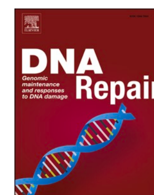
DNA Repair 125 (2023) 103484

Contents lists available at ScienceDirect

DNA Repair

journal homepage: www.elsevier.com/locate/dnarepair



A novel role for Mms2 in the control of spontaneous mutagenesis and Pol3 abundance

Michal Krawczyk [1], Agnieszka Halas, Ewa Sledziewska-Gojska *

Laboratory of Mutagenesis and DNA Damage Tolerance, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02-106 Warsaw, Poland

ARTICLE INFO

Keywords:
Spontaneous mutagenesis
Mms2
Pol3
DNA polymerase zeta
DNA polymerase delta
S. cerevisiae

ABSTRACT

Mms2 is a ubiquitin E2-variant protein with a very well-documented function in the tolerance pathway that protects both human and yeast cells from the lethal and mutagenic effects of DNA damage. Interestingly, a high expression level of human *MMS2* is associated with poor survival prognosis in different cancer diseases. Here we have analyzed the physiological effects of Mms2 overproduction in yeast cells. We show that an increased level of this protein causes a spontaneous mutator effect independent of Ubc13, a cognate partner of Mms2 in the PCNA-polyubiquitinating complex responsible for the template switch. Instead, this new promutagenic role of Mms2 requires Ubc4 (E2) and two ubiquitin ligases of HECT and RING families, Rsp5 and Not4, respectively. We have established that the promutagenic activity of Mms2 is dependent on the activities of error-prone DNA polymerase ζ and Rev1. Additionally, it requires the ubiquitination of K164 in PCNA which facilitates recruitment of these translesion polymerases to the replication complex. Importantly, we have established also that the cellular abundance of Mms2 influences the cellular level of Pol3, the catalytic subunit of replicative DNA polymerase δ. Lack of Mms2 increases the Pol3 abundance, whereas in response to Mms2 overproduction the Pol3 level decreases. We hypothesize that increased levels of spontaneous mutagenesis may result from the Mms2-induced reduction in Pol3 accumulation leading to increased participation of error-prone polymerase ζ in the replication complex

1. Introduction

Spontaneous mutations, on the one hand, play an important role in the evolution of species and adaptation processes, but, on the other hand, contribute to ageing, the development of cancer, and genetic diseases. Therefore, recognition of the endogenous cellular processes influencing the level of spontaneous mutagenesis is of great importance.

Yeast Mms2, as well as its homologues in other eukaryotes, function in the DNA damage tolerance (DDT) pathway which limits the level of mutagenesis, both spontaneous and induced by DNA damage. The main activity of Mms2 is connected to the formation of specific polyubiquitin chains serving in posttranslational protein modification [1]. Protein ubiquitination requires consecutive activities of several classes of enzymes: ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3) [2]. In this process, the ubiquitin is sequentially transferred from one enzyme to the next and finally to a target protein. Proteins can be modified by addition of a single ubiquitin or chain of ubiquitins. Ubiquitins can form a chain by conjugation to one of the seven lysine (K) residues in the acceptor ubiquitin. Best characterized and most frequent ubiquitin chains, which form a signal for proteasomal degradation of modified protein, are linked through K48 [3]· Less frequent polyubiquitin chains are linked via other lysines in the ubiquitin. Functions of these alterative chains are associated with altering protein interactions or subcellular localization and are often unrelated to proteasomal degradation [4].

Mms2 belongs to a family of ubiquitin conjugating-like proteins called UEVs (ubiquitin E2-variant proteins). These proteins contain a ubiquitin-conjugating catalytic fold, a structure typical for E2 enzymes, and are able to bind to ubiquitin. However, UEVs lack the active cysteine residue responsible for the attachment of ubiquitin's carboxyl group of C-terminal glycine to an amino group within the modified protein. UEVs function predominantly as heterodimers with Ubc13 (E2), and these complexes are responsible for the assembly of polyubiquitin chains linked via K^{63} [1,4]. K^{63}- linked polyubiquitin chains play important signaling functions in various aspects of the DNA damage response, endocytosis, protein sorting, translation, and inflammatory response

* Corresponding author.
E-mail address: esg@ibb.waw.pl (E. Sledziewska-Gojska).
[1] Current address: Genome Stability Research Group, Institute of Enzymology Research Centre for Natural Sciences, 1117 Budapest, Hungary.

https://doi.org/10.1016/j.dnarep.2023.103484
Received 21 December 2022; Received in revised form 27 February 2023; Accepted 9 March 2023
Available online 13 March 2023
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[5–8].

Mms2 is the only representative of the UEV-family in the budding yeast. The best characterized and ubiquitously conserved function of Mms2 is associated with the error-free DNA damage tolerance (DDT) pathway. DDT allows the completing DNA replication perturbed by a template damage or defects of replicating machinery. The activities of DNA damage tolerance pathways are orchestrated by the ubiquitination of the replication processivity clamp PCNA [8]. In response to replication stress, K164 of PCNA is monoubiquitinated by the Rad6-Rad18 (E2-E3) ubiquitination complex which facilitates the recruitment of DNA damage-tolerant polymerases to stalled replication forks [9]. These polymerases, called translesion synthesis (TLS) polymerases, often function in an error-prone manner [10]. Polyubiquitination of the monoubiquitinated PCNA by the attachment of a chain of ubiquitins linked via K^{63} initiates a second pathway of DDT called damage avoidance or template switch (TS). In this pathway the replicative DNA polymerase avoids synthesis through DNA damage by a transient use of the intact sister chromatid strand as the replicated template. This pathway of DDT is error-free. PCNA polyubiquitination is mediated by the E2 complex Mms2-Ubc13 in concert with the RING ubiquitin ligase Rad5 [7,11]. Consistent with the role of TS in damage tolerance, the expression of the genes encoding Mms2 and Ubc13 is induced in response to DNA damage [12]. In addition to the initiation of TS, the ubiquitination activity of the Mms2-Ubc13-Rad5 complex is postulated to modulate homologous recombination in yeast [13–15]. Moreover, a specialized ubiquitin conjugating activity of Mms2-Ubc13 complex has been shown to mediate a delay of G2/M cell cycle transition by stimulating autoubiquitination of the RING ubiquitin ligase, Chf2 [16]. There are also indications that Mms2 can physically interact with HECT ubiquitin ligase Rsp5 [17,18]. However, the significance of this interaction has not been revealed so far.

A dysfunction of the yeast Mms2, as well as of Ubc13 and Rad5, causes a DNA damage sensitivity and increases both spontaneous and DNA damage-induced mutagenesis [19]. This mutator effect results from a take-over of the replication of damaged DNA templates by error-prone TLS polymerase ζ (Pol ζ) in the absence of TS. Similarly, defects of human homologs of Mms2, Ubc13 and Rad5 abolish the PCNA modification by attachment of the K63-linked polyubiquitin chain and cause genomic instability [20]. Interestingly, a high expression level of hMms2 (UBE2V2) is associated with a poor survival prognosis in renal cancer (The Human Protein Atlas), estrogen receptor-positive /HER--negative breast cancer [21], melanoma [22], and lung adenocarcinoma [23]. The mechanism of these effects is not fully understood.

Here we show that overexpression of Mms2 in the budding yeast causes increased spontaneous mutagenesis. This mutator effect is Ubc13-independent, but it requires Ubc4, Rsp5 and Not4 ubiquitinating enzymes. Additionally, it depends on Pol ζ and PCNA monoubiquitination. We also show that the cellular abundance of Mms2 inversely correlates with the level of Pol3, the catalytic subunit of replicative polymerase delta (Pol δ). Our findings suggest that the promutagenic activity of Mms2 results from the increased participation of Pol ζ in DNA synthesis associated with limited Pol3 accumulation.

2. Material and methods

2.1. *S. cerevisiae* strains and plasmid construction

The *S. cerevisiae* strains used in this study are listed in Table S1. Yeast strains were constructed by targeted gene disruption via direct transformation of relevant host cells with PCR-amplified disruption cassettes. The desired integrants were verified by PCR. All *kanMX4* disruption cassettes were amplified through PCR, using genomic DNA from appropriate BY4741 derivatives (Euroscarf) carrying deletions of ORFs of interest, with the corresponding primers A and D from the Saccharomyces Genome Deletion Project. Strains disrupted with *natMX4* or *hphMX4* cassettes were constructed by replacing the *kanMX4* marker in the respective BY4741 *kanMX4* strains (Euroscarf) with the *natMX4* or *hphMX4* marker according to the procedure of [24]. The *siz1::URA3* cassette was constructed as previously described [14]. The construction of *rsp5* mutant strains had been done by integrating plasmids using the procedure described in Gajewska et al. [25]. The *ubc4* mutant strains were constructed with the use of the integrating plasmids described in Stoll et al. [26]. The YFP-tagging of Rfa1 protein was done with a cassette amplified from pRYL24 plasmid described in Jedrychowska et al. [27]. The SC2757 strain (Euroscarf) carrying the *MMS2-TAP* fusion was used to estimate the level of Mms2 produced from its native locus. A DNA extract from this strain was used also as a template for PCR to construct the plasmid expressing *MET25-MMS2-TAP* fusion.

The plasmids carrying *MMS2* and *MMS2-TAP* under *MET25* or *GPD* promoter were constructed by cloning *MMS2* or *MMS2-TAP* ORF from genomic DNA. The primers used for amplifying *MMS2* and *MMS2-TAP* include sequence for restriction sites for *BamHI* or *HindIII* or *EcoRI* (Table S2) which enables the introduction of PCR products into p425*MET*, p423*MET* and p416*GPD* plasmids. Other plasmids, derivatives of p306 and p414 with *MMS2* and *MMS2-TAP* under *GPD* promoter, were obtained by subcloning *MMS2* and *MMS2-TAP* from the plasmids mentioned above. The plasmid carrying *UBC13* under *MET25* promoter was constructed by cloning *UBC13* ORF from genomic DNA. The primers used for amplifying *UBC13* include sequence for restriction sites for *EcoRI* or *XhoI* (Table S1) which enables the introduction of PCR product into p423*MET* plasmid. The plasmid carrying the *pol30K107R* variant was constructed by cloning the *POL30* gene under native promoter from the pBL230 plasmid [28] with oligonucleotides introducing a mutation changing K107 to arginine and primers including restriction sites for *PstI* and *XhoI*.

2.2. Growth conditions

Yeast cultures were grown at 30 °C in standard media: non-selective yeast rich medium (YPD) (1 % yeast extract, 2 % peptone and 2 % glucose) and minimal medium (SD) (0.67 % yeast nitrogen base without amino acids, 2 % glucose) supplemented with required amino acids and nitrogenous bases.

2.3. Spontaneous mutagenesis assay

To determine *trp1–1* reversion rates, yeast strains were cultured at 30 °C to the logarithmic growth phase (OD_{600} 0.8) in minimal SD medium supplemented according to the nutritional requirements of a strain. The number of mutant cells was estimated by plating 100 μl of the undiluted cultures on minimal plates supplemented with all nutritional requirements except tryptophan. For the detection of Can^R forward mutants, cultures were plated on a complete minimal medium (lacking arginine) containing 30 μg/ml L-canavanine sulfate (Sigma Aldrich). Canavanine-resistant colonies were counted following incubation of the plates for 4–5 days at 30 °C. To estimate the number of all viable cells, serial dilutions of the cultures were plated on fully supplemented minimal plates and incubated at 30 °C for 2–3 days. Mutation rates were calculated using the Maximum Likelihood Estimate (MLE) method [29, 30] with a Newton-Raphson-type algorithm modified to account for a partial plating, available in a free R package rSalvador [31,32]. This calculator also computes 95 % confidence intervals and employs Likelihood Ratio Test to calculate the statistical significance of the differences between mutation rates of various strains [33]. To account for multiple comparisons, the P-values were adjusted using the Benjamini-Hochberg procedure [34]. Data from 3 to 6 independent experiments with at least 6 cultures in each experiment were used. At least two independently isolated strains of each genotype were used in the assays.

2.4. Determination of UV radiation sensitivity and UV-induced mutagenesis

To determine the UV sensitivity of yeast strains a series of tenfold dilutions of each of the overnight yeast cultures were made in sterile distilled water and 3 μl was withdrawn from each tube to spot on fully supplemented minimal plates. To determine the frequency of UV-induced mutagenesis, the assay was performed similarly to that applied for spontaneous mutagenesis. After plating on minimal plates supplemented with all nutritional requirements except tryptophan (for estimation of TRP^+ reversions) or complete minimal medium (for survival estimation) cells were exposed to specified doses of UV radiation (254 nm) using a UV cross-linker (UVP model CL-1000), and incubated at 30 °C in the dark for 2–5 days.

The mutation frequency was calculated by dividing the number of TRP^+ colonies by the viable cell number. The results for TRP^+ reversion represent the mean values from 3 independent experiments with at least 5 cultures in each experiment. At least two independently isolated strains of each genotype were used in the assays. *P*-values for statistical differences of mutant frequencies between analyzed strains were determined using the unpaired Student's t-test.

2.5. Cell synchronization

Yeast strains were grown in minimal SD medium supplemented according to the nutritional requirements at 30 °C until they reached an OD_{600} 0.7. Cell growth was arrested in the G1-phase by treatment with α factor for 3 h at the same temperature. The α factor was added to cultures in two doses: at 1 mg/ml for C10–15a-derived strains strain, or, for strains with Δ*bar1* mutation, 100 ng/ml; first dose was added at the beginning, and the second dose after 90 min of synchronization. For cell cycle progression experiments, α factor was washed away three times with water, and the cells were resuspended in fresh SD medium containing pronase (75 μg/ml) and incubated for desired time.

2.6. Cell cycle monitoring by flow cytometry

Cell synchronization and progression of the cell cycle were monitored by flow cytometry. 1 ml of a yeast culture was centrifuged (13,000 rpm for 1 min) and harvested cells were subjected to permeabilization and fixation via suspension in 1 ml of chilled (−20 °C) 70 % ethanol (Polmos, Warsaw, Poland). Ethanol-fixed cells were harvested, washed, and resuspended in 1 ml of sodium citrate (50 mM, pH 7,0). After brief sonication, they were treated with RNaseA (0.25 mg/ml) at 50 °C for 1 h and with proteinase K (1 mg/ml) for another hour at 50 °C. Then, samples were diluted in sodium citrate containing propidium iodide (16 μg/ml) and incubated overnight at 4 °C. The DNA content was identified by measuring the propidium iodide fluorescence signal (FL2) using Becton Dickinson FACSCalibur and the CellQuest software (BD Bioscience).

2.7. Western blotting

For Western blotting, 2 ml of cells were collected by centrifugation and whole-cell extracts were prepared as previously described by Knop et al. [35]. Finally, pellet was resuspended in 50 μl of Laemmli buffer with Yeast/Fungal Protease Arrest 1000x (GBiosciences) and stored at − 20 °C. Equal volumes of cell extracts were separated by electrophoresis by SDS-PAGE (8 % polyacrylamide gel for POL3-HA and 12 % polyacrylamide gel for PCNA, Rad53, Mms2-TAP) and the proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Amersham) overnight. Blots were blocked for 2 h in 5 % (w/v) non-fat dried milk in TBST (25 mM Tris–HCl (pH 7.5), 137 mM NaCl, 27 mM KCl, 0.1% (v/v Tween-20) before probing with primary antibodies.

The following primary antibodies were used: rabbit peroxidase-anti-peroxidase affinity-isolated antibody (PAP, Sigma-Aldrich) to detect the protein A tag, mouse monoclonal anti-Pgk1 primary antibody (Abcam), mouse anti-actin monoclonal antibody (Millipore), mouse anti-HA (Abnova), goat anti-Rad53 (Santa Cruz), rabbit anti-Clb2 (Santa Cruz). PCNA was detected by rabbit polyclonal antibodies kindly provided by Bruce Stillman. Then, after washing with TBST buffer, the following secondary antibodies were used: goat anti-mouse HRP-conjugated immunoglobulin (Dako), goat anti-rabbit HRP IgG (Santa Cruz), and donkey anti-goat HRP IgG (Santa Cruz). The signal was detected using a chemiluminescent substrate for HRP (Clarity Western ECL substrate- Bio Rad) using a CCD gel imager. The resulting bands were quantified using ImageJ 1.47 software (NIH, USA). The quantification procedure always included normalization to the levels of Pgk1 or Act1. Loading control and target protein band intensities were quantified within the linear range of detection (unsaturated WB signal).

2.8. Fluorescence microscopy

Yeast strains containing the chromosomal fusion of *RFA1-YFP* were grown at 30 °C to exponential phase in SD medium supplemented with the required amino acids and nitrogenous bases. The Rfa1-YFP foci were examined using a Zeiss Axio Imager2. Images were processed with the AxioVision software. At least 2000 cells were screened for each of two biological repeats.

3. Results

3.1. Overexpression of Mms2 causes an increased level of spontaneous mutagenesis

To analyze the cellular effects of Mms2 overexpression we transformed yeast cells with pMET25MMS2, a multicopy expression plasmid, bearing the *MMS2* ORF under the *MET25* promoter. In parallel, to compare the cellular levels of the Mms2 produced from either the multicopy plasmid or the native *MMS2* locus, we constructed a plasmid expressing *MET25-MMS2-TAP* fusion. The presence of the multicopy expression plasmid pMET25MMS2-TAP caused an over 30-fold increase in the level of Mms2 as compared to the level produced from the *MMS2-TAP* fusion in the native *MMS2* locus (Fig. S1). The overproduction of Mms2 had no effect on either the cell cycle progression or sensitivity to UV radiation (Fig. 1 A and B). However, the elevated level of Mms2 caused up to 5-fold increase in the reversion rate of *trp1–1* nonsense mutation and 3-fold increase in the rate of forward mutations resulting in canavanine resistance ($CAN1^R$) (Fig. 1 C). Interestingly, an increase in the cellular abundance of Mms2 did not influence the level of UV-induced mutagenesis Fig. 1D).

3.2. The mutator effect of Mms2 overproduction is entirely dependent on Pol ζ activity and PCNA ubiquitination

To shed some light on the origin of mutations associated with an increased cellular level of Mms2, we studied the role of error-prone Pol ζ in the mutagenesis. Deletion of the *REV3* gene encoding the enzymatic subunit of Pol ζ entirely abolished the mutagenic effect of Mms2 overproduction (Fig. 2 A). The same effect was observed in cells devoid of Rev7, the accessory subunit of Pol ζ The Mms2-mediated mutagenesis turned out to be also dependent on Pol32 (Fig. 2 A), suggesting that this mutagenesis required the activity of the four-subunit form of Pol ζ (Pol ζ_4) In agreement with this suggestion, introduction of the *rev3–cysB* (*rev3–CC1449,1473SS*) allele, that specifically compromises the formation of Pol ζ_4 [38], abolished the Mms2-induced mutagenesis (Fig. 2B). The TLS activity of Pol ζ also requires its interaction with Rev1, which is responsible for the recruitment of Pol ζ to a stalled replication complex [39], and the promutagenic activity of Mms2 was eliminated in cells devoid of Rev1 (Fig. 2 A). The Pol ζ recruitment requires also PCNA-K164 monoubiquitination [9]. To examine the role of PCNA modification in the Mms2-induced mutagenesis, we used the

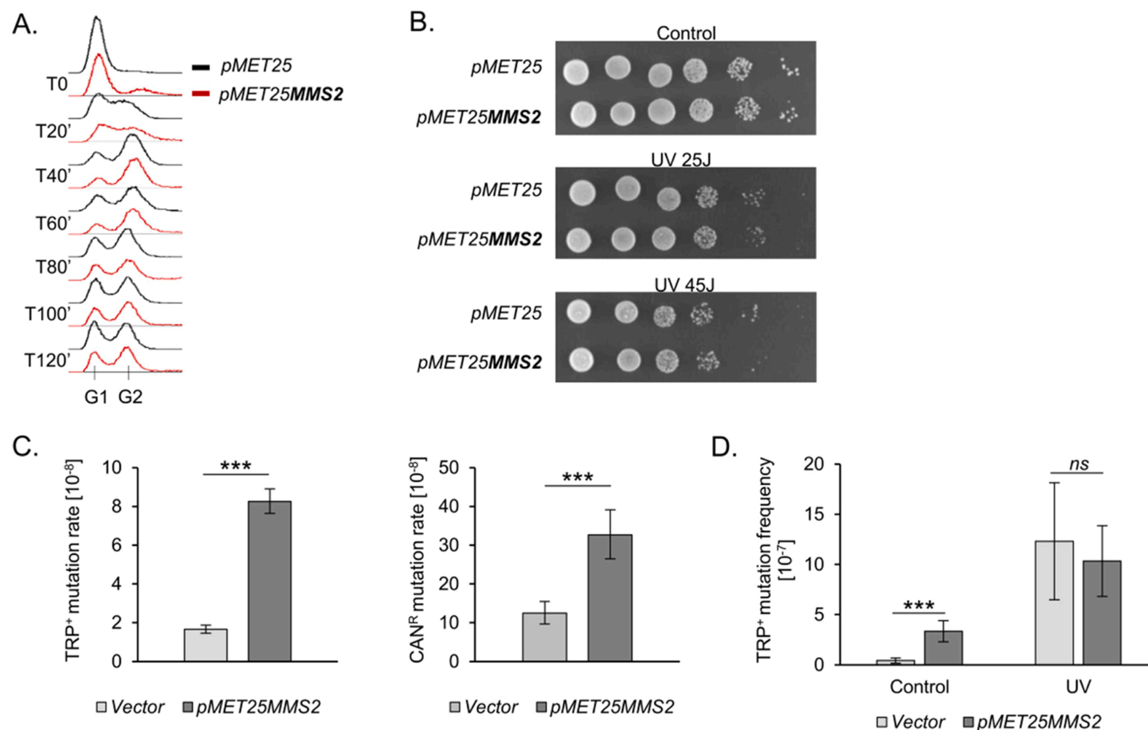

Fig. 1. Mms2 overproduction increases the rate of spontaneous mutagenesis. A. Mms2 overproduction does not influence the cell cycle progression. Representative FACS analysis of DNA content in cells carrying the pMET25MMS2 plasmid (red) vs cells transformed with the control vector (pMET25) (black). B. Mms2 overproduction does not influence yeast growth. Representative spotting plate test of ten-fold serial dilutions of the C10–15a strain transformed with the pMET25MMS2 plasmid in comparison to cells carrying the control vector (pMET25). The spotted cells were untreated (top), or UV-irradiated [25 and 45 J/m^2] (bottom). C. Mms2 overproduction stimulates spontaneous mutagenesis. The rates of mutations leading to *trp1–1* reversion (left panel) and canavanine resistance (CAN^R) (right panel) in C10–15a [36] or GIL104 [37] cells, respectively, transformed with pMET25MMS2 or control vector (pMET25). Mutation rates with 95 % confidence intervals (95 % CI) were calculated from data obtained from at least three independent experiments. D. Mms2 overproduction does not influence UV-induced mutagenesis. The frequency of spontaneous (control) and UV [5 J/m^2]-induced reversions of *trp1–1* in C10–15a transformed with pMET25MMS2 or control vector (pMET25). Mean values of 3 independent experiments ± SD. *P- values*: * ** <0.001, *ns* ≥ 0.05.

DF5-POL30 strain and its derivative carrying the *pol30K164R* mutation [8,40]. In this genetic background we investigated the rate of mutations leading to Can^R phenotype (as the *trp1–1*Δ mutation in DF5-POL30 genome is nonreversible). Mms2 overproduction caused 4-fold increase in the CAN^R mutation rate and this mutator effect was largely suppressed by the *pol30K164* mutation (Fig. 2 C).

In addition to preventing the PCNA ubiquitination, the *pol30K164R* mutation abolishes also the SUMOylation of K164 by the Ubc9-Siz1 complex. This modification is involved in modulating the level of homologous recombination during S-phase and, under specific conditions, can affect the level of spontaneous mutagenesis [41]. To distinguish which of PCNA modifications, ubiquitination or/and SUMOylation, is/are involved in the Mms2-mediated mutagenesis, we used a derivative of the C10–15a strain devoid of Siz1 [42]. We established that Mms2 overproduction caused a similar increase in the rate of TRP$^+$ in the *siz1* deletion mutant strain as in the control strain (Fig. 2D). This result pointed to ubiquitination as the PCNA modification required for the Mms2-mediated mutagenesis. Since the determination of the requirement for PCNA-K164 in the Mms2-induced mutagenesis was based on the CAN^R assay and PCNA SUMOylation on the TRP$^+$ assay, we decided to further corroborate the role of PCNA ubiquitination in the promutagenic activity of Mms2. We examined the role of Rad18, the ubiquitin ligase responsible for PCNA-K164 ubiquitination, in Mms2-induced reversions of *trp1–1*. The deletion of *RAD18* itself causes a spontaneous mutator effect [43]. That is why an estimation of the level of mutations induced by Mms2 overproduction in the absence of Rad18 was difficult. Therefore, we took advantage of a previous finding that the mutator effect of Δ*rad18* is partially suppressed by a deletion of the *SIZ1* gene [41] and analyzed the effect of Δ*rad18* on the Mms2-mediated mutagenesis in the background of a Δ*siz1* derivative of C10–15a. In Δ*siz1* strains the Mms2-overproduction caused a mutator effect only when Rad18 was intact. However, in an isogenic strain devoid of Rad18 the Mms2-overproduction did not cause a significant increase in the *trp1–1* reversion rate in comparison to the respective control (Fig. 2D). Altogether, the results indicated that PCNA monoubiquitination is required for the Mms2-induced mutagenesis leading to both Can^R and Trp$^+$ phenotypes.

3.3. Overproduction of Mms2 does not activate DNA damage response

Recruitment of Pol ζ to a replication complex is predominantly a consequence of DNA damage or other perturbations in DNA synthesis. Therefore, we investigated if Mms2 overproduction activated DNA damage responses. First, we checked if increased Mms2 abundance stimulated foci formation by the single-stranded DNA binding replication protein A (RPA). An increase of RPA foci number reflects an accumulation of single-stranded DNA due to DNA replication obstacles. Measurements of the numbers of cells accumulating YFP-labeled Rfa1, a subunit of heterotrimeric RPA, in cells transformed with pMET25MMS2, or the control plasmid, showed no increase in RPA foci accumulation due to Mms2 overproduction (Fig. 3 A and B). Consistently, increased cellular abundance of Mms2 did not elevate the phosphorylation level of H2A histone, that provides evidence of occurrence of DNA double-strand breaks (Fig. 3 C). Additionally, phosphorylation of Rad53, the central signal transmitter in the DNA damage and replication stress response, has not been changed upon MMS2 overexpression (Fig. 3 C). We also did not detect a significant increase in the level of PCNA ubiquitination at K164 (Fig. 3D). Altogether, the results suggest that the Mms2-mediated

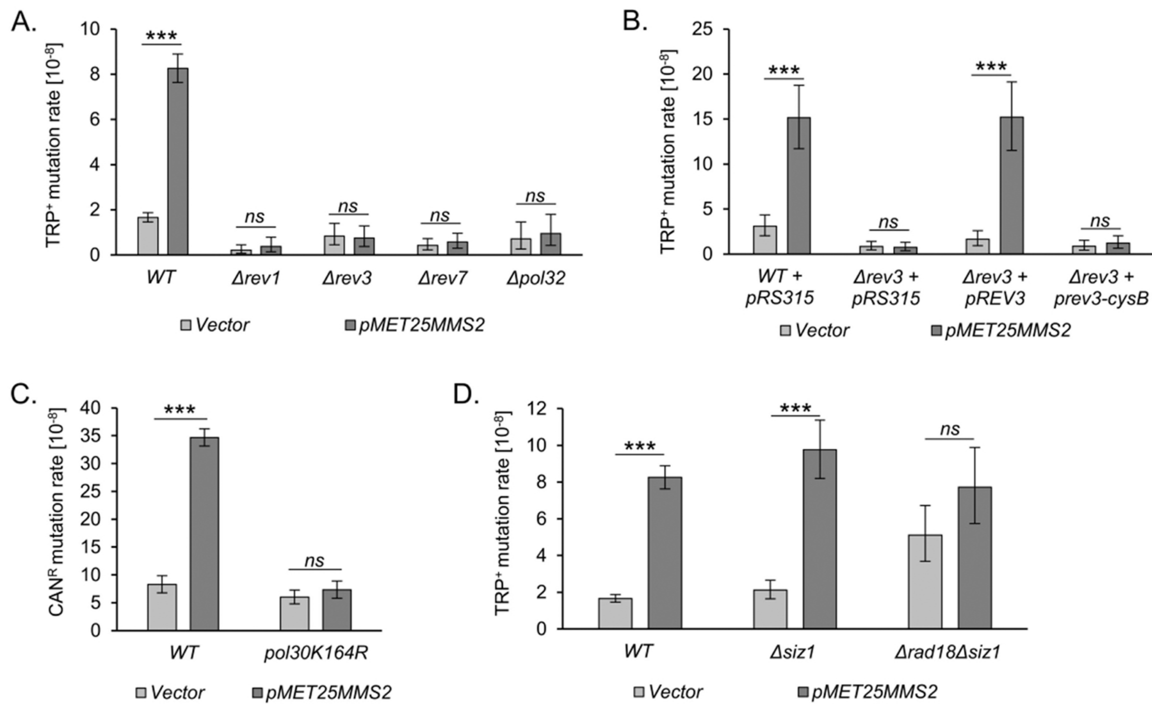

Fig. 2. Mutagenesis induced by Mms2 overproduction depends on Pol ζ, Rev1 and PCNA-K164 ubiquitination. The rates of *trp1–1* reversions were analyzed in derivatives of C10–15a carrying alleles indicated on the diagrams (A, B), transformed with plasmids: pMET25 (vector) or pMET25MMS2 (overexpressing Mms2). B. Cells were additionally transformed with vector pRS315 or its derivatives carrying the *REV3* gene or the *rev3-cysB* allele. C. The rates of CAN^R forward mutations were analyzed in the DF5-POL30 strain (WT), and its derivative carrying *pol30K164R* mutation, transformed with plasmids: pMET25 (vector) or pMET25MMS2 (for overexpression of *MMS2*). D. The rates of reversions of the *trp1–1* mutation in derivatives of the C10–15a strain, carrying Δ*siz1* or Δ*siz1*Δ*rad18*, transformed with pMET25MMS2 or pMET25 (control). Mutation rates with 95 % confidence intervals (95 % CI) were calculated from data obtained from at least 3 independent experiments. *P-values*: * ** <0.001; *ns* ≥ 0.05.

mutator effect is not connected to the significant activation of replication stress or response to DNA damage.

3.4. Mms2-mediated spontaneous mutator effect is Ubc13-independent

The best known cellular functions of both yeast Mms2, and its homologs in higher organisms, require the interaction between Mms2 and Ubc13 (E2). We asked if the promutagenic activity of Mms2 overproduction requires also Ubc13. Defects of the error-free TS pathway, as a consequence of Ubc13 deficiency, result in a strong mutator effect. For this reason, it was difficult to determine the level of mutagenesis induced by Mms2 overproduction in a *ubc13* deletion background, similarly to the situation described for Δ*rad18*. Therefore, to study Mms2-mediated mutagenesis, as previously, we used strains devoid of the Siz1 activity, in which the mutator effect caused by TS deficiency was partially suppressed [14,41]. Surprisingly, we found that the Mms2 overproduction caused a significant increase in the mutation rate in cells devoid of Ubc13, indicating that the Mms2-dependent mutator effect was Ubc13-independent (Fig. 4 A).

In addition, we investigated the possibility that the mutator effect of Mms2 overproduction was a consequence of an imbalance between the cellular level of Mms2 and its natural interaction partner Ubc13. For this purpose, cells carrying pMET25MMS2 were additionally transformed with the multicopy plasmid p423MET25UBC13, allowing the expression of *UBC13* from the *MET25* promoter on the p423 vector. Overproduction of Ubc13 together with that of Mms2 did not affect Mms2-induced mutagenesis (Fig. 4B), indicating that the mutator effect of Mms2 overproduction was not due to overabundance of Mms2 relative to Ubc13 in the cell.

3.5. Mms2-mediated spontaneous mutator effect requires Ubc4, Rsp5 and Not4

It has been previously described that in response to a DNA ligase1 deficiency PCNA is polyubiquitinated at K107 and this phenomenon requires Mms2 but not Ubc13 [44]. Instead, the PCNA-K107 ubiquitination was dependent on another ubiquitin conjugating enzyme Ubc4 [44]. Interestingly, we established that the Mms2 overproduction dependent mutagenesis required Ubc4 as well. As shown in Fig. 5 A, the promutagenic activity of Mms2 was suppressed by a deletion of the *UBC4* gene, but not by a deletion of the *UBC5* gene encoding the closest paralog of Ubc4 which can replace Ubc4 in many cellular processes. Despite the similarities between the proteins involved in PCNA-K107 ubiquitination and the Mms2-mediated mutagenesis, we found that the mutagenic effect of Mms2 was not correlated with PCNA K107 ubiquitination as the *pol30K107R* allele did not affect the mutator effect (Fig. 5B).

To better understand the role of Ubc4 in the Mms2-dependent mutagenesis, we investigated the involvement of different domains of this ubiquitin conjugating enzyme in the mutagenesis in cells overproducing Mms2. Introduction of the *ubc4C86A* point mutation in the active site of Ubc4, which eliminates its ability to conjugate ubiquitin [26], abolished the Mms2-dependent mutagenesis (Fig. 7 C) consistently with the requirement of Ubc4 enzymatic activity in the mutagenic process. Introduction of the *ubc4N78S* mutation that eliminates the RING E3-catalyzed isopeptide formation, but not HECT E3 transthiolation [26], caused also a significant decrease in the level of Mms2-induced mutations pointing to the participation of a RING-type ubiquitin ligase in stimulating the mutagenesis (Fig. 5 C). However, the mutation causing Ubc4's disability to cooperate with RING-type ligases did not inhibit the Mms2-induced mutagenesis to the same extent as Δ*ubc4*, suggesting an involvement of additional functions of Ubc4 in

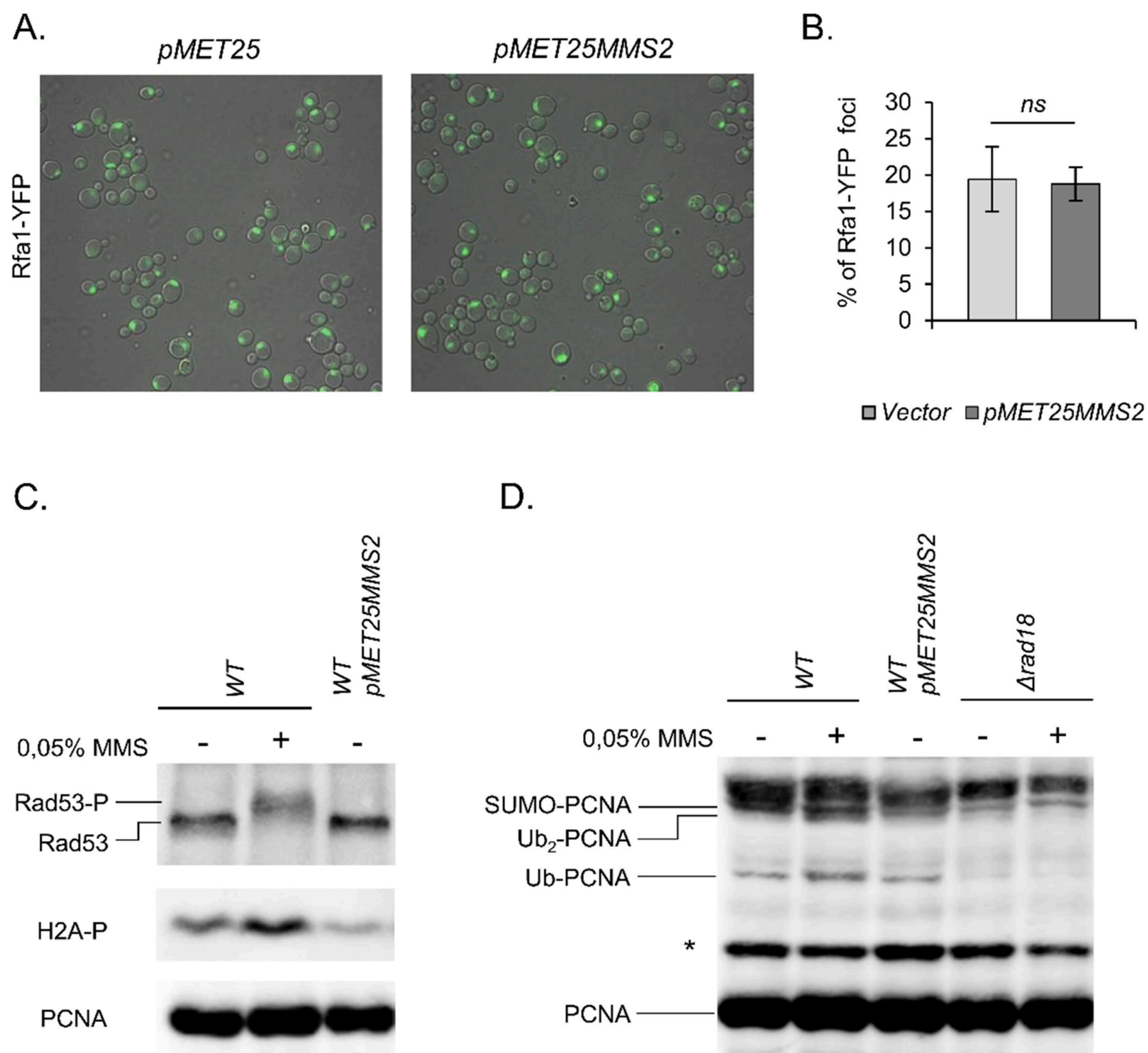

Fig. 3. Overproduction of Mms2 does not induce DNA damage response. A. Comparison of the Rfa1-YFP foci formation in cells expressing pMET25MMS2 vs control vector (pMET25); B. Rfa1 foci quantification. Mean value from 3 independent experiments ± SD. *ns*- not significant (*P-value* ≥0.05) C. Mms2 overproduction does not increase the phosphorylation of Rad53 and H2A histone. Representative western blot of cell extracts of C10–15a carrying control vector (pMET25) untreated (negative control) or treated (positive control) with 0.05 % MMS for 1 h, or pMET25MMS2. D. Mms2 overproduction does not increase the PCNA ubiquitination level. Representative western blot of cell extracts of C10–15a (WT) and YAH18 (*Δrad18*) carrying control vector (pMET25) untreated (negative control) or treated (positive control) with 0,05 % MMS for 1 h and C10–15a carrying pMET25MMS2. * unspecific band.

the Mms2-induced mutagenesis. Consistently, the *ubc4A97D/F63A* mutation that abrogates the interaction of Ubc4 with ubiquitin ligases of both RING and HECT types [26] has entirely suppressed the mutator effect caused by Mms2 overproduction (Fig. 5 C). Altogether, the results of this analysis have confirmed that the Ubc4-dependent ubiquitination process is involved in the induction of the Mms2-induced mutations and suggested the role of cooperation of both RING and HECT types of ubiquitin ligases with Ubc4 in this process.

In the search for ubiquitin ligases that could cooperate with Ubc4 in the promutagenic activity of Mms2 we have found that Not4, a RING E3-ubiquitin ligase involved together with Ubc4 in a degradation of the catalytic subunit of DNA polymerase α [45], was required for the Mms2-dependent mutagenesis (Fig. 6A). Looking for a HECT-type ubiquitin ligase, we examined whether the major enzyme of this type in yeast, Rsp5, was required for the Mms2-induced mutagenesis. Rsp5 enzyme is encoded by an essential gene and its deletion or a mutation leading to a replacement of the fully conserved active cysteine in its HECT domain lead to lethality [46]. Therefore, to study the role of Rsp5 in the mutagenic effect of Mms2, we have used cells carrying a conditional mutation in the HECT domain, *rsp5–1*, which abrogates a significant part of ubiquitin ligase activity, or mutations in WW domains, *rsp5-ww2* or *rsp5-ww3*, responsible for Rsp5 interactions with other proteins [47]. The results confirmed that the HECT domain of Rsp5 was engaged in the Mms2-dependent mutagenesis. Additionally, we established that this effect required also the WW3 interacting domain of Rsp5 (Fig. 6B).

3.6. Mms2-overproduction decreases Pol3 accumulation in the cell cycle

The requirement of Pol ζ, as well as factors involved in its recruitment to the replication complex, for Mms2-induced mutagenesis strongly suggests that in Mms2-overproducing cells the contribution of this error-prone polymerase in DNA replication is increased. Since known cellular responses leading to increased recruitment of Pol ζ to replication forks are not activated in cells overexpressing Mms2, as shown above, we checked if increased participation of Pol ζ in DNA replication might result from a decreased abundance of the catalytic subunit of replicative polymerase δ (Pol δ) in these cells. To detect Pol3, we used the previously described YUS609 strain [48] carrying a Pol3 variant tagged with HA epitope at its C-terminus. These cells were

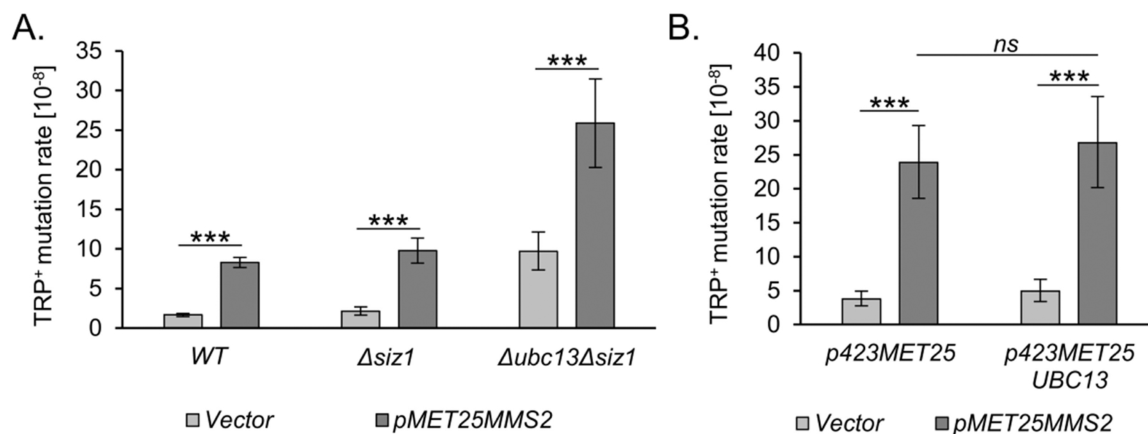

Fig. 4. The role of Ubc13 in the mutagenesis induced by Mms2 overproduction. The rates of reversions of the *trp1–1* mutation in derivatives of the CA10–15a strain A. carrying Δ*siz1*or *Δubc13*Δ*siz1*, transformed with the pMET25MMS2 or pMET25 (control vector). B. transformed with the p423MET25 or p423MET25UBC13 and additionally transformed with pMET25 (control vector) or pMET25MMS2. Mutation rates with 95 % confidence intervals (95 % CI) were calculated from data obtained from at least 3 independent experiments. Mutation rates with 95 % confidence intervals (95 % CI) were calculated from data obtained from at least 3 independent experiments. *P-values*: * <0.05, * ** <0.001.

transformed with pMET25 or pMET25MMS2. Surprisingly, we found for both plasmids that transformed cells grew poorly on selective media. This growth reduction was independent on Mms2 overproduction and suggested that the tagged Pol3 is not able to efficiently replicate the multicopy plasmids.

Therefore, we cloned the *MMS2* ORF under control of the *GPD* promoter on centromeric vectors p414 and p416 (derived from pRS414 and pRS416) and established that expression of *GPD-MMS2* increased the cellular abundance of Mms2 circa 10-fold (Fig. S1). The presence of centromeric plasmids did not affect growth of cells carrying the tagged Pol3, which allowed us to analyze the effect of Mms2 overproduction on the cellular level of Pol3. We examined the accumulation of Pol3-HA after the release of cells from the arrest in G1 phase. In control cells, Pol3-HA accumulated over time during cell cycle progression, reaching the maximum level 60 min after the start of S phase. Overproduction of Mms2 caused a significant reduction of this accumulation, so that the level of Pol3 at time 60' was 2-fold lower than in control cells (Fig. 7 A and Fig. S2), indicating that Mms2 might affect Pol3 accumulation during the cell cycle. We tested also how the level of Mms2 obtained from the centromeric plasmid construct, which is lower than that from the multicopy plasmid (Fig. S1), affects spontaneous mutagenesis. While we found no statistically significant increase in the mutation rate leading to *trp1–1* reversion (Fig. 7B), a 50 % increase in the rate of CAN^R mutations in cells expressing Mms2 from pGPDMMS2 plasmid was found to be significant (Fig. 7 C).

To determine the effect of further increase in Mms2 abundance on Pol3 level and the mutator effect, we integrated the *GPD-MMS2* fusion into the genomes of YUS609 and C10–15a strains, and transformed cells of these new strains with pGPDMMS2 centromeric plasmid or the control vector. This resulted in the 17-fold increase in the level of Mms2 (Fig. S1) and maximum 10-fold decrease in Pol3 accumulation at time 60' from the start of S phase (Fig. 7D and Fig. S3). This limited Pol3 accumulation was accompanied with a significant increase in mutation rate (Fig. 7E and F).

These results indicate that the effects of Mms2 overproduction, Pol3 downregulation and spontaneous mutator effect, are in an inverse relationship with each other. As already mentioned, we were not able to establish the level of Pol3 in cells overproducing Mms2 from pMET25MMS2 due to reduced growth of cells carrying tagged Pol3 in the presence of multicopy plasmids, however, given the inverse correlation with the Mms2-induced mutagenesis, the inhibition of Pol3 accumulation in these cells should be quite severe.

To distinguish if the Mms2-mediated inhibition of Pol3 accumulation reflects only the artificial situation of Mms2 overproduction or Mms2 is more generally involved in the regulation of Pol3 abundance, the level of Pol3 was analyzed in cells devoid of Mms2 (Δ*mms2*). The results shown in Figs. 8 and S4 confirm a general involvement of Mms2 in the limitation of Pol3 accumulation during cell cycle, as the level of this enzyme is substantially higher in cells devoid of Mms2 in comparison to control cells producing Mms2 at the native level.

4. Discussion and conclusions

Here we have examined the physiological effects of Mms2 overproduction in yeast cells. This approach has led to an unexpected discovery of a novel role for Mms2 in control of genetic stability. Namely, we found that Mms2, in addition to its known antimutator function, may also have a promutagenic activity when overexpressed in yeast cells, leading to increased levels of spontaneous mutagenesis.

4.1. Genetic requirements for promutagenic activity of Mms2 overproduction

We have shown that the promutagenic activity of Mms2 is independent of Ubc13, the cognate E2 partner of Mms2, responsible for the synthesis of polyubiquitin chains linked through K63. Recently, the new Ubc13-independent Mms2 activities have been described. It has been shown that Mms2, independently of Ubc13, is involved in the cellular response to a defect in DNA ligase1 in the budding yeast [44] and in large chromosomal rearrangements mediated by Rad52 in fission yeasts [50]. Interestingly, similarly to the promutagenic effect of Mms2 we found, those Ubc13-independent functions of Mms2 require the E2 activity of Ubc4 and PCNA ubiquitination. However, the mechanism of Mms2-induced mutagenesis requires ubiquitination of PCNA-K164 and not K107, which plays the key role in the cellular response to a defect of DNA ligase1 and causes the Rad52-mediated gross chromosomal rearrangements (27,36). This indicates that the promutagenic activity of Mms2 employs different mechanisms than those previously described for the Mms2-dependent and Ubc13-independent response.

The requirement of Ubc4 in Mms2-induced mutagenesis may suggest the existence of a Ubc4-Mms2 complex, as previously proposed for fission yeast [50]. However, a direct interaction between Ubc4 and Mms2 has never been shown. Results presented in this work indicate the dependence of Mms2-mediated mutagenesis on Rsp5. This HECT ubiquitin ligase was previously postulated to interact with Mms2 [17,18]. Although the results indicating this interaction come only from two

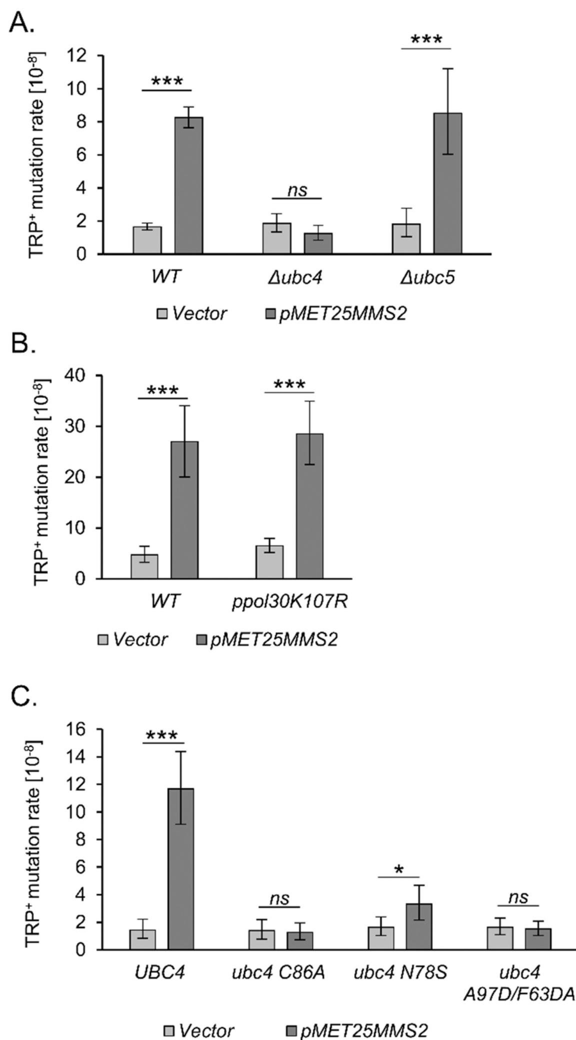

Fig. 5. Mutagenesis induced by Mms2 overproduction is independent of the K107 ubiquitination in PCNA but requires Ubc4 activities. The rates of *trp1–1* reversions were analyzed in derivatives of C10–15a carrying mutations, as indicated in the diagrams (A, B, and C), transformed with plasmids pMET25 (vector) or pMET25MMS2 (overproducing Mms2). Mutation rates with 95 % confidence intervals (95 % CI) were calculated from data obtained from at least 3 independent experiments. *P-values*: *ns* $\geq$ 0.05, * <0.05, * ** <0.001.

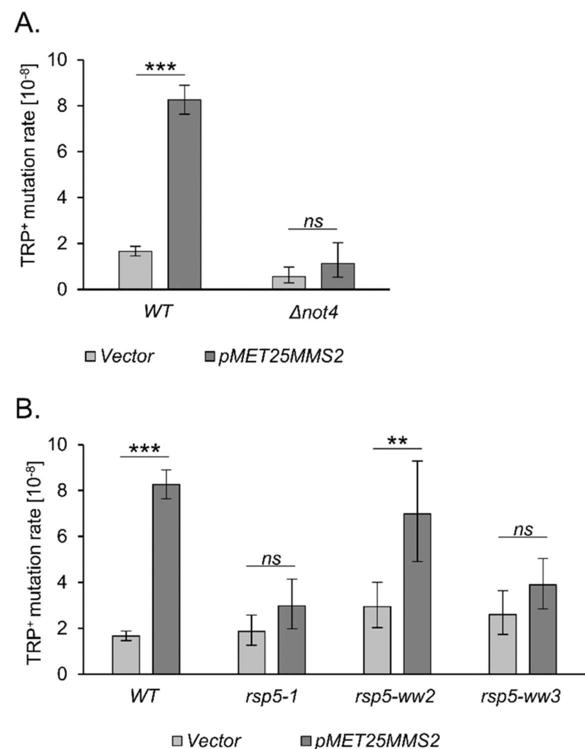

Fig. 6. Ubiquitin ligases involved in the Mms2-induced mutagenesis. The rates of *trp1–1* reversions were analyzed in derivatives of C10–15a carrying a deletion of the *NOT4* gene (A) or point mutations affecting Rsp5 activities (B). All strains were transformed with pMET25 (control vector) or pMET25MMS2 (to overproduce Mms2). Mutation rates with 95 % confidence intervals (95 % CI) were calculated from data obtained from at least 3 independent experiments. *P-values*: * ** < 0.001, * * < 0.01, *ns* $\geq$ 0.05.

sources, a published PhD thesis [17] and high throughput experiments [18], the intriguing possibility of an indirect interaction between Mms2 and Ubc4 via Rsp5, a well-known interactor of Ubc4 [26,51], is worth considering. We established that the promutagenic activity of Mms2, in addition to the E3 activity of Rsp5, required its WW3 domain, which had been previously postulated to physically interact with Mms2 [17]. We have also shown that, in addition to Rsp5, the promutagenic activity of Mms2 depends on the RING ubiquitin ligase Not4, which, like Rsp5, is known to interact with Ubc4 [45]. Altogether, the genetic requirements of the Mms2-induced mutagenesis strongly suggest a role of ubiquitination in this process. However, the identification of the ubiquitinating complexes involved and their specific targets requires further detailed studies.

4.2. Mms2-dependent inhibition of Pol3 accumulation and the role of Pol ζ

PCNA-K164 ubiquitination plays a central role in the recruitment of TLS polymerases to the DNA replication complex [9]. Consistently, we have shown that the mutator effect of Mms2 overproduction is entirely dependent on Pol ζ_4, the error-prone polymerase that shares the accessory subunits with Pol δ [38,48,52]. In addition to PCNA ubiquitination, the Mms2-mediated mutagenesis requires the master regulator of TLS, Rev1, which targets Pol ζ to the replication fork. The increased recruitment of Pol ζ to the replication complexes is often connected with the activation of checkpoint pathways in response to DNA damage or other replication stressors [27,53,54]. However, the Pol ζ-dependent mutagenesis resulting from Mms2 overproduction does not appear to be associated with a significant induction of the checkpoint response. Instead, we have found that Mms2 is involved in regulating the accumulation of Pol3, the catalytic subunit of Pol δ, during cell cycle. Deletion of the *MMS2* gene causes an increase in the Pol3 level, whereas overproduction of Mms2 results in reduced accumulation of this Pol δ subunit. It has been previously shown that decreased level of Pol3 resulted in increased mutagenesis [55]. It is also well documented that in yeast mutant strains with defective replisome 80–90 % of arising mutations are attributed to Pol ζ replicating undamaged DNA [54, 56–59]. Consistently, our results indicate that the Pol ζ activity is responsible for the Mms2-induced mutagenic effect which is accompanied with decreased Pol3 level.

In addition to increased mutagenesis, the previous studies of reduced Pol3 abundance, either through inhibition of POL3 transcription or increased degradation of the degron-fused Pol3 variant, have shown inhibition of cell growth and activation of the checkpoint pathway [55, 60]. Mms2 overproduction did not cause those deleterious effects even in cells with a high Mms2 level. However, both inhibition of *POL3* transcription and the use of degron-fused Pol3 resulted in a stable reduction in the level of this protein throughout the cell cycle, while in cells overproducing Mms2, Pol3 accumulation in S/G2-phase was still observed. Although the levels of this accumulation were significantly

Fig. 7. Mms2 overproduction reduces the Pol3 accumulation in the cell cycle. A, D. Representative western blots of cell extracts from (A) YUS609 (expressing *POL3-HA*) transformed with control vector (pGPD) or pGPDMMS2, (D) YAH269 (YUS609 with the integrated vector p306) transformed with control vector (pGPD) (left) and YAH271 (YUS609 with integrated *GPD-MMS2* fusion) transformed with pGPDMMS2 (right). Cells synchronized in the G1 phase with α-factor were released (by α-factor removal) to S-phase progression at time 0'. The cell cycle progression was monitored by Clb2 accumulation and FACS analysis. Densitometrically quantified Pol3 band levels normalized to the corresponding Pgk1 bands and to time 0' are shown below Pol3 bands [%]. B, E. The rates of mutations leading to *trp1–1* reversion in (B) C10–15a transformed with pGPDMMS2 or control vector (pGPD), (E) YAH270 (C10–15a with integrated vector p306) transformed with control vector (pGPD) or YAH273 (C10–15a*GPD-MMS2*) transformed with pGPDMMS2. C, F. CAN^R mutation rates in (C) WCG-4a [49] transformed with pGPDMMS2 or control vector (pGPD), (F) YAH278 (WCG4–4a with the integrated vector p306) transformed with control vector (pGPD) and YAH279 (WCG-4a*GPD-MMS2*) transformed with pGPDMMS2. Mutation rates with 95 % confidence intervals (95 % CI) were calculated from data obtained from at least three independent experiments. *P- value*: ns ≥ 0.05, * *< 0.01, * ** <0.001.

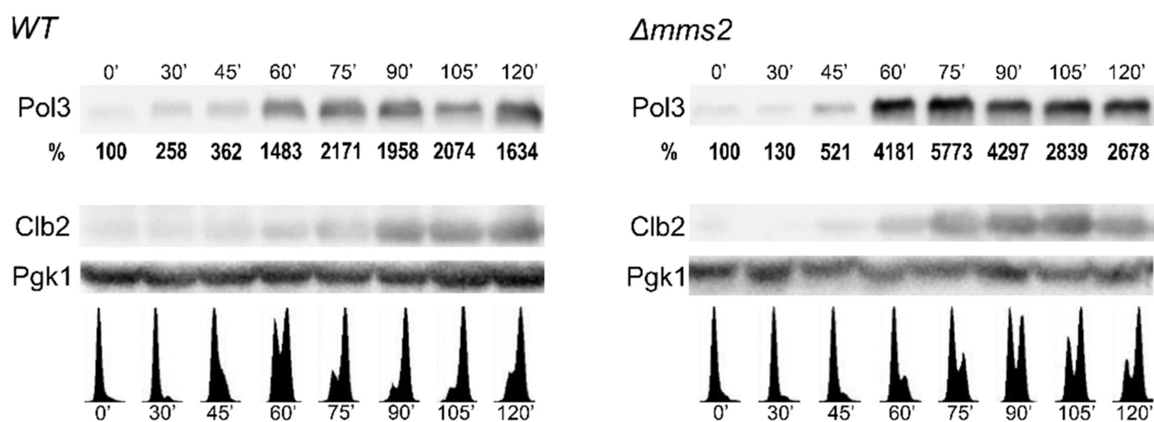

Fig. 8. Increased accumulation of Pol3 in yeast lacking Mms2. A. Representative western blots of cell extracts from YUS609 (expressing *POL3-HA*) and its derivative carrying Δ*mms2::kanMX4*. Cells synchronized in the G1 phase with α-factor were released (by α-factor removal) to the S-phase progression at time 0'. The cell cycle progression was monitored by Clb2 accumulation and FACS analysis. Densitometrically quantified Pol3 band levels normalized to the corresponding Pgk1 bands and to time 0' are shown below Pol3 bands [%].

lower than in cells with native *MMS2* expression, this may have been enough to maintain the integrity of the replication fork. It also cannot be ruled out that the downregulation of Pol3 by Mms2 is accompanied by an additional regulatory pathway that protects the integrity of the replication fork. Pol ζ recruitment to replication complex could be also involved in protection of the complex functionality when the level of Pol3 is reduced. Intriguingly, while the increased recruitment of Pol ζ is often associated with the increased level of PCNA monoubiquitination [9], this is not a case in response to Mms2 overproduction. Nevertheless, the native level of PCNA monoubiquitination and Rev1 support are still required for the Mms-induced mutagenesis mediated by Pol ζ. It may suggest that the recruitment of Pol ζ is dependent on already existing ubiquitinated PCNAs, which are formed at DNA sites where replicative DNA polymerase is temporarily inhibited due to various natural obstacles in the replicated template. It is possible that Pol3 abundance at these sites could be a crucial factor modulating the frequency of Pol δ replacement by Pol ζ in the replication complex. Thus, reduced Pol3 levels, as a result of Mms2 overproduction, could promote the targeting of Rev3 to the replication apparatus at these DNA sites, resulting in increased spontaneous mutagenesis.

Recently, it has been shown that in response to UV damage, recruitment of Pol ζ to the replication complex also requires an active reduction of Pol3 abundance [61]. However, that induced polymerase exchange, which was accompanied by increased PCNA ubiquitination and activation of DNA damage checkpoint, was Mms2-independent. Consistently, we have shown, that Mms2 overproduction does not influence UV-induced mutagenesis. This result may suggest that DNA damage response induced by UV radiation can somehow replace or neutralize Mms2-mediated mechanisms leading to increased mutagenesis.

In conclusion, we have found that the level of the catalytic subunit of Pol δ depends on the cellular abundance of Mms2 in the absence of external stressors. On the other hand, an increased expression of Mms2 results in induction of Pol ζ-dependent spontaneous mutagenesis that is associated with the reduction of Pol3 level.The results brought us to propose a working model that the reduced level of Pol3 could lead to increased recruitment of the error-prone Pol ζ to the DNA replication complex, which would result in enhanced spontaneous mutagenesis. This process would require the activity of the ubiquitin conjugating enzyme Ubc4 as well as Not4 and Rsp5 ubiquitin ligases, but not the cognate Mms2 interactor Ubc13. Thus, our study implicates a new function for Mms2 in control of spontaneous mutagenesis that goes beyond its canonical role in TS.

Funding

This work was supported by the National Science Centre [grant number 2016/23/N/NZ3/01550].

CRediT authorship contribution statement

MK: Funding acquisition, Investigation, Methodology, Visualization, Writing – review & editing. **AH:** Investigation, Methodology, Formal analysis, Writing – review & editing. **ESG:** Conceptualization, Formal analysis, Visualization, Supervision, Writing – original draft, Writing – review and editing.

Conflict of interest

The authors declare that they have no conflict of interest.

Data availability

No data was used for the research described in the article.

Acknowledgements

We thank Drs R. Woodgate, A. Kaniak-Golik, I. Unk, H. Ulrich and T. Zoladek for kindly providing the yeast strains and Dr R.E. Klevit for plasmids. We would like to express our great appreciation to Krystian Lazowski for help in the statistical analysis. We are also grateful to Drs A. Bebenek, J. McIntyre, A. Kaniak-Golik and I. Fijalkowska for critical reading of the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.dnarep.2023.103484.

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