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...
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; Stephenkova 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 55Fe 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 in vivo DNA binding, and for Polδ exonuclease activity allowing proofreading (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 δ 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 2C 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 47G containing chromosomal pol3-13 and dre2 F221S mutations, and 113D 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 TRPI 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.
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...
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

dpol3-13 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 dreg F36L-K153STOP (Soler et al. 2011) and tah18-S15 (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 dreg F36L-K153STOP mutations, or pol3-13 and tah18-S15 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 dreg F36L-K153STOP 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 dreg or pol3-13 tah18 strains, when overexpressed. Briefly, strains holding the two chromosomal mutations pol3-13 dreg, or pol3-13 tah18 were transformed by a multicopy 2 µ-b 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 pol3-13. 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).

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 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

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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 resuspension 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 come 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, XR52 (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 (Vipse 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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**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), mms19 (allele 114H) | Not tested | Chanet and Heude (2003) |
| dre2 F361-K153STOP (Soler et al. 2011) | Reduced | This work |
| dre2 173-348 (Soler et al. 2011) | Reduced | This work |
| tah18 S15 (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-NBF35  | Partial (>70%)      | Not tested          |

Whether plasmids (first column) allow the growth of yeast strains 15A2 and 15A4 on SFOA-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.
was assessed using drop tests. As shown in Figure 4, overexpressing either Rad51 or Rad52 strongly improved the thermostensitivity 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, 55Fe 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
unsuccessful. However, following the reasonable hypothesis that C1074S and C1074A effects are comparable in vitro as they are in vivo, 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).

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 complexes, 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).

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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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