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Investigation

Fe-S coordination defects in the replicative DNA polymerase delta cause deleterious DNA replication *in vivo* and subsequent DNA damage in the yeast *Saccharomyces cerevisiae*

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Abstract

B-type eukaryotic polymerases contain a [4Fe-4S] cluster in their C-terminus domain, whose role is not fully understood yet. Among them, DNA polymerase delta (Polδ) plays an essential role in chromosomal DNA replication, mostly during lagging strand synthesis. Previous *in vitro* work suggested that the Fe-S cluster in Polδ is required for efficient binding of the Pol31 subunit, ensuring stability of the Polδ complex. Here, we analyzed the *in vivo* consequences resulting from an impaired coordination of the Fe-S cluster in Polδ. We show that a single substitution of the very last cysteine coordinating the cluster by a serine is responsible for the generation of massive DNA damage during S phase, leading to checkpoint activation, requirement of homologous recombination for repair, and ultimately to cell death when the repair capacities of the cells are overwhelmed. These data indicate that impaired Fe-S cluster coordination in Polδ is responsible for aberrant replication. More generally, Fe-S in Polδ may be compromised by various stress including anti-cancer drugs. Possible *in vivo* Polδ Fe-S cluster oxidation and collapse may thus occur, and we speculate this could contribute to induced genomic instability and cell death, comparable to that observed in *pol3-13* cells.

Keywords: DNA polymerase delta; iron-sulfur cluster; pol3-13; DNA replication; DNA damage

Introduction

Based on sequence homology, DNA polymerases are divided into different classes. In *Saccharomyces cerevisiae*, the three essential DNA polymerases Pol alpha (α), Pol delta (δ), and Pol epsilon (ε), involved in DNA replication, as well as Pol zeta (ζ), involved in translesion synthesis, belong to class B DNA polymerases. These polymerases are conserved among eukaryotes, and their catalytic subunits exhibit eight conserved cysteine (Cys) residues in their carboxy terminal domain (CTD). Those residues are split into two groups of four, namely CysA and CysB motifs. As opposed to CysA motif, CysB motif actually coordinates a [4Fe-4S] cluster (Netz *et al.* 2011).

The role of the [4Fe-4S] cluster in class B polymerases is not fully understood. In addition, the consequences for the cells of Fe-S impairment in DNA polymerases remain difficult to handle experimentally as, for example, the clusters in the three essential DNA polymerases α, ε, and δ are essential for viability. In Polα, the [4Fe-4S] cluster has been quite extensively studied in human cells as compared to yeast. This evolutionary conserved cluster is deeply buried within the human protein core of the regulatory subunit p58C C-terminal domain, and plays essential structural roles as mutations of the cysteines involved in cluster coordination provoked p58C folding disruption (Klinge *et al.* 2007; Weiner *et al.* 2007; Liu and Huang 2015). It was also suggested to be involved in organizing the protein surface to facilitate the binding of DNA (Vaithiyalingam *et al.* 2010). The cluster is essential for initiating primer synthesis *in vitro* (Klinge *et al.* 2007; Weiner *et al.* 2007) and *in vivo* (Liu and Huang 2015). Actually, the redox state of the [4Fe-4S] cluster functions as a reversible switch for DNA binding ("redox switching") as shown *in vitro* (Liu and Huang 2015); this allows primase function by fine-tuning protein binding to DNA in yeast and human cells despite pretty strong structural dissimilarities between the two proteins (O'Brien *et al.* 2018). More broadly, a cluster-dependent redox regulation of B-type DNA polymerases has been extensively explored by the Barton's laboratory [23–26], revealing the major role of redox regulation of DNA polymerases activities *in vivo*, through oxidation/reduction of the cluster. This fine redox signaling is proposed to regulate polymerases handoff during DNA replication, but also during repair.

In Pol ε, recent reports indicated that the [4Fe-4S] cluster initially identified as binding the CysB domain within the CTD of

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Pol2 (catalytic subunit) (Jain *et al.* 2014), rather binds CysX motif, located in the upstream-moiety of the protein, within the catalytic domain of Pol2 (Ter Beek *et al.* 2019). Interestingly, the catalytic domain of Pol2 is not essential for DNA replication despite containing all known DNA polymerase and exonuclease motifs (Kesti *et al.* 1999; Kunkel and Burgers 2008; Pavlov and Shcherbakova 2010); it is also dispensable for DNA repair and viability in the yeasts *S. cerevisiae* (Kesti *et al.* 1999) and *S. pombe* (Feng and D'Urso 2001). Noticeably, in its absence, yeast cells are sick and display thermosensitive growth and replication elongation defects at permissive temperatures (Ohya *et al.* 2002), underscoring a pronounced deleterious context.

Pol ζ was initially identified as a two protein-complex composed of Rev7 and Rev3, the latter including a [4Fe-4S] cluster of unknown function (Netz *et al.* 2011). Numerous studies in yeast have demonstrated since a long time a role of Rev3 in translesion synthesis and mutagenesis induced by DNA damage (Lemontt 1971; Cassier-Chauvat and Moustacchi 1988; Morrison *et al.* 1989; Johnson *et al.* 2003). Additional findings identified another Pol ζ complex, namely Pol ζ(4) as it includes two additional subunits, Pol31 and Pol32 (Makarova *et al.* 2012). Interestingly, Pol31 and Pol32 are components of the Polδ complex (Gerik *et al.* 1998), which may indicate possible cross-talks between both Pol ζ(4) and Polδ complexes (Baranovskiy *et al.* 2012; Stepchenkova *et al.* 2017). The Fe-S cluster in Rev3 revealed to be essential for the formation of Pol ζ(4) and damage-induced mutagenesis (Makarova *et al.* 2012). No functional role has been clearly evidenced for this cluster until now, even though a redox regulation of Pol ζ involving the cluster was recently suggested (Malik *et al.* 2020).

Pol δ includes three subunits: Pol3, Pol31, and Pol32 (Gerik *et al.* 1998; Baranovskiy *et al.* 2008), among which only the catalytic subunit Pol3 is essential for viability and binds a [4Fe-4S] cluster in its C-terminus part (Netz *et al.* 2011). Pol3 is responsible for both polymerizing and 3'–5'exonuclease activity allowing proofreading. *In vitro* experiments showed that mutations in several cysteines residues coordinating the cluster obviously impaired cluster binding; the C1074A mutation is responsible for decreased ^{55}Fe bound to purified Pol3-CTD, and the combined mutations C1059S and C1074S result in decreased binding of Fe-S cluster to purified Pol3-CTD (Netz *et al.* 2011). Due to a compromised Polδ complex stability in the C1059S C1074S mutant, authors suggested that the cluster was necessary for complex integrity, through Pol3 interaction with Pol31. Still, basal DNA polymerase activity of Polδ is unaffected by the C1059S C1074S double mutation *in vitro* (Netz *et al.* 2011). Similarly, the deletion of the last four residues of Pol3 was earlier proposed not to affect DNA replication *in vivo* but rather to alter Pol3 binding to Pol31 (Brocas *et al.* 2010). These results are in accordance with a previous proposal that the polymerase catalytic domain is actually located within the N-terminal part of the CTD (Swan *et al.* 2009). The [4Fe-4S] cluster in the very C-terminus end of the CTD may play an important structural role as the functional integrity of purified Polδ complex depends on the binding of an Fe-S cluster to Pol3 (Netz *et al.* 2012). Although POLD1, the human catalytic subunit of Polδ, also binds a [4Fe-4S] cluster, its presence does not seem required for Polδ assembly *in vitro* (Jozwiakowski *et al.* 2019), as opposed to yeast. Nevertheless, the cluster presence revealed necessary for PCNA-dependent primer extension, for DNA binding, and for Polδ exonuclease activity (Jozwiakowski *et al.* 2019).

The previously described *pol3-13* yeast mutant (Giot *et al.* 1997) exhibits a mutation of the very last cysteine in Cys B motif, which has been replaced by a serine (C1074S) (Figure 1A). As a consequence, the mutated yeast strain exhibits thermo-sensitive growth from 30°C, and cells accumulate as large budded cells with a 2 C DNA content after shifting to nonpermissive temperature (36°C) (Giot *et al.* 1997). Aiming to characterize the physiological consequences of the C1074S mutation *in vivo*, we demonstrate in this study that *pol3-13* DNA replication is defective, even though S phase kinetics is not dramatically affected. In addition, DNA damage could be evidenced, as well as the requirement of homologous recombination for *pol3-13* viability. Finally, increased Fe-S cluster biogenesis partially restored *pol3-13* defects. Our results suggest that Fe-S in Pol δ allows DNA replication through stabilizing the Polδ complex, as DNA damage accumulate in the replicated DNA of *pol3-13* cells but the elongation kinetics did not appear majorly decreased. Requirement of DNA repair genes for insuring *pol3-13* mutant viability suggests that the DNA damage generated needs to be fixed using homologous recombination pathways.

Materials and methods

Plasmids and DNA constructs

pRS426-DRE2

A 1610 bp insert containing the *DRE2* gene under the control of its own promoter was purified from the previously described pRS416-*DRE2* plasmid (Vernis *et al.* 2009) after restriction by *XhoI* and *NotI*, and subcloned into pRS426, giving rise to pRS426-*DRE2*.

pRS426-TAH18

A 2410 bp insert containing the *TAH18* gene under the control of its own promoter was purified from the previously described pRS416-*TAH18* plasmid (Vernis *et al.* 2009) after restriction by XhoI and NotI, and subcloned into pRS426, giving rise to pRS426-*TAH18*.

Yeast media

Unless plasmid selection was needed, yeast strains were grown in rich YPD medium (1% yeast extract, 2% bacto peptone, and 2% glucose). For YPD plates, 2% agar was added. For *LEU2*- containing plasmid selection, yeast cells were grown on Synthetic Complete medium lacking leucine (SC–LEU) (0.67% yeast nitrogen base, adenine, histidine, lysine, uracil, tyrosine, methionine, tryptophan, 40 mg/L for each, 2% glucose, and 3% agar). For *URA3*-containing plasmid selection, yeast cells were grown on Synthetic Complete medium lacking uracil (SC–URA) (0.67% yeast nitrogen base, adenine, histidine, lysine, leucine, tyrosine, methionine, tryptophan, 40 mg/L for each, 2% glucose, and 3% agar). 5-Fluoroorotic acid (5-FOA) was used at 1 mg/mL in SC medium (0.67% yeast nitrogen base, adenine, histidine, lysine, uracil, leucine, tyrosine, methionine, tryptophan, 40 mg/L for each, 2% glucose, and 3% agar).

Yeast strains

Yeast strains 15A2 and 15A4

Yeast strains 47 G containing chromosomal *pol3-13* and *dre2* F221S mutations, and 113 D containing chromosomal *pol3-13* and *tah18* mutations (Chanet and Heude 2003), are viable thanks to the presence of the p96 multicopy plasmid, a YRp7-derivative plasmid (Struhl *et al.* 1979) containing a wild-type *POL3* allele, in addition to *ADE3* and *TRP1* genes. Both strains have been transformed by plasmids pRS426-*DRE2* and pRS426-*TAH18*, respectively. Transformants that were able to lose p96 plasmid were selected, and named respectively 15A2 and 15A4. All strains used in this study are congenic (except strain 3B9 used as a control) and listed in Table 1.

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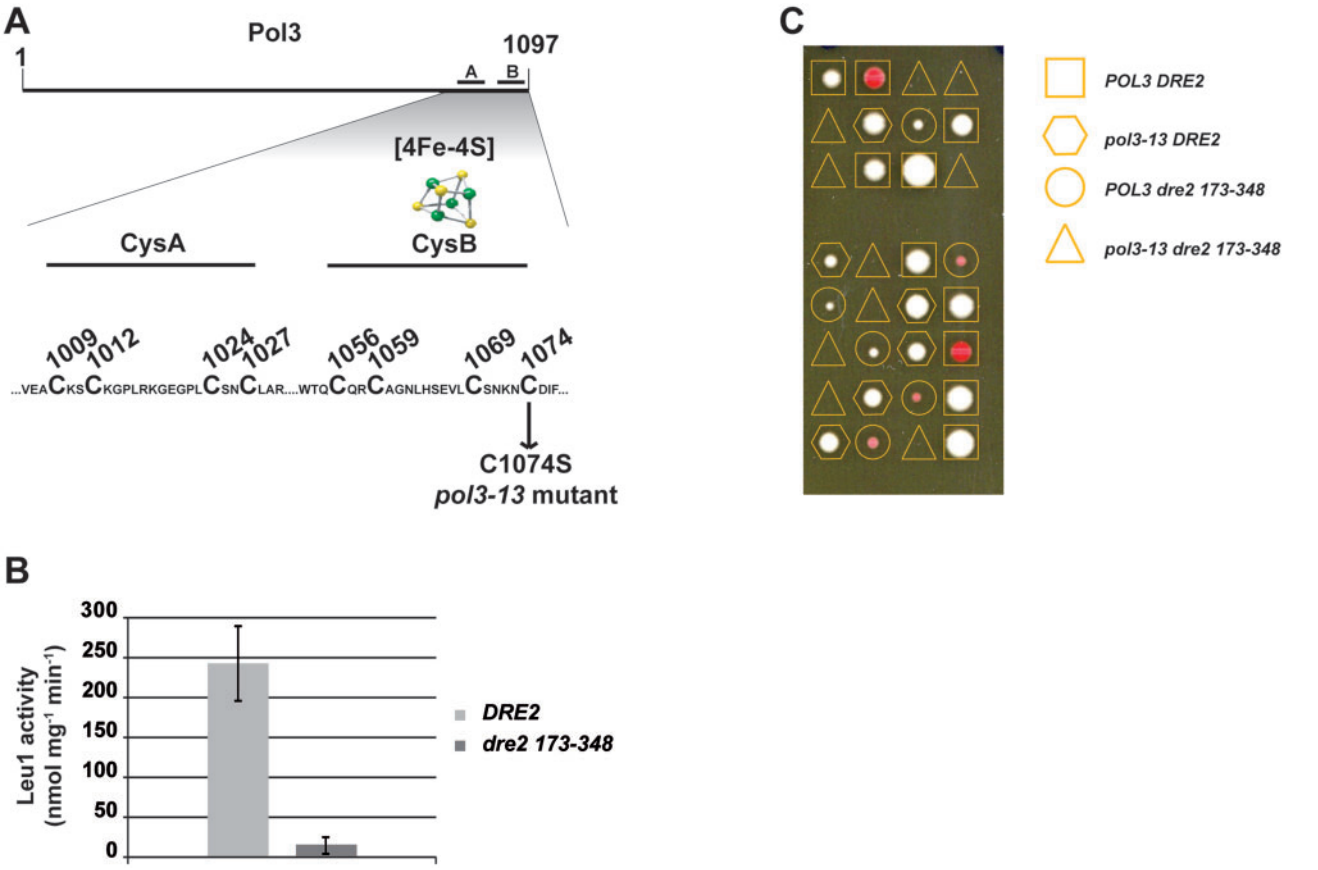

Figure 1 Pol3-13 mutant is not viable in presence of altered Fe-S biogenesis. (A) Schematic drawing showing conserved cysteine residues in Pol3 carboxy-terminus. C1074S mutation is the *pol3-13* mutation. (B) Leu1 activity measurement as a reporter of cytosolic Fe-S cluster biogenesis (Kispal *et al.* 1999). Citraconate was used as a substrate of Leu1. Leu1 activity (nmol.mg^{-1}.min^{-1}) was calculated from the decrease in OD at 235 nm due to citraconate isomerization. The genotype of the 2 yeast strains used is indicated. Reported values are the means of three independent experiments ± SD. (C) *pol3-13* and *tah18-5I5* mutations are synthetic lethal. Dissection plate from sporulated diploid *pol3-13-/POL3 tah18-5I5/TAH18* shows that spores harboring both *pol3-13* and *tah18-5I5* mutations are unable to grow. Similar results were obtained when combining *pol3-13* and *dre2 F36I-K153STOP* mutations, or *pol3-13* and *dre2 173-348* mutations.

Table 1 Yeast strains used in this study

Strain	Genotype	Source
3B9	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rad52Δ::KANR*	Euroscarf
5C4	*MATa ura3 leu2 LYS2 his3 dre2Δ::TRP1-CYH2S pRS416-DRE2-URA3*	Vernis *et al.* (2009)
5I5	*MATa his3 leu2 LYS2 trp1 ura3 cyh2R tah18-5I5*	Vernis *et al.* (2009)
8C2	*MATalpha HIS3 leu2 lys2 trp1 ura3 cyh2R*	Soler *et al.* (2011)
11C9	*MATalpha HIS3 leu2 lys2 trp1 ura3 pol3-13*	This study
11D1	*MATa his3 LYS2 pol3-13*	This study
12G3	*MATalpha his3 leu2 LYS2 trp1 ura3 cyh2R tah18-5I5*	Vernis *et al.* (2009)
14D6	*MATalpha HIS3 leu2 lys2 trp1 ura3 cyh2R pRS426-YAP1-URA3*	This study
15A2	*MATalpha trp1 dre2 F221S pol3-13 pRS426-DRE2-URA3*	This study
15A4	*MATalpha trp1 tah18 pol3-13 pRS426-TAH18-URA3*	This study
19B4	*MATa ura3 leu2 LYS2 his3 dre2Δ::dre2 173-348*	This study
21H3	*MATalpha his3 lys2 LEU2-GAL-RAD52 trp1 pol3-13*	This study
21H5	*MATa his3 lys2 LEU2-GAL-RAD52 trp1 pol3-13 rad52Δ::KANR*	This study
21H6	*MATa HIS3 lys2 URA3-GAL-RAD51 trp1 pol3-13 rad51Δ::KANR*	This study

All strains are congenic except 3B9 (Euroscarf).

Percentage of growth

Percentage of growth in Table 3 was calculated as follows: 15A2 and 15A4 cells were transformed by the plasmids of the first column, and plated on SC–LEU medium, with an approximate density of 100–300 transformants per plate. Colonies were counted before being replica plated on 5FOA-containing medium, in order to counter select the presence of the pRS426-*DRE2-URA3* plasmid in 15A2 cells or the pRS426-*TAH18-URA3* plasmid in 15A4 cells. The number of growing colonies on 5FOA-containing medium was counted then divided by the number of colonies counted before replica plating.

Isopropylmalate isomerase (Leu1) activity measurement

The activity of yeast isopropylmalate isomerase was measured on semi-purified protein extract by successive ammonium sulfate precipitation following a method previously described by

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Kohlhaw (1988). Cells were grown until mid-log phase and were harvested. Crude extracts were prepared in 50 mM phosphate buffer (pH 7.1) containing 50 mM NaCl and the 50%–65% ammonium sulfate fraction was resuspended in 50 mM phosphate buffer (pH 7.1) containing 1.6 M ammonium sulfate and 30% glycerol. The artificial substrate citraconate was used to determine total Leu1 activity by monitoring consumption of citraconate at 235 nm using increasing amounts of protein. Protein concentrations were measured using a Quant-iT protein assay kit in a Qubit fluorimeter (Life Technologies).

Flow cytometry

One milliliter of yeast cells culture was spun down for 5 minutes at maximum speed (14,000 rpm) and supernatant was removed. Cells were immediately resuspended in 1 mL 75% ethanol and kept overnight at 4°C. Fixed cells were spun down and pellet was washed in 1 ml 50 mM Tris-HCl pH 7.5 before resuspension in 1 ml 50 mM Tris-HCl pH 7.5 containing 1 mg/mL RNAse (Sigma). Suspensions were incubated for 4 hours at 37°C, before resuspending cells in 500 µl PBS buffer containing propidium iodide at 0.05 mg/mL. Cell suspension was sonicated for 5 seconds at medium power, before diluting cells at 1/10th in 50 mM Tris-HCl pH 7.5 and analyzing samples on a Becton Dickinson FACS Calibur. Fifty thousand cells were counted for each panel.

Western blotting

Protein extracts were prepared using TCA method (Foiani *et al.* 1994). Proteins were separated on 10% acrylamide gels and transferred onto ECL membrane (Amersham). The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20. Proteins were detected using a polyclonal anti-Rad53 antibody (1/10,000, generous gift from JFX. Diffley), and secondary horseradish peroxidase-conjugated antibody (Jackson), and ECL Western blot detection reagents (Amersham).

Pulsed field gel electrophoresis

Preparation of yeast chromosomes for pulsed field gel electrophoresis (PFGE) was done as described previously with slight modifications (Vezinhet *et al.* 1990; Casaregola *et al.* 1998). The gels were run in a BioRad CHEF Mapper apparatus in 0.5x TBE running buffer at 14°C. Chromosomes were separated following a 25-hour migration with pulses from 40 to 120 seconds in 1% Seakem GTG agarose (FMC, USA) gel at 6 V/cm (200 V).

Gel coloration and ImageJ quantification

Agarose gels were soaked in water containing 0.5 µg/mL ethidium bromide for 15 minutes, with gentle agitation. After rinsing with water, gels were exposed to UV light and densitometric analysis of chromosomal bands was performed using ImageJ.

Data availability

Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables.

Results

pol3-13 mutant needs fully functional Fe-S cluster biogenesis for viability

Several years ago, genetic interaction between *POL3* and several actors implicated in cytosolic Fe-S biogenesis was previously identified in our laboratory, including *DRE2*, *TAH18*, and *NBP35* (Chanet and Heude 2003).

In this study, mutated *dre2 F36I-K153STOP* (Soler *et al.* 2011) and *tah18-5I5* (Vernis *et al.* 2009) alleles, otherwise previously reported to strongly decrease cytosolic Fe-S cluster biogenesis (Soler *et al.* 2011; Huang *et al.* 2016), were introduced into a yeast mutant carrying the *pol3-13* mutation (Chanet and Heude 2003). Strains carrying both *pol3-13* and *dre2 F36I-K153STOP* mutations, or *pol3-13* and *tah18-5I5* mutations were unviable, consistent with previously identified synthetic lethal relations between *POL3* and *DRE2/TAH18* (Chanet and Heude 2003). Furthermore, the coding sequence of the Dre2 truncated version *dre2 173-348* (Soler *et al.* 2011) was integrated into yeast chromosome under the control of the native *DRE2* promoter, replacing wild-type *DRE2* allele. This new strain exhibited no obvious phenotype regarding growth, even though cytosolic Fe-S biogenesis was altered, as shown in Figure 1B by measuring the activity of the reporter protein Leu1 (Kispal *et al.* 1999). Synthetic lethal interaction with the *pol3* C1074S mutation was further evidenced (Figure 1C), arguing, together with previous results, that cells need fully functional cytosolic Fe-S biogenesis to deal with *pol3-13* associated defects. Synthetic lethality links identified are summarized in Table 2.

Increasing cytosolic Fe-S biogenesis improves *pol3-13* growth defects

To find new clues regarding the functional interaction between *POL3* and Fe-S biosynthesis machinery, we performed a multicopy suppressor screen to identify genes capable of suppressing lethality of *pol3-13 dre2* or *pol3-13 tah18* strains, when overexpressed. Briefly, strains holding the two chromosomal mutations *pol3-13 dre2*, or *pol3-13 tah18* were transformed by a multicopy 2 µ-based yeast DNA library, and transformants growing in the absence of wild-type *DRE2* or *TAH18* allele were selected. Plasmids were retrieved and further tested by re-transforming test strains. DNA sequencing of the retrieved plasmids revealed several genes implicated in Fe-S biogenesis as *DRE2*, *TAH18*, *NBP35*, and *NFS1*, but also *YAP1*, encoding a major redox transcription factor in yeast (Moye-Rowley *et al.* 1988, 1989), and involved in activating the transcription of Fe-S biogenesis genes (Huang *et al.* 2016). Results of the multicopy suppressor screen are presented in Table 3. Altogether, they argue that not only proficient Fe-S biogenesis is needed for *pol3-13* viability, but that increasing Fe-S overall biogenesis is of benefit for *pol3-13* cells. This result may indicate an overall decreased Fe-S occupation in *pol3* C1074S *in vivo*. These *in vivo* data are also in line with previous *in vitro* findings that Fe-S cluster content in purified *pol3* C1074A CTD is indeed strongly decreased (Netz *et al.* 2011). Further analysis of DNA replication in *pol3-13* cells is thus of interest in an attempt to analyze the consequences of *in vivo* Fe-S defects in Pol δ, still mild enough not to be lethal at the permissive temperature.

pol3-13 cells achieve DNA replication at nonpermissive temperature but passage through S phase is toxic

S phase kinetics was analyzed by flow cytometry. Growing yeast cells were synchronized in late G1 at 24°C (permissive temperature) using the mating pheromone alpha factor and then released into S phase, either at 24°C or 37°C (nonpermissive temperature). Samples were analyzed every 10 minutes (Figure 2A). At 24°C, S phase kinetics was comparable in *POL3* and *pol3-13* mutated cells (Figure 2B), even though somewhat slower in the mutated cells as compared to wild-type, considering S phase entry and duration. This indicates that impaired Fe-S cluster coordination in the *pol3-13* mutant does not dramatically prevent replication proceeding until completion. Surprisingly, at 37°C, S phase occurred

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Table 2 Synthetic lethality identified between *pol3-13* allele and several alleles of genes involved in Fe-S biogenesis

Alleles exhibiting synthetic lethality with *pol3-13* allele	Leu1 activity	Reference
dre2 (alleles 47 G and 113 A), *tah18* (alleles 113 D and 209 D), *nbp35* (allele 32B), *mms19* (allele 114H)	Not tested	Chanet and Heude (2003)
dre2 F36I-K153STOP (Soler *et al.* 2011)	Reduced	This work
dre2 173-348 (Soler *et al.* 2011)	Reduced	This work
tah18 5I5 (Vernis *et al.* 2009)	Reduced	This work

Table 3 Genetic links identified between *POL3* and several genes involved in Fe-S biogenesis

Plasmids used	15A2 (*dre2 pol3-13*)	15A4 (*tah18 pol3-13*)
pRS425	No	No
pRS425-*DRE2*	Yes	Partial (<90%)
pRS425-*TAH18*	Yes	Yes
pRS425-*NFS1*	Yes (poor growth)	Partial (>80%)
pRS425 *dre2 173-348*	Yes	Partial (>70%)
pRS425-*YAP1*	Yes	Partial (>70%)
pRS425-*NBP35*	Partial (>70%)	Not tested

Whether plasmids (first column) allow the growth of yeast strains 15A2 and 15A4 on 5FOA-containing medium, *i.e.*, in the absence of *DRE2* or *TAH18* wild-type alleles, is indicated. Percentage of growth is calculated as described in the Material and Methods section.

similarly in *POL3* and *pol3-13* cells as well, and replication kinetics was not dramatically affected, even though S phase was moderately slower in the mutated cells as compared to wild-type. Nevertheless, we observed that *pol3-13* cells stayed arrested at the G2/M stage and failed to enter the next cycle after resuspending cells into fresh medium without nocodazole and shifting the temperature to 24°C, as opposed to *POL3* cells (Figure 2C).

We next monitored cell viability during passage through S phase. *Pol3-13* and *POL3* cells were synchronized using alpha factor at the permissive temperature (24°C). Alpha factor was then removed and cells were released into S phase in fresh medium at either 24°C or 37°C. Samples were taken every 10 minutes and plated onto agar plates at 24°C; colony formation was assessed after 3 days. By the end of S phase at the restrictive temperature (37°C), only 10–15% of the *pol3-13* cells remained viable as compared to *POL3* cells (Figure 2D). It is very likely that cells that remain viable by the end of the experiment correspond to cells that did not came out of the block, and did not actually perform S phase, as it is commonly estimated that release efficiency from alpha factor block, even after several washes with fresh medium, is roughly 90%. We concluded that the passage through S phase is toxic for *pol3-13* cells and leads to cell death. Interestingly, cell viability decreased as a linear function of time spent in S phase. This distribution is compatible with irreversible and lethal damage occurring during S phase, generated randomly during the replication process.

pol3-13 cells accumulate DNA defects during S phase

The nature of the lethal events that are generated during S phase of *pol3-13* cells at nonpermissive temperature is unknown. One possibility is that aberrant DNA structures that cannot be solved are created, ultimately leading to cell death. PFGE has been described as a method of choice to evaluate yeast chromosomal integrity (Hennessy and Botstein 1991). Appropriate electrophoresis conditions allow intact chromosomes to enter the agarose gel, as opposed to chromosomes containing particular DNA structures, such as replicating DNA or any branched structure, due to less compact organization. A decreased amount of DNA entering the gel is expected in those conditions. Chromosomes of replicating yeast cells at nonpermissive temperature were separated by PFGE, and further stained using ethidium bromide. Fluorescence under UV was recorded. As shown in Figure 3, A and B, fluorescence of yeast chromosomes decreases as S phase proceeds, due to replication intermediates. By the end of S phase, fluorescence intensity of *POL3* chromosomes increases again as expected, whereas it remains low in *pol3-13* cells, indicating that chromosomes of *pol3-13* cells still exhibit particular DNA structures that prevent entering the gel after S phase completion, in contrast to *POL3* cells. These PFGE data are consistent with our cell cycle and cell viability analyses described above, indicating the presence of aberrant DNA structures in the chromosomes of *pol3-13* cells by the end of S phase that cannot be solved.

We then asked whether the events occurring at the nonpermissive temperature in *pol3-13* yeast cells can activate the S phase checkpoint. Rad53 is an essential protein kinase that is phosphorylated and activated in a MEC1- and TEL1-dependent manner in response to DNA damage (Sanchez *et al.* 1996). We thus monitored Rad53 hyperphosphorylation in response to temperature shift during S phase. Yeast cells were synchronized in G1 with alpha-factor at 24°C, before being released into S phase at 24°C, 30°C, or 37°C. Rad53 was not hyperphosphorylated in an asynchronous *pol3-13* culture, in contrast to hydroxyurea-treated cells used as a positive control (Figure 3C). Noteworthy, Rad53 was hyperphosphorylated in *pol3-13* cells replicating at 30°C and 37°C, as indicated by the appearance of slower migrating forms. These results show that despite normal S phase kinetics, passage through S phase activates S phase checkpoint in *pol3-13* cells at the nonpermissive temperature, further confirming the presence of abnormal DNA structures generated during the replication process.

Increased DNA repair in *pol3-13* cells improves cell viability

In addition to the fact that alleles such as *dre2* and *tah18* involved in Fe-S biogenesis are synthetic lethal with *pol3-13*, early work in our laboratory indicated that deletions of genes implicated in homologous recombination including *RAD51*, *RAD52*, and *RAD54* (Giot *et al.* 1997), as well as mutated alleles of *RAD55*, *RAD57*, *RAD59*, *MRE11*, *XRS2* (Chanet and Heude 2003), are also synthetic lethal with *pol3-13*. Thus, homologous recombination turns to be essential for *pol3-13* cells viability, whereas it is dispensable in a *POL3* context. We asked whether increasing homologous recombination in *pol3-13* cells might improve cell viability. Previous studies indicated that Rad51, involved in strand exchange between homologous DNA molecules, can increase homologous recombination by 20-fold when overexpressed 2–3 times (Vispe *et al.* 1998). Studies also indicated the stimulation of homologous recombination in case of Rad52 overexpression in human cells (Yanez and Porter 2002; Nogueira *et al.* 2019). We overexpressed Rad51 or Rad52 in *pol3-13* cells and thermosensitivity of the cells

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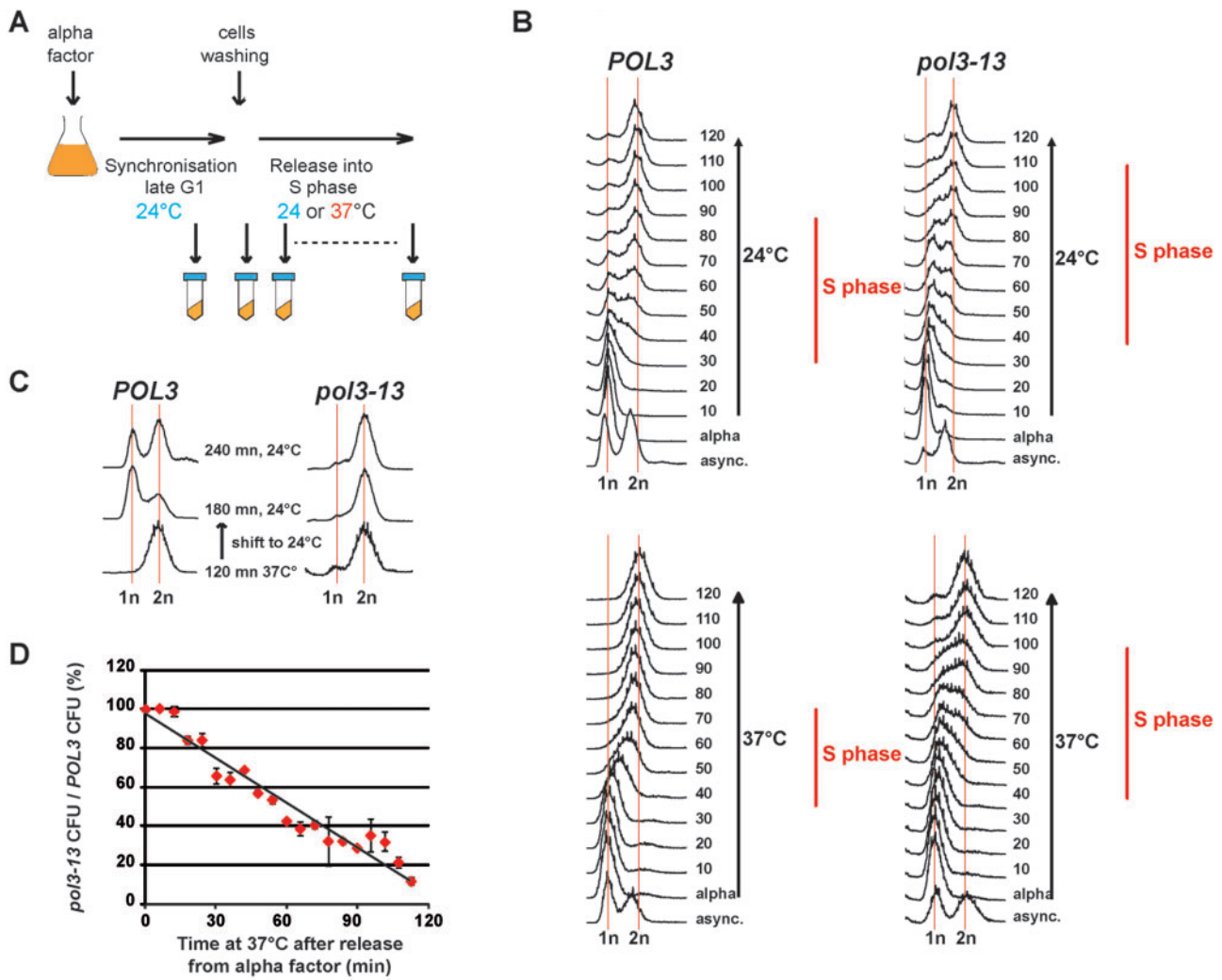

Figure 2 *pol3-13* yeast cells proceed through S phase at nonpermissive temperature and encounter lethal events. (A, B) Yeast cells were cultivated in rich medium at 24°C and arrested in G1 phase in the presence of alpha factor. Cells were then released into YPD medium containing nocodazole to prevent next mitosis, at 24°C (upper panel) or 37°C (lower panel) as indicated. Cells were collected every 10 minutes, and the DNA content of yeast cells was measured by flow cytometry. (C) *pol3-13* cells fail to enter the next cell cycle after S phase at the nonpermissive temperature. Cells were taken at the end of S phase at 37°C (Figure 2B, lower panel, 120 mn) and released into fresh complete medium at 24°C, without nocodazole at 24°C. Samples were analyzed by flow cytometry at the indicated times. (D) S phase at 37°C alters *pol3-13* cells viability as a linear function of time. Yeast cells were taken from experiment A (lower panel) and plated onto YPD plates at 24°C. Percentage of *pol3-13* colony forming units (CFU) among *POL3* CFU were plotted as a function a time. Data shown are the mean ± standard deviation of two independent experiments.

was assessed using drop tests. As shown in Figure 4, overexpressing either Rad51 or Rad52 strongly improved the thermosensitivity of *pol3-13* cells, enabling cell growth until 32°C, as opposed to 28°C in the absence of overexpression. Upregulating homologous recombination repair is of clear benefit to *pol3-13* mutated cells, supporting our hypothesis that DNA lesions are causative of poor cell viability at higher temperatures.

Discussion

This work provides evidence that *in vivo* impairment of yeast Polδ Fe-S cluster coordination provokes major genomic instability and cell death. Our *in vivo* data indicate that DNA replication during S phase is defective and generates dramatic DNA damage in the *pol3* C1074S (*pol3-13*) mutant. Aberrant DNA structures are likely produced during S phase but not resolved, and reveal toxic for the cells. If not rescued by Rad51- and Rad52- dependent repair, those structures may persist and lead to cell death.

Fe-S cluster status in *pol3* C1074S remains unknown *in vivo* but several lines of evidence strongly suggest a decreased occupation rate. We showed here that overexpressing genes involved in Fe-S biogenesis improves *pol3-13* cells condition. Pioneering work based on purified Pol3 CTD indicated that *in vitro* binding of Pol31 to *pol3* C1074S was impaired (Netz *et al.* 2011). Also, in another *pol3* mutated version, namely C1074A, ^{55}Fe incorporation into plasmid-encoded mutated HA– *Pol3* C1074A –CTD was decreased, suggesting a lower amount of cluster in the purified protein, as compared to wild-type. Furthermore, cluster binding in *pol3* C1074A purified CTD was not improved by Pol31 overexpression, as opposed to the situation in wild-type *POL3* purified CTD (Netz *et al.* 2011). Supportive to those *in vitro* data, 2-hybrid analysis in yeast indicated that the C1074A and C1074S mutations have comparable effects on preventing Pol3 binding to Pol31 (Sanchez Garcia *et al.* 2004). Finally, *in vivo* overexpression of Pol31 in *pol3-13* cells did not improve cellular growth defects in our hands (data not shown). Our own attempts to solve the question of Fe-S occupation in the purified mutated *pol3* C1074S CTD were

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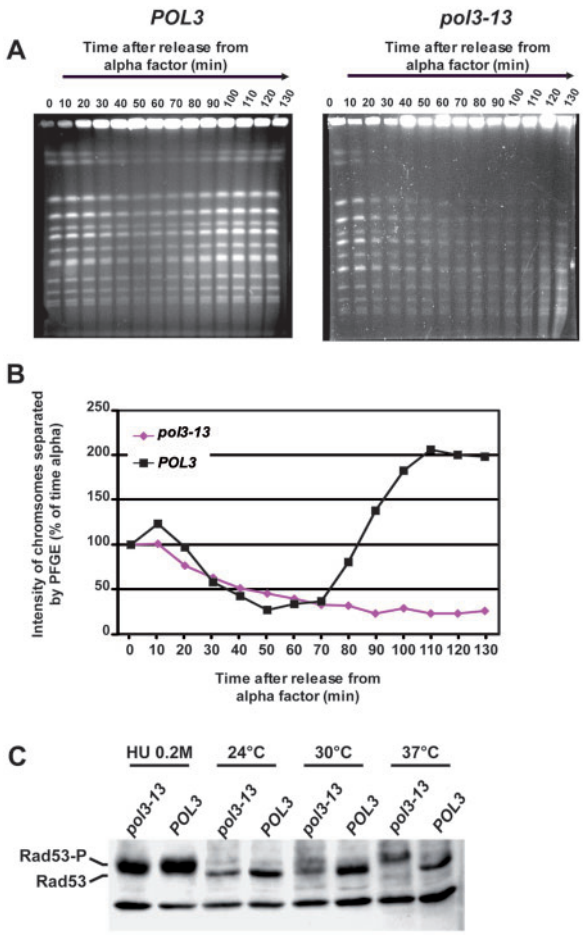

Figure 3 Evidence of persistent branched DNA structures produced during S phase that can activate DNA replication checkpoint. (A) Pulsed-field gel electrophoresis of yeast chromosomes after release from alpha factor block. *POL3* and *pol3-13* yeast cells were cultivated in rich medium at 24°C and arrested in G1 in presence of alpha factor. Cells were then released into YPD medium containing nocodazole to prevent next mitosis at 37°C. Time 0 corresponds to cells blocked in alpha factor just before release into fresh medium. Cells were collected every 10 minutes, and were further processed for PFGE analysis. Separated chromosomes were stained with ethidium bromide. (B) Intensity of chromosomes bands under UV light was analyzed by densitometry using ImageJ software. Densitometry value in alpha factor arrested cells was set to 100 (alpha column). Time 0 corresponds to cells blocked in alpha factor just before release into fresh medium. (C) Western blot analysis of Rad53 phosphorylation. Yeast cells were cultivated in rich medium at 24°C and arrested in G1 in the presence of alpha factor. Cells were then released into YPD medium at 24°C, 30°C, or 37°C, or at 24°C in the presence of 200 mM hydroxyurea (HU, positive control). Samples were taken at 60 minutes after release, and processed for immunoblotting to detect Rad53 with a polyclonal antibody.

unsuccessful. However, following the reasonable hypothesis that C1074S and C1074A effects are comparable *in vivo* as they are *in vitro*, our results might indicate that the amount of functional Polδ complex, actually containing a Fe-S cluster, is decreased in *pol3-13* cells. As shown previously, absence of Fe-S in Pol3 highly decreases Polδ stability (Netz *et al.* 2011). Absence of Fe-S in a fraction of Polδ (apo-Pol3) could lead to rapid turn-over and consequent decreased Pol3 levels. In accordance, absence of Fe-S in purified Pol3 CTD rendered the protein sensitive to degradation, as frequently seen for Fe-S proteins (Netz *et al.* 2011). Consistently, it has been documented that decreased amount of Polδ is a cause of major instability in yeast (Zheng and Petes 2018). Interestingly, recent work identified that reduced cellular levels of Polδ in yeast cells precisely lead to dramatic phenotypes, close to those found in *pol3-13* cells: dependence on checkpoint signaling and recombination-mediated repair for cellular viability, pathological DNA replication due to a defect in Pol δ-mediated Okazaki fragment processing, and overall genome stability defaults (Koussa and Smith 2021). It is thus possible that DNA damage generated during S phase in *pol3-13* cells may be due to decreased levels of Fe-S-containing Polδ, related to an increased instability of the Polδ complex in absence of Fe-S.

In yeast, Tsa1 is a key peroxiredoxin involved in reducing peroxides and diminishing oxidative insults within cells. Its absence is the cause of major genomic instability as shown previously (Iraqui *et al.* 2009). In addition, its human homolog Prx1 is capable of suppressing yeast genomic instability when expressed heterologously, underlining the conserved peroxiredoxin function among organisms (Iraqui *et al.* 2008). Interestingly, cells devoid of Tsa1 rely on Rad51-dependant repair for aerobic viability (Iraqui *et al.* 2009), but not on base-excision repair, nucleotide excision repair nor DNA mismatch repair pathways that are usually expected to intervene in case of DNA oxidation (M-E Huang, unpublished results). These data suggest that, in *tsa1* mutated cells, double-strand breaks and flawed DNA structures are present that need to be repaired by the Rad51-dependent pathway, as in *pol3-13* cells. In accordance, cells devoid of superoxide dismutase 1, another antioxidant enzyme, rely on Rad51 for viability (Choi *et al.* 2018). Our work may provide a mechanistic insight to these observations that cells defective in anti-oxidant systems need homologous recombination for their viability: either the oxidation of the Pol3 Fe-S cluster or the *pol3-13* mutation may have the same consequence, that is a reduced level of Fe-S-containing Polδ within cells, with dramatic consequences on DNA integrity and cell death. This proposal was also suggested recently by other authors, based on Polδ structural data (Jain *et al.* 2019). It is also supported by previous findings, that [4Fe-4S] clusters in yeast cells are critically oxidized and degraded *in vivo* in presence

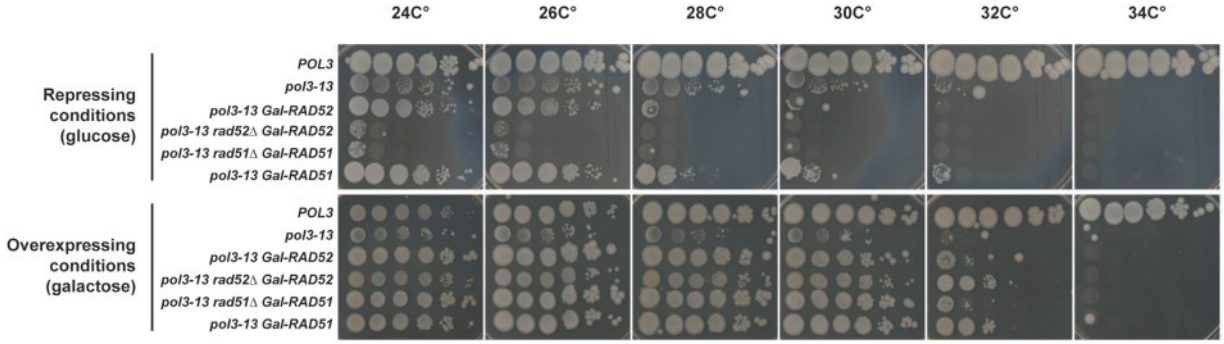

Figure 4 Increasing homologous recombination improves *pol3-13* growth at nonpermissive temperature. Drop tests of serial 10-fold dilutions of yeast cell cultures on glucose-containing medium (upper raw of panel, repressing conditions) and on galactose-containing medium (lower raw of panel, inducing conditions). Yeast genotypes are indicated on the left. Five-microliter drops were spotted on the top of the agar plates, and cell growth was assessed after 3–4 days at indicated temperatures.

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of chronic oxidative stress, leading to enzyme inactivation (Srinivasan *et al.* 2000). Fe-S cluster defect due to endogenous mitochondrial dysfunction was also previously linked to genomic instability (Veatch *et al.* 2009). We speculate that genomic instability observed in cells exposed to reactive oxygen species-generating anticancer drugs, such as doxorubicin and cisplatin, for example, might originate, at least in part, in Fe-S cluster oxidation and collapse in DNA-transacting proteins containing Fe-S clusters, including replicative polymerases. This might contribute to some extent to drugs-induced cell death.

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Conflicts of interest

None declared.

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