A mutated dph3 gene causes sensitivity of Schizosaccharomyces pombe cells to cytotoxic agents

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Original Article

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Abstract Dph3 is involved in diphthamide modification of the eukaryotic translation elongation factor eEF2 and in Elongator-mediated modifications of tRNAs, where a 5-methoxycarbonyl-methyl moiety is added to wobble uridines. Lack of such modifications affects protein synthesis due to inaccurate translation of mRNAs at ribosomes. We have discovered that integration of markers at the msh3 locus of Schizosaccharomyces pombe impaired the function of the nearby located dph3 gene. Such integrations rendered cells sensitive to the cytotoxic drugs hydroxyurea and methyl methanesulfonate. We constructed dph3 and msh3 strains with mutated ATG start codons (ATGmut), which allowed investigating drug sensitivity without potential interference by marker insertions. The dph3-ATGmut and a dph3::loxP-ura4-loxM gene disruption strain, but not msh3-ATGmut, turned out to be sensitive to hydroxyurea and methyl methanesulfonate, likewise the strains with cassettes integrated at the msh3 locus. The fungicide sordarin, which inhibits diphthamide modified eEF2 of Saccharomyces cerevisiae, barely affected survival of wild type and msh3Δ S. pombe cells, while the dph3Δ mutant was sensitive. The msh3-ATG mutation, but not dph3Δ or the dph3-ATG mutation caused a defect in mating-type switching, indicating that the ura4 marker at the dph3 locus did not interfere with Msh3 function. We conclude that Dph3 is required for cellular resistance to the fungicide sordarin and to the cytotoxic drugs hydroxyurea and methyl methanesulfonate. This is likely mediated by efficient translation of proteins in response to DNA damage and replication stress.

Keywords DNA damage response · Elongator · Genome stability · Sordarin · Transcriptional interference · Translation elongation factor eEF2 · tRNA modifications

Introduction

Dph3 is involved in the Dph pathway for a unique diphthamide modification of eukaryotic translation elongation factor 2 (eEF2) (Schaffrath et al. 2014). Modified eEF2 ensures correct movement of tRNAs and mRNAs along ribosomes and allows rapid addition of amino acids to growing peptide chains during translation. Diphthamide modification by Dph1–Dph7 is best understood in Saccharomyces cerevisiae (Schaffrath et al. 2014). Dph3 provides electrons to Dph1–Dph2 for the catalysis of 3-amino-carboxypropyl on a specific histidine of eEF2 (Liu et al. 2004; Dong et al. 2014). Subsequently, Dph5 tetra-methylates the 3-amino-carboxypropyl-histidine, which is then converted to diphthine by Dph7. Finally, Dph6 modifies diphthine to diphthamide (Lin et al. 2014; Schaffrath et al. 2014). Diphthamide
modified eEF2 is a target of bacterial toxins, such as diphtheria toxin, and fungicides such as sordarin (Van Ness et al. 1980; Chen et al. 1985; Domínguez and Martin 1998; Jablonowski and Schaffrath 2007). Diphtheria toxin is an ADP ribosylase of Corynebacterium diphtheria that ADP-ribosylates the diphthamide modification of eEF2; thereby inhibiting its function, which ultimately leads to cell death and causes diphtheria in humans (Schaffrath et al. 2014). Sordarin is a metabolite produced by the fungus Sordaria araneosa and inhibits diphthamide modified eEF2 in S. cerevisiae through a sordarin specificity region located N-terminal to the diphthamide (Shastry et al. 2001). Inhibition by sordarin occurs by blocking ribosomal translocation through stalling of eEF2, thereby preventing translation of mRNAs (Justice et al. 1998; Domínguez et al. 1999). S. cerevisiae and some other fungi are sensitive to sordarin, whereas deletions of any of the DPH genes confer resistance (Domínguez et al. 1999; Shastry et al. 2001; Bär et al. 2008; Uthman et al. 2013).

Dph3 also plays a role in the first step of tRNA wobble uridine modifications carried out by the Elongator complex to form 5-carbonylmethyl-uridine (cm5U34) (Huang et al. 2005; Bär et al. 2008; Greenwood et al. 2009). The cm5U34 modification is methylated to 5-methoxycarbonyl-methyl-uridine (mcm5U34) by Trm9–Trm112 and to 5-carbamoylmethyl-uridine (ncm5U34) by an unknown enzymatic activity (Karlsborn et al. 2014; Deng et al. 2015). The mcm5 modified uridine can be further thiolated to mcm5s2 by the Urm1 pathway (Nakai et al. 2008; Leidel et al. 2009). Both, tRNA modifications and diphthamide-modified eEF2 ensure optimal translation of mRNAs to proteins (Svejstrup 2007; Schaffrath et al. 2014; Gu et al. 2014; Nedialkova and Leidel 2015; Thivaille and de Crécy-Lagard 2015).

In this study, we discovered that replacement of the open reading frame of S. pombe msh3 by gene disruption cassettes interfered with dph3 functions, which rendered cells sensitive to hydroxyurea (HU) and methyl methanesulfonate (MMS). Msh3 is a eukaryotic homologue of bacterial MutS. MutS is a DNA mismatch binding protein that initiates removal of mismatched and unpaired nucleotides, which were incorporated into the nascent strand during replication (Marti et al. 2002; Jiríčková 2013). We verified with dph3 and msh3 strains with mutated ATG start codons (ATGmut) that drug sensitivity was indeed due to an impaired dph3 function. Thus, Dph3 plays a role in response to DNA damage and replication stress, likely through modifications of tRNA and/or eEF2, which allow efficient biosynthesis of DNA damage response proteins.

### Results

**Gene disruptions of msh3 caused HU and MMS sensitivity by interference with functions of the flanking dph3 gene**

The S. pombe msh3 and dph3 genes share an intergenic region of only 268 base pairs (bp) between the two ATG start codons (Wood et al. 2002; http://www.pombase.org/). Data obtained by functional genomics further revealed that the intergenic region constitutes the 5′ untranslated regions (5′ UTR) of the msh3 and dph3 mRNAs, with divergent and likely overlapping promoters (Li et al. 2015) (Fig. 1a). Our initial work aimed to analyse functions of msh3 in genome stability. The original msh3 disruption was constructed before the sequence of the gene was determined (Fleck et al. 1992), thereby deleting parts of both dph3 and msh3; hence termed dph3–msh3::ura4.
(or dph3–msh3Δ) from here on (Fig. 1b). Our previous data revealed that S. pombe Msh3 does not have a function in mismatch repair (MMR) of base–base mismatches and loops of one to four nucleotides (Tornier et al. 2001; Mansour et al. 2001; Marti et al. 2002; Villahermosa et al. 2017). To further understand the functions of MMR factors and particularly of Msh3, we examined sensitivity of dph3–msh3::ura4 and the MMR mutants msh2Δ, msh6Δ, pms1Δ and mlh1Δ to HU and MMS. HU inhibits its ribonucleotide reductase, thereby depleting the dNTP pools, which affects DNA replication and repair. MMS is an alkylating agent that causes DNA breaks. The dph3–msh3::ura4 deletion strain, but none of the MMR mutants msh2Δ, msh6Δ, pms1Δ and mlh1Δ turned out to be sensitive to either drug (Fig. 1c).

Because ura4 in the dph3–msh3::ura4 mutant disrupts two genes, we decided to construct a clean msh3 deletion by replacing the entire open reading frame and no other parts with a kanMX cassette (Fig. 1b). The resulting msh3::kanMX deletion mutant was sensitive to HU and MMS like the original dph3–msh3::ura4 mutant (Fig. 1d). However, when we replaced the open reading frame of msh3 either with an hphMX cassette or with ura4 flanked by lox sites (Fig. 2a), we noticed that drug sensitivity was not necessarily due to loss of Msh3. The msh3::hphMX mutant showed some sensitivity to MMS, but barely to HU, whereas the msh3::loxP-ura4-loxM was not sensitive to either drug (Fig. 2b). The observed effects on drug sensitivity can be explained by the presence of mutations in the intergenic region and by positional effects of the inserted markers, which interfere with dph3 transcription. Sequencing of the 5′ UTR of the msh3::kanMX and msh3::hphMX strains revealed the wild-type sequence, ruling out the first possibility.

**A dph3-ATGmut strain, but not msh3-ATGmut, was sensitive to HU and MMS**

We then decided to follow two approaches for testing whether drug sensitivity was caused by an impaired function of dph3 or of msh3. We treated wild type, dph3Δ and msh3Δ with the fungicide sordarin and we constructed strains with mutated ATG start codons and tested them for sensitivity to HU and MMS. In the case of the msh3-ATGmut strain, we also mutated the fourth and the 31st codons, which both code for methionine (see also Fig. 2a). With the ATG mutations, we avoided that cassette integrations affected the promoters of the genes. The dph3::loxP-ura4-loxM and dph3-ATGmut strains, but not the msh3-ATGmut strain, were sensitive to HU and MMS (Fig. 2b). Thus, defective dph3, but not msh3, clearly caused drug sensitivity. Furthermore, it became evident that integration of kanMX or hphMX at the msh3 locus interfered with dph3 function.

**S. pombe Dph3 confers resistance to sordarin**

Parallel to the experiment with the ATGmut strains, we tested the effect of sordarin on S. pombe strains. We tested the msh3::kanMX strain, which was sensitive to HU and MMS (Fig. 1d), the msh3::loxP-ura4-loxM strain, which was not sensitive to HU or MMS (Fig. 2b), the dph3–msh3::ura4 double deletion strain, and the dph3::loxP-ura4-loxM strain and compared them with wild type. Our original expectation was that an impaired dph3 function causes resistance to sordarin as it has been described for mutations in *S. cerevisiae DPH* genes (Bär et al. 2008; Uthman et al. 2013). However, we learned that S. pombe wild type cells were highly resistant to 50 μg/mL sordarin (Fig. 3a), whilst an *S. cerevisiae* wild type strain was extremely sensitive, even at a low concentration of 20 μg/mL (Fig. 3b), consistent with previous results (ShasTry et al. 2001; Bär et al. 2008; Uthman et al. 2013). At these concentrations, dph3Δ was slightly more sensitive than wild type, while deletions of msh3 had no effect and
the double mutant dph3Δ–msh3Δ behaved like the dph3Δ single mutant (Fig. 3a). When testing sensitivity to high doses of sordarin, it became evident that dph3Δ was more sensitive than wild type, while the msh3Δ single mutant was not more sensitive than wild type. The msh3 deletion strain used was msh3::loxP-ura4-loxM. An msh3::kanMX disruption strain was tested in an independent experiment, which also behaved like wild type (Supplemental Fig. S1). An S. cerevisiae wild type strain was extremely sensitive to sordarin. dph3Δ was sensitive to a high dose of sordarin when compared to wild type. Sordarin treated S. pombe cells were elongated. Wild type and dph3Δ mutant cells, taken from plates with the indicated amounts of sordarin and from the control plate, were analysed by microscopy. The sordarin specificity region of eEF2′s of various yeasts as specified by Shastry et al. (2001). Numbers below the alignment indicate the position of the region within the two identical S. pombe eEF2 proteins encoded by eft201 and eft202. Amino acid residues that differ from the S. cerevisiae sequence are underlined. S. c. Y521S is a mutated form of S. cerevisiae eEF2 (Shastry et al. 2001). S and R to the right of the sequences indicate sordarin sensitivity and resistance, respectively. (R) indicates intermediate resistance.

msh3::kanMX did not cause reduced dph3 expression

Since cassette integrations at the msh3 locus interfered with dph3 function, we were interested to know whether mRNA levels of dph3 were affected in the msh3::kanMX mutant. mRNA levels of wild type and msh3::kanMX strains, either untreated or treated with HU, were determined by semi-quantitative PCR using primers dph3q-F and dph3q-R (Fig. 4a; Supplemental Fig. S2). The forward primer dph3q-F spans intron I, and thus prevents amplification of genomic DNA or unspliced pre-mRNA. We could not find major differences in dph3 expression between wild type and the msh3::kanMX mutant (Fig. 4b, c). Thus, altered dph3

**Fig. 3** S. pombe is resistant to sordarin. a dph3Δ mutants were more sensitive to sordarin than wild type. Deletion of dph3, either alone or in combination with msh3Δ, caused sordarin sensitivity when compared to wild type, while the msh3Δ single mutant was not more sensitive than wild type. The msh3 deletion strain used was msh3::loxP-ura4-loxM. An msh3::kanMX disruption strain was tested in an independent experiment, which also behaved like wild type (Supplemental Fig. S1). b An S. cerevisiae wild type strain was extremely sensitive to sordarin. dph3Δ was sensitive to a high dose of sordarin when compared to wild type. d Sordarin treated S. pombe cells were elongated. Wild type and dph3Δ mutant cells, taken from plates with the indicated amounts of sordarin and from the control plate, were analysed by microscopy. e The sordarin specificity region of eEF2′s of various yeasts as specified by Shastry et al. (2001). Numbers below the alignment indicate the position of the region within the two identical S. pombe eEF2 proteins encoded by eft201 and eft202. Amino acid residues that differ from the S. cerevisiae sequence are underlined. S. c. Y521S is a mutated form of S. cerevisiae eEF2 (Shastry et al. 2001). S and R to the right of the sequences indicate sordarin sensitivity and resistance, respectively. (R) indicates intermediate resistance.
transcription in msh3::kanMX cells occurred in a way not detectable with the methods applied. Although our results were negative, we could not come up with alternative explanations other than that transcriptional interference is responsible for the observed drug sensitivity.

Integration of the ura4 marker at the dph3 locus did not affect the Msh3 function in mating-type switching

Since msh3 disruptions interfered with Dph3 function, we asked whether also the opposite occurred, i.e. whether the dph3::loxP-ura4-loxM cassette integration affected Msh3 function. For this we tested the mating-type switching competence of an h80 dph3::loxP-ura4-loxM mutant. Msh3, formerly known as Swi4, is involved in mating-type switching (Fleck et al. 1990; Egel 2005). Homothallic h80 msh3 mutants form so-called mottled colonies and frequently produce duplications in the mating-type region, leading to heterothallic h+ cells (Egel et al. 1984; Fleck et al. 1990, 1992). Wild-type colonies turned homogeneously brown after iodine staining (Fig. 5a) for explanation of the mating-type switching assay see “Materials and methods”). The h80 msh3::loxP-ura4-loxM and msh3-ATGmut strains formed mottled and iodine-negative (approximately 60%) colonies, indicative for a switching defect. The h80 dph3::loxP-ura4-loxM and h80 dph3-ATGmut mutants formed iodine positive colonies, although less homogeneously stained than wild type (Fig. 5a). The dph3 mutants mainly produced four-spored asci, but unlike wild type, also had many empty zygotes without spores (Fig. 5b). Importantly, mottled and iodine-negative colonies with heterothallic cells were not observed among thousands of colonies. Thus, mating-type switching was not affected, indicating that Msh3 is functional in dph3Δ background.

Discussion

We have discovered that replacement of the S. pombe msh3 gene by kanMX or hphMX interfered with the function of the adjacent dph3 gene. Such msh3 gene disruptions rendered cells sensitive to HU and MMS. The dph3 and msh3 genes are closely linked and transcribed in opposite directions from divergent and likely overlapping promoters or by one bidirectional promoter (Li et al. 2015) (Fig. 1a). It is unlikely that the cassettes replacing msh3 delete part of the 5' UTR or vital promoter elements of dph3, since loxP-ura4-loxM, which replaced essentially the same DNA portion as kanMX and hphMX, was not sensitive to HU or MMS (Fig. 2b). The 34-nucleotide long sequence between the two supposed transcription start sites contains 5'-TTTA-TAT-3', which can be used as TATA box from both directions. Interestingly, many DNA repair genes in the human
genome, including MSH3, share a short promoter region with a flanking gene (Adachi and Lieber 2002). In the case of MSH3, the flanking gene is DHFR, which is involved in the synthesis of nucleic acid precursors (Fujii and Shimada 1989). The DHFR enzyme is a target of the chemotherapeutic agent methotrexate (Goodsell 1999). A methotrexate resistant cell line has been shown to have simultaneously up-regulated DHFR and MSH3, which led to imbalanced MMR complexes and thereby reduced the efficiency of repair of base–base mismatches (Drummond et al. 1997). It is, therefore, conceivable that expression of dph3 and msh3 in S. pombe is also co-regulated.

The cassettes kanMX and hphMX contain the strong TEF promoter of the Ashbya gossypii TEF gene encoding translation elongation factor 1 alpha (Steiner and Philippsen 1994; Bähler et al. 1998; Hentges et al. 2005). In both cases, the TEF promoter is in close proximity to the dph3 promoter. In the msh3::loxP-ura4-loxM disruption, ura4 is expressed by its own promoter, which is also located next to dph3. However, this construction did not cause HU or MMS sensitivity (Fig. 2b). Approximately, five mRNA molecules of the ura4 gene are present in vegetative growing cells (Marguerat et al. 2012). It is, therefore, conceivable that the presence of the strong TEF promoter, but not of the weaker ura4 promoter, interfered with transcription of the dph3 gene. Transcriptional interference of flanking genes can occur naturally and often reflects a regulatory role in gene expression. Transcriptional interference can also be caused by integration of foreign DNA, e.g. by transposons and viruses, or artificially by genetic manipulations (Shearwin et al. 2005). For example, cassette integrations at the mouse MRF4 gene interfered with the function of the nearby Myf5 gene when cassettes were transcribed towards Myf5, but not when transcribed in the opposite direction (Olson et al. 1996). When testing dph3 mRNA levels and size, we could not detect any obvious difference between wild type and the msh3::kanMX mutant (Fig. 4). Thus, although transcriptional interference...
by cassette integration is the likely cause for the observed phenotypes, we were not able to support this by experimental evidence. A study in *S. cerevisiae* revealed that it is a common feature that cassette integrations affect the function of a neighbouring gene (Ben-Shitrit et al. 2012). Among nine selected genes, only five showed a significantly altered mRNA level. Thus, transcriptional interference of the remaining four genes, as for the *S. pombe dph3* gene, likely occurred by subtle changes in expression or by structural changes of the mRNAs, which were not detectable with the methods applied.

We noticed that colonies of the *h90 dph3::loxP-ura4-loxM* and *h90 dph3-ATGmut* strains were less homogeneously stained by iodine vapour than *h90* wild-type colonies (Fig. 5a). Both *dph3* mutants contained many zygotes without spores (Fig. 5b). On the other hand and in contrast to *msh3* mutants, mottled and iodine negative (heterothallic) colonies were not formed. Thus, replacement of *dph3* by the *ura4* marker did not disrupt the Msh3 function in mating-type switching. The phenotype of reduced sporulation of *dph3* mutants is unrelated to Msh3 functions, as *dph3-ATGmut* does not have an integrated cassette that may interfere with *msh3* expression.

Mutated *dph3* (*kit11), like the other *dph* mutated genes in *S. cerevisiae* causes resistance to diphertheria toxin and sordarin due to the lack of the diphthamide modification of eEF2 (Schaffrath et al. 2014). *dph3* mutants are also resistant to the killer toxin of *Kluyveromyces lactis*, like Elongator (*elp*) mutants and unlike the other *dph* mutants (Fichtner and Schaffrath 2002; Bär et al. 2008). In this case, resistance was due to the absence of a mcm5_U34 modification of tRNAs, which the killer toxin requires for cleavage of the tRNAs. We have found that *S. pombe* wild type cells were highly resistant to the fungicide sordinar, but became sensitive when *dph3* was deleted (Fig. 3). This is in contrast to *S. cerevisiae*, where wild type cells are extremely sensitive and deletions of any of the *DPH* genes confer resistance (Bär et al. 2008; Uthman et al. 2013). The different impact of sordinar on wild type cells of both yeasts is likely due to differences in the sordinar specificity region of the eEF2 proteins as defined by Shastry et al. (2001) (Fig. 3e). The reason why *S. pombe dph3Δ* is sensitive to sordinar is currently unknown and should be investigated in future studies. One possibility is that lack or reduction of tRNA modifications exacerbate the effect of sordinar, which stalls eEF2 on ribosomes and thereby blocks ribosomal translocation during translation of mRNAs (Justice et al. 1998; Domínguez et al. 1999). However, this would mean that high sordinar concentrations can inhibit eEF2 without the diphthamide modification. We further found that wild type and *dph3Δ* mutant cells were elongated when treated with sordinar (Fig. 3d), indicating a defect in cell cycle progression, where cells were able to grow but had problems to divide.

In *S. pombe elp3* mutants, protein levels of Cdr2, Atf1, and Pcr1 are low (Bauer et al. 2012; Fernández-Vázquez et al. 2013). The Cdr2 kinase positively regulates the G2/M transition of the cell cycle, while Atf1-Pcr1 controls cellular stress responses on the transcriptional level. *elp3* mutants were sensitive to hydrogen peroxide, rapamycin and to incubation at 36 °C. These phenotypes could be rescued by elevated levels of tRNA_Lys expressed from multi-copy plasmids (Bauer et al. 2012; Fernández-Vázquez et al. 2013). This demonstrates that the inability to modify this tRNA species in *elp3* cells caused the observed phenotypes. Lack of the mcma_U34 modification leads to inefficient translation of proteins with a high number of AAA codons (vs. AAG) for lysine and low protein levels in *elp3* mutants (Bauer et al. 2012; Fernández-Vázquez et al. 2013). Indeed, when the AAA codons of *cdr2* or *atf1* were changed to AAG, protein levels returned to the wild-type level (Bauer et al. 2012; Fernández-Vázquez et al. 2013). Since Dph3 acts together with Elp3 in tRNA modifications, it is plausible that lack of such modifications affect synthesis of specific proteins required for a proper response of the cells to HU and/or MMS treatment.

We have found in the present study that *S. pombe dph3* mutations caused sensitivity to the cytotoxic agents HU and MMS. Thus, Dph3 acts in response to DNA damage and replication stress, likely by tRNA and/or eEF2 modifications, which ensure efficient translation of proteins required for such processes. To our knowledge, a function for Dph3 in response to DNA damaging drugs has not been described yet. An *S. cerevisiae elp3* mutant showed some sensitivity to HU and MMS, which was further increased in double mutants additionally defective in the histone acetyltransferase Rtt109, the histone chaperones Asf1 and Cac1 or mutated in PCNA (Li et al. 2009). Future work should address how other *S. pombe* mutants of the Dph pathway and of the Elongator complex react to DNA damaging agents and which phenotypes are due to impaired tRNA modifications or loss of the eEF2 diphthamide modification. Such studies might give valuable information about the choice of drugs in cancer chemotherapy in relation to the status of the Dph and Elongator pathways in tumour cells. It has been found that approximately 80% of ovarian cancers have *DPH1* gene deletions and that ELP3 is up-regulated in breast cancer cells, which in turn leads to increased levels of the oncoprotein DEK (Chen and Behringer 2004, 2005; Kong et al. 2011; Delaunay et al. 2016). *DPH3* promoter mutations are present in up to 16% of melanomas and silencing of *DPH3*
in mouse melanoma cells impairs metastasis (Wang et al. 2012; Denisova et al. 2015; Fredriksson et al. 2014). Thus, a combination of drug treatment with targeting specific factors of the modification pathways appears to be a sensible approach to sensitize rapidly growing cancer cells. Finally, it is important to better understand the antifungal mode of action of drugs such as sordarin for selective and efficient killing of pathogenic fungal strains, which is especially crucial for immunocompromised patients, for example for those suffering from AIDS or cancer.

Materials and methods

Yeast media, genetic methods and strains

Yeast media were YEA (yeast extract agar), YEL (yeast extract liquid), MEA (malt extract agar) and MMA (minimal medium agar) as described (Gutz et al. 1974) with the following modified composition of MMA: 0.17% yeast nitrogen base without amino acids/ammonium sulphate/thiamine, 0.5% ammonium sulphate, 1% glucose, 1.8% granulated agar (Formedium). The supplements adenine, histidine, leucine and uracil, were added at concentrations of 100 mg/L to complex media and MEA, and to MMA where required. G418 (100 mg/L) and 5-fluoroorotic acid (1 g/L) were added to MM. G418 (100 mg/L) to complex media and MEA, and to MMA (200 mg/L) and 5-fluoroorotic acid (1 g/L) were added to YEA after autoclaving. Drug containing plates were prepared 2 days before use and until then stored at room temperature. Strains were grown overnight to stationary phase prepared 2 days before use and until then stored at room temperature. Strains were grown overnight to stationary phase and cell titres were determined with a haemocytometer. Cultures were diluted to 10^7 cells/mL in sterile H2O and three consecutive 1:10 dilutions were prepared. Ten micro-liter of all four dilutions were spotted on drug containing plates and on control plates without drugs. Images were taken with a Gel Doc 2000 system (Bio-Rad) after 3 days of incubation at 30 °C. Drugs used were HU (Formedium), drug containing liquid), MEA (malt extract agar) and MMA (mini-
Primers for amplification of \textit{msh3} were \textit{msh3\_pAW8\_For} 5’-GCTATGGCGGCGCCGATACTCGTATAGCATACT ATATAACCTAAGTTATAGCACCACCCCACACCGCA CGAAGGGAGAAAGGAGAATGAGGATAACTATG-3’ and \textit{msh3\_pAW8\_Rev} 5’-TTCGCGGCGGCGCATACCTCGT ATATAAATTACATGCAATGGATTACGCTCTTCC AAAGGCTAAG-3’. Nucleotides underlined in \textit{msh3\_pAW8\_For} and the second codon of \textit{msh3}. Nucleotides underlined in \textit{msh3\_pAW8\_Rev} 5’ changed the two last codons of \textit{msh3} from GAA ATC (encoding the amino acids glutamic acid and isoleucine) to GAG CTC (encoding glutamic acid and leucine), thereby introducing a \textit{SacI} restriction site, allowing further manipulation if desired.

**Construction of \textit{msh3} and \textit{dph3} strains with mutated ATG start codons**

Strain DE5 (h\(^{-}\) \textit{dph3-ATGmut} \textit{ura4-D18}) was constructed by transformation of DE4 with a PCR fragment obtained with primers \textit{dph3\_ATGmut\_For} 5’-TAGCTT GTAGTTTTTCTATGTGCGTCTCCACATATAAAAACATTTTTGTTGAGGCGACGCACCT TCTGGCACTGATGCTATGGAAGCCGGCAAA-TGA TCATTTCAGCAAGAATTCG-3’ and \textit{dph3\_Rev} 5’-TCAC GATGTAAGAGTAGCCTCTACTCTTCTGTAATTTCAAGATATTTGGAGAATAATAGAGTT AAAACACAAATAGAAATATAGGAAAAATTT TTGCTGCAATGATTATAGGTAAGATAGATG-3’ and as template genomic DNA of strain RO144 (\textit{smt}0). “-” in primer \textit{dph3\_ATGmut\_For} indicates deletion of an A of the wild type sequence, which causes an in-frame TGA stop codon instead of the ATG start codon. By this procedure, the \textit{lox} sites flanking \textit{dph3} in DE4 were replaced in DE5. Sequencing confirmed the correct mutation in DE5 and an additional T155A point mutation, which is in intron II of the \textit{dph3} gene.

DE7 (\textit{smt}0 \textit{msh3-ATGmut} \textit{leu1-32} \textit{ura4-D18}) originated from the transformation of KK83 with a PCR fragment obtained from a mutagenized pAW8-\textit{msh3} plasmid as template. This plasmid contains the \textit{msh3} gene, with the 31st codon mutated from ATG to ATC by site directed mutagenesis with a QuickChange lightizing site-directed mutagenesis kit (Agilent Technologies) using primers \textit{msh3\_M31I\_S} 5’-GGAGAACATATCAGAAGATATCGTGTCCATGAGCAG-3’ and \textit{msh3\_M31I\_AS} 5’-CTGGACACCTGACGGGCAAGCTATCTTCTGTATTTGCCTCC-3’. Nucleotides deviating from the wild-type sequence are underlined. Primers for amplifying the 5’ part of the open reading frame of \textit{msh3} with ATG mutations at codons 1 and 4 were \textit{msh3\_ATGmut\_For} 5’-TTCGCGCTATATTCATTTAGA ACTATAAATGCGTTCACAAATCTATGTGAGCA AATTTGATTGTCAGTTTTATTTGCTGTTTATTCA TCGAGAGGATAGGATATGTCAGTTACATTACCTAGT-3’ and \textit{msh3\_Rev} 5’-CTTAAACTCTGATAGTGTGCTTAA ATTTATTTATACAGTAAATCTTTATGTTGTGAA GTATCTCATTATGATATATATGCTGATTATG-3’. Primer \textit{msh3\_ATGmut\_For2} contained base substitutions (underlined), which changed the ATG start codon of \textit{msh3} to TCG and the fourth codon from ATG to TAG. After integration into the genome, the mutated \textit{msh3} gene was amplified by PCR and sequencing confirmed the three desired ATG mutations. In addition, we found A555G, C696T and T1112C mutations. The primers used for PCR to construct an ATG mutated \textit{msh3} strain were designed in such a way that the (His)\(_6\) tag, the glycine linker and the \textit{lox} sites of plasmid pAW8-\textit{msh3-M31I} were not present in the genome of the resulting DE7 strain.

**\textit{dph3} expression**

10 mL cultures were grown in YEL at 30 °C to a density of approximately 10\(^8\) cells/mL and 5 mL were taken out, pelleted, frozen in liquid nitrogen and stored at −80 °C. 20 mM HU was added to the remaining cultures, which were further incubated for 4 h. Cells were pelleted, frozen in liquid nitrogen and stored at −80 °C. RNA was isolated from untreated and HU-treated cells using a MasterPure Yeast RNA Purification kit (epicentre) including DNase I treatment. 2 µg of RNA were reverse transcribed with oligo (dT)\(_{18}\) primers using a Tetro cDNA Synthesis kit (Bioline). The resulting cDNA was subjected to 22–35 cycles of 30 s at 94 °C, 30 s at 57 °C and 30 s at 72 °C and a final extension for 10 min at 72 °C. For amplification of \textit{dph3} cDNA the forward primer \textit{dph3q-F} 5’-AGATTTCACTGTTT GACGCCG-3’ (spanning intron I of \textit{dph3}, and thus prevents amplification of contaminating genomic DNA) and the reverse primer \textit{dph3q-R} 5’-CTTGGAACCAAGGCAAACATC-3’ was used. In a pilot experiment (Supplemental Fig. S3), we used primers \textit{dph3\_cPCR\_For} 5’-ATGATGAAGGTTCTGAATGGAAGTTG-3’ and \textit{dph3\_cPCR\_Rev} 5’-GAACAAACCAAAGCTACTTGCCCAATTCAAAACC-3’. Other reverse primers for amplification of \textit{dph3} cDNAs of various sizes were \textit{dph3q-R\#2} 5’-TCACCCGGACATCAGCGCTG-3’, \textit{dph3q-R\#3} 5’-AGGGTGCTCTGAAGACATCTG-3’, \textit{dph3q-R\#4} 5’-GTA GCCCTCCTATCCCTCGT-3’, \textit{dph3q-R\#5} 5’-TGGAAA ATGCGTACGCTCAGAA-3’, \textit{dph3q-R\#6} 5’-AGGTGGGCTTTTATTTTTCGAGT-3’, \textit{dph3q-R\#7} 5’-ACGTTGGGCAACAAGTACGGA-3’ and \textit{dph3q-R\#8} 5’-CCACTGTGGTCGTTGAC-3’. Primers for amplification of act1 cDNA were act1-1For 5’-AAGTCACCCCATGTCAGCACGG-3’ and
act1-Rev 5’-CAGTCAACAAGCAAGGGTGC-3’. PCR products were separated on 1.5% agarose gels in Tris–Borate-EDTA buffer and images taken with a Gel Doc 2000 system (Bio-Rad). Band intensities of PCR products were quantified with the ImageJ software (NIH). dph3-specific bands were normalised to blanks to subtract background and to act1 cDNA.

Mating-type switching assay

Iodine vapour stains spores but not vegetative S. pombe cells (Forsburg and Rhind 2006). On sporulation medium, colonies of a switching competent h90 wild-type strain turn homogeneously brown when stained with iodine, while colonies of non-sporulating heterothallic strains are yellow. h90 msh3 mutants form mottled colonies and frequently segregate iodine-negative h+ colonies due to duplications in the mating-type region (Egel et al. 1984; Fleck et al. 1990). Images of iodine-stained colonies were taken with a LEICA MZ10F microscope using LEICA Application Suite V3 software. To determine the frequency of heterothallic colonies, three h90 colonies of each strain were streaked on MEA and grown for 3 days at 30 °C. From each original h90 colony, approximately 300–500 colonies were inspected after iodine staining.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

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