotides. In addition, Mlh1, and by extrapolation MutLα, is involved in removal of four-nucleotide loops. Since four-nucleotide deletions, detectable with (GACC)6, and four-nucleotide insertions, detectable with (GACC)7ΔT, were the predominant reversion events in *msh2* and *msh6* mutants, slippage of one repeat can occur in the template and in the nascent strand during replication, and both types of events are corrected by MMR mediated by MutSα and MutLα.

**Msh3 has an MMR-independent function in repeat stability**

Our previous data showed that *msh3* mutants had no significant defects in repair of base-base mismatches and of loops with one unpaired nucleotide in a Tn7 repeat and in nonrepetitive DNA (Tornier et al. 2001). Inactivated Msh2, Msh6, and Mlh1 caused instability of the tetranucleotide repeats, while defective Msh3 and Exo1 rather made the repeats slightly more stable, although this was not significantly different to wild type (Table 2). The (GACC)7 repeat reverted in wild type by insertions of eight nucleotides, while in *msh2* and *msh6* mutants, mainly deletions of four nucleotides occurred (Table 1). Thus, MutSα of *S. pombe* is capable to initiate MMR of loops with four unpaired nucleotides, whereas MutSβ is not.

The assay with the (GACC)7ΔT repeat allowed distinguishing deletions from insertions by the color of Ade+ revertants (Figure 2A). We found that wild type, msh3, and exo1 mainly reverted by eight-nucleotide deletions, and msh2, msh6, and mlh1 mainly by four-nucleotide insertions (Figure 2B and Table 1). Thus, this assay also revealed that MutSα but not MutSβ initiates MMR of loops with four nucleotides. In addition, Mlh1, and by extrapolation MutLα, is involved in removal of four-nucleotide loops. Since four-nucleotide deletions, detectable with (GACC)6, and four-nucleotide insertions, detectable with (GACC)7ΔT, were the predominant reversion events in *msh2* and *msh6* mutants, slippage of one repeat can occur in the template and in the nascent strand during replication, and both types of events are corrected by MMR mediated by MutSα and MutLα.
Intriguingly, the mutant showed a lower mutation rate than wild type (Table 3), indicating that the Msh3 function is related to recombination mechanisms but not in MMR (Pinto et al. 2005; Burby and Simmons 2017). MutS2 of Helicobacter pylori binds to DNA structures that resemble recombination intermediates and inhibits strand exchange in vitro (Pinto et al. 2005). In this regard, S. pombe Msh3 may be functionally similar, although structurally, it lacks the endonuclease domain of MutS2, and homology of its amino acid sequence clearly allocates it to the group of eukaryotic Msh3 proteins.

Structural studies with human MutS heterodimers showed that mismatch binding largely occurs by Msh3 or Msh6, while Msh2 has few contacts with the DNA backbone of correctly paired nucleotides in the vicinity (Warren et al. 2007; Gupta et al. 2011). The human Msh6 protein interacts directly with mismatched bases via a phenylalanine, which is conserved in eukaryotic Msh6 orthologs and bacterial MutS. In contrast, human Msh3 lacks this residue and instead interacts with phosphate groups of the unpaired nucleotides (Gupta et al. 2011). Work by Lee et al. (2007) demonstrated that deletion of the mismatch binding domain of S. cerevisiae Msh2 causes loss of MutSβ—dependent MMR activity and revealed that the domain in Msh2 is required for general DNA binding, and in Msh3 for binding to DNA loops. In complex with Msh2, a chimeric Msh6 protein of S. cerevisiae containing the mismatch binding domain of Msh3 showed substrate specificity of Msh3, i.e., high affinity to loops with one to four unpaired nucleotides (Shell et al. 2007). The amino acid sequence within the mismatch binding domain of S. pombe Msh3 is very similar to that of human Msh3. However, in contrast to S. cerevisiae and human Msh3 and orthologs of other eukaryotes, S. pombe Msh3 lacks a canonical PIP box, which mediates interaction with PCNA. The PIP box of human Msh3 overlaps with the Mlh1 binding domain (Iyer et al. 2010). Thus, it is also conceivable that amino acid residues required for interaction with Mlh1 are not present in S. pombe Msh3. It is currently not known whether S. pombe Msh3 can interact with PCNA or MutLα. If it does not, this may explain that it does not participate in MMR.

**Role of MutSα and MutSβ in MMR**

The Escherichia coli homodimer MutS enables repair of base-base mismatches and loops with up to four nucleotides (Iyer et al. 2006).

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**Table 4 Reversion spectra of the (CTGCC)₄ repeat**

| Relevant Genotype | (CTGCC)₄ −10 bp | (CTGCC)₄ +5 bp | (CTGCC)₄ +20 bp |
|-------------------|-----------------|----------------|-----------------|
| Wild type         | 1               | 15             | 0               |
| msh2             | 1               | 9              | 0               |
| msh3             | 0               | 10             | 0               |
| msh6             | 2               | 7              | 1               |
| msh1*          | 6               | 10             | 0               |
| exo             | 2               | 8              | 0               |

Repeat tract changes were determined from random independent revertants.

*Distribution of deletions and insertions in msh1 background significantly different to wild type (χ² = 4.57; p = 0.033). Reversion spectra of all other mutants were not significantly different to wild type.

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**Table 5 Reversion rates of hepta-nucleotide repeats**

| Relevant Genotype | (ATCGTCC₆)ΔT | Rate | Fold Increase | p-Value | (ATCGTCC₆)ΔT | Rate | Fold Increase | p-Value |
|-------------------|--------------|------|---------------|---------|--------------|------|---------------|---------|
| Wild type         | 7.2 ± 3.2 × 10⁻⁶ | 1.00 | 1.00 | 1.00 | 7.5 ± 2.9 × 10⁻⁶ | 1.00 | 1.00 | 1.00 |
| msh2             | 7.3 ± 1.8 × 10⁻⁶ | 1.00 | 1.00 | 1.00 | 6.8 ± 0.9 × 10⁻⁶ | 1.00 | 1.00 | 1.00 |
| msh3             | 1.4 ± 0.7 × 10⁻⁵ | 1.90 | 0.13 | 0.13 | 2.1 ± 0.6 × 10⁻⁵ | 2.80 | 0.0008 | 0.0008 |
| msh6             | 5.2 ± 3.2 × 10⁻⁶ | 0.70 | 0.40 | 0.40 | 1.0 ± 0.4 × 10⁻⁵ | 1.30 | 0.34 | 0.34 |
| msh1*          | 6.2 ± 2.3 × 10⁻⁶ | 0.90 | 0.66 | 0.66 | 4.7 ± 0.9 × 10⁻⁶ | 0.60 | 0.15 | 0.15 |
| exo             | 1.2 ± 0.4 × 10⁻⁵ | 1.70 | 0.12 | 0.12 | 2.2 ± 0.9 × 10⁻⁵ | 2.90 | 0.0066 | 0.0066 |

*Numbers are mean values with SDs.

**Relative to wild type.

**p-values were calculated by a two-tailed Student’s t-test in comparison to wild type.
Reconstituted MMR with *S. cerevisiae* proteins in *vitro* revealed that both MutSα and MutSβ could initiate repair of base-base mismatches and of loops with one, two, or four nucleotides (Bowen et al. 2013). Human MutSα binds to base-base mismatches and to loops with up to eight nucleotides, while MutSβ allows repair of loops with two to about eight nucleotides (Genschel et al. 1998). Our genetic data imply that *S. pombe* Msh6 as part of the MutSα heterodimer is able to bind to loops with up to four unpaired nucleotides. Thus, the substrate spectrum of bacterial MutS and MutSα of *S. pombe* appears to be similar. During evolution, the spectrum had been extended to enable recognition of larger loops in humans. On the other hand, the substrate spectrum of MutSβ considerably differs between species. Human MutSβ supports repair of loops with two to eight nucleotides (Genschel et al. 1998), whereas *S. cerevisiae* MutSβ is also involved in repair of some base-base mismatches besides loop repair (Harrington and Kolodner 2007), and *S. pombe* MutSβ apparently does not have a function in MMR. In addition, some eukaryotes, such as *Caenorhabditis elegans* and *Drosophila melanogaster* do not have an Msh3 ortholog (Marti et al. 2002) and likely carry out MMR with MutSα and MutLα and no other MutS and MutL heterodimers, like *S. pombe* does.

Harrington and Kolodner (2007) interpreted mutation spectra of base substitutions in *S. cerevisiae* msh3 mutants that were different to wild type as a role of MutSβ in MMR of base-base mismatches. We observed differences of *msh3* in mutation spectra for a (GT)$_n$ repeat (Mansour *et al.* 2001), a reduction of recombination events (Tornier *et al.* 2001), and of reversion rates at the (CTGCC)$_6$ repeat (Table 3), an altered reversion spectrum for (GACC)$_8$ (Table 1) and repeat instability of (ATCGTCC)$_5$ (Table 5). We interpret these differences as phenotypes caused by loss of MMR-independent functions of Msh3.

**Does *S. pombe* Exo1 have a function in MMR?**

Exo1 of *S. pombe* was the first eukaryotic exonuclease to be identified as having a function in repair of mismatches (Szankasi and Smith 1995). Further studies with *S. pombe* showed that Exo1 contributes to MMR of base-base mismatches (Rudolph *et al.* 1998), modulates MMR of two-nucleotide loops in nonrepetitive DNA (Marti *et al.* 2003), and has an MMR-independent function in dinucleotide repeat stability (Mansour *et al.* 2001). In the present study, we did not find any evidence for a role of Exo1 in tetra- and penta-nucleotide repeat stability (Table 2 and Table 3). However, we observed that loss of Exo1 caused instability of hepta-nucleotide repeats, in contrast to the MMR mutants *msh2*, *msh6*, and *mlh1* (Table 5). Exo1 also acts in recombination and double-strand break repair (Fiorentini *et al.* 1997; Tsubouchi and Ogawa 2000; Kirkpatrick *et al.* 2000; Cejka 2015). Thus, a defect in a

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**Table 6** Reversion spectra of hepta-nucleotide repeats

| Relevant Genotype | (ATCGTCC)$_5$ΔT $-$14 bp | (ATCGTCC)$_5$ΔT $+$14 bp | (ATCGTCC)$_5$ΔT $-$7 bp | (ATCGTCC)$_5$ΔT $+$7 bp |
|------------------|-----------------|-----------------|-----------------|-----------------|
| Wild type        | 7.5 ± 2.1 $10^{-4}$ | 1               | 7.5 ± 2.1 $10^{-4}$ | 1               |
| msh3             | 1.5 ± 0.6 $10^{-5}$ | 2.8             | 1.5 ± 0.6 $10^{-5}$ | 2.8             |
| exo1             | 1.7 ± 0.9 $10^{-5}$ | 2.9             | 1.7 ± 0.9 $10^{-5}$ | 2.9             |
| msh3 exo1        | 5.7 ± 0.5 $10^{-6}$ | 0.8             | 5.7 ± 0.5 $10^{-6}$ | 0.8             |
| rad2             | 1.5 ± 0.7 $10^{-5}$ | 2               | 1.5 ± 0.7 $10^{-5}$ | 2               |
| rad50            | 1.4 ± 0.4 $10^{-5}$ | 1.9             | 1.4 ± 0.4 $10^{-5}$ | 1.9             |
| rad51            | 1.7 ± 0.6 $10^{-5}$ | 2.3             | 1.7 ± 0.6 $10^{-5}$ | 2.3             |
| msh3 rad2        | 2.1 ± 0.5 $10^{-5}$ | 2.8             | 2.1 ± 0.5 $10^{-5}$ | 2.8             |
| msh3 rad50       | 2.6 ± 0.7 $10^{-5}$ | 3.5             | 2.6 ± 0.7 $10^{-5}$ | 3.5             |
| msh3 rad51       | 2.2 ± 0.1 $10^{-6}$ | 0.3             | 2.2 ± 0.1 $10^{-6}$ | 0.3             |
| exo1 rad50       | 2.7 ± 0.8 $10^{-5}$ | 3.6             | 2.7 ± 0.8 $10^{-5}$ | 3.6             |
| exo1 rad51       | 7.0 ± 3.1 $10^{-4}$ | 0.9             | 7.0 ± 3.1 $10^{-4}$ | 0.9             |

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**Table 7** Reversion rates of (ATCGTCC)$_5$ repeats in msh3, exo1, and rad mutants

| Relevant Genotype | Rate$^a$ | Fold Increase$^b$ | p-Values$^c$ |
|------------------|----------|-----------------|--------------|
| Wild type        | 7.5 ± 2.1 $10^{-4}$ | 1               | vs. WT | vs. msh3 | vs. exo1 | vs. rad |
| msh3             | 2.1 ± 0.6 $10^{-5}$ | 2.8             | 0.0008       |
| exo1             | 2.2 ± 0.9 $10^{-5}$ | 2.9             | 0.0066       |
| msh3 exo1        | 5.7 ± 0.5 $10^{-6}$ | 0.8             | 0.0049       | 0.032 
| rad2             | 1.5 ± 0.7 $10^{-5}$ | 2               | 0.043        |
| rad50            | 1.4 ± 0.4 $10^{-5}$ | 1.9             | 0.016        |
| rad51            | 1.7 ± 0.6 $10^{-5}$ | 2.3             | 0.0087       |
| msh3 rad2        | 2.1 ± 0.5 $10^{-5}$ | 2.8             | 0.98         | 0.24     |
| msh3 rad50       | 2.6 ± 0.7 $10^{-5}$ | 3.5             | 0.26         | 0.033   |
| msh3 rad51       | 2.2 ± 0.1 $10^{-6}$ | 0.3             | 0.0018       | 0.01    |
| exo1 rad50       | 2.7 ± 0.8 $10^{-5}$ | 3.6             | 0.24         | 0.025   |
| exo1 rad51       | 7.0 ± 3.1 $10^{-4}$ | 0.9             | 0.011        | 0.013   |

$^a$Numbers are mean values with SDs. Values from wild type, msh3, and exo1 derived from Table 5 and are shown for comparison.

$^b$Relative to wild type.

$^c$p-values were calculated by a two-tailed Student’s t-test in comparison to the indicated strains. WT, wild type; rad, rad2FEN1, rad50, or rad51 single mutants.
recombination mechanism might cause hepta-nucleotide instability of the S. pombe exo1 mutant rather than MMR deficiency, as discussed below.

Although a nuclease is essential for removal of unpaired nucleotides during MMR, Exo1 does seem to be dispensable for MMR-mediated loop repair in S. pombe. This may be attributed to redundancy with other nucleases. MutLα of S. cerevisiae and humans has endonuclease activity, which is sufficient for completing MMR in the absence of Exo1 (Kadyrov et al. 2006, 2007; Smith et al. 2013; Goellner et al. 2014, 2015). Thus, it is also likely that Exo1 of S. pombe participates in MMR, but that MutLα and maybe other nucleases can replace its function. In fact, the amino acids required for MutLα nuclease activity are all highly conserved between eukaryotes, including S. pombe (Smith et al. 2013), supporting the idea that having endonuclease activity is a general feature of eukaryotic MutLα. In S. cerevisiae, exo1 mutants exhibit weak defects in MMR (Tishkoff et al. 1997; Amin et al. 2001; Smith et al. 2013; Goellner et al. 2014), likely because MutLα and Exo1 nuclease activities are largely redundant. exo1 deletion strains and pmst1 strains with mutations causing endonuclease deficiency generally showed subtle increases of mutation rates, which strongly increased when both mutations were combined (Smith et al. 2013).

Rad51 and Exo1 are involved in error prone repair at (ATCGTCC)₅ repeats in msh3 mutants

Stability of the hepta-nucleotide repeat (ATCGTCC)₅ was influenced by processes involving Msh3, Exo1, Rad27, Msh5, Rad50, and Rad51. Deletions of any of the genes caused a ~2–3-fold increase of reversion rates, which was predominantly due to expansions by one repeat unit and therefore by insertions in the nascent strand (Table 6 and Table 7). Rates were not further increased in the msh3 rad27, msh3 rad50, and exo1 rad50 double mutants. Instead, the msh3 exo1, msh3 rad51, and exo1 rad51 double mutants had lower rates than the respective single mutants. In S. cerevisiae, CAG trinucleotide repeats were unstable in rad51, rad52, and mre11 single mutants (Sundararajan et al. 2010). However, increased rates of repeat expansions in mre11 were largely suppressed by additional mutation of rad52. These data suggest that the MRN complex plays a role in maintaining repeat stability, and that downstream steps of HR in mre11, but not in wild-type background, can carry out error prone recombination at repeats (Sundararajan et al. 2010). In summary, the (ATCGTCC)₅ repeat analyzed in our study might be stabilized by Msh3 and slipped-out loops correctly processed by HR requiring Rad50, Exo1, and Rad51, thereby preventing aberrant events. In the absence of Msh3, the Exo1 and Rad51 proteins might carry out error prone processes, such as misalignment of repeats after strand resection catalyzed by Exo1 and during strand invasion mediated by Rad51.

Conclusions

We conclude from our studies that S. pombe Msh6, as part of MutSo, recognizes base-base mismatches and loops with one to four unpaired nucleotides, while Msh3 does not play a significant role in MMR, but rather maintains repeat stability independently of MMR. Consequently, S. pombe MMR cannot repair loops with five or more nucleotides, in contrast to human MMR (Genschel et al. 1998). Microsatellites with five or six iterated nucleotides are rare in S. pombe (hepta-nucleotide repeats were not analyzed) (Karaoğlu et al. 2005), but are relatively abundant in the human genome (Lander et al. 2001). Thus, to ensure genome stability, humans require repair of larger loops that occur by strand slippage in microsatellites, while larger loops may be formed rarely in S. pombe microsatellites. It is therefore critical for humans, but not for S. pombe, to have an MMR system that can deal with larger loops.

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