

Tdp1 protects against oxidative DNA damage in non-dividing fission yeast

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In humans, a mutation in the tyrosyl-DNA phosphodiesterase (Tdp1) is responsible for the recessively inherited syndrome spinocerebellar ataxia with axonal neuropathy (SCAN1). Tdp1 is a well-conserved DNA repair enzyme, which processes modified 3' phospho-DNA adducts *in vitro*. Here, we report that in the yeast *Schizosaccharomyces pombe*, *tdp1* mutant cells progressively accumulate DNA damage and rapidly lose viability in a physiological G0/quiescent state. Remarkably, this effect is independent of topoisomerase I function. Moreover, we provide evidence that Tdp1, with the polynucleotide kinase (Pnk1), processes the same naturally occurring 3'-ends, produced from oxidative DNA damage in G0. We also found that one half of the dead cells lose their nuclear DNA. Nuclear DNA degradation is genetically programmed and mainly depends on the two DNA damage checkpoint responses, ATM/Tel1 and ATR/Rad3, reminiscent to programmed cell death. Diminishing the respiration rate or treating cells with a low concentration of antioxidants rescues the quiescent *tdp1* mutant cells. These findings suggest that mitochondrial respiration causes neuronal cell death in the SCAN1 syndrome and in other neurological disorders.

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Introduction

Single-strand breaks (SSBs) are a physiological common source of DNA lesions. They are often associated with 'dirty' ends and hinder essential biological processes. Endogenous reactive oxygen species (ROS), such as hydroxyl radicals and superoxide, are natural by products and if not rapidly neutralized, they may chemically attack the DNA and induce SSBs with 3'-phosphate or 3'-phosphoglycolate termini. Topoisomerase I (Top I) also introduces transient SSBs to relax the DNA during DNA replication and transcription. If a DNA polymerase or RNA polymerase encounters the DNA-Top I intermediate complex before DNA religation, then Top1 remains covalently trapped to the 3'-ends of the DNA breaks (Pommier *et al*, 2003).

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Cells have evolved several specialized and partially overlapping efficient DNA repair processes to handle a broad range of SSB DNA lesions. However, it was estimated that about 1% of the SSBs escape from the DNA repair machineries and are transformed into double-strand breaks (DSBs) during DNA replication (Vilenchik and Knudson, 2003). Such DSBs are efficiently repaired by the homologous recombination (HR) machinery and errors in this process can be detrimental to cell viability or contribute to genetic instability. (Vilenchik and Knudson, 2003; Roseaulin *et al*, 2008). Taken together, it appears that SSB production and their repair are two critical parameters for genetic stability.

Tyrosyl-DNA phosphodiesterase 1 (TDP1) is a well-conserved SSB repair enzyme (Pouliot *et al*, 1999) cleaving *in vitro* the phosphodiester bond linking Top 1 to the 3'-end of DNA ends (Yang *et al*, 1996; Pouliot *et al*, 2001; Inamdar *et al*, 2002; Liu *et al*, 2002; Interthal *et al*, 2005b). Additionally, Tdp1 hydrolyses a variety of 3' lesions, including 3'-phosphoglycolate (Interthal *et al*, 2001, 2005a; Debethune *et al*, 2002; Inamdar *et al*, 2002) a DNA adduct produced by H₂O₂, the drug bleomycin (BLM) and ionizing radiation. Following the Tdp1 reaction, a 3'-phosphate and a 5'-hydroxyl remain at the ends of the SSB, which are further processed by the polynucleotide kinase 3' phosphatase (PNKP), restoring the conventional 3'-hydroxyl and 5'-phosphate DNA ends (Vance and Wilson, 2001). In mammals, Tdp1 interacts with ligase III and in concert with PNKP, the scaffold protein XRCC1 and the SSB sensor PARP1, repair the SSBs (Plo *et al*, 2003; El-Khamisy *et al*, 2005).

Consistent with this, lymphoblastoid cell lines from spinocerebellar ataxia with axonal neuropathy 1 (SCAN1) patients and budding yeast cells, mutated in *tdp1*, are sensitive to the anticancer drug camptothecin (CPT), which specifically traps Topo1 on DNA (Pouliot *et al*, 1999; Vance and Wilson, 2002; El-Khamisy *et al*, 2005; Miao *et al*, 2006). Furthermore, SCAN1 mutant cells are sensitive to H₂O₂, and low doses of ionizing radiation and cellular extracts from these cells were found deficient in the removal of 3'-phosphoglycolate adducts (Zhou *et al*, 2005; El-Khamisy and Caldecott, 2007). Current models propose that physiological Topo1 or ROS-induced SSB is responsible for neuronal dysfunction in the absence of the DNA end processing enzyme, Tdp1, but there is as yet no conclusive evidence for this hypothesis (El-Khamisy and Caldecott, 2007; Rass *et al*, 2007).

Although DSBs and blocked replication forks are known to alert the DNA damage checkpoints, including the ataxia telangiectasia mutated (ATM or Tel1 in *Schizosaccharomyces pombe*) and ATM- and Rad3-related (ATR or Rad3 in *S. pombe*) checkpoint kinases (Bartek and Lukas, 2007; Lavin, 2008), little is known about whether and how simple lesions, such as SSBs, alert the DNA damage checkpoints. However, it was recently reported in human cells that XRCC1 is phosphorylated in an ATM-dependent manner, suggesting that SSBs can also trigger checkpoint responses in G1 (Chou *et al*, 2008).

In this study, we studied the function of *tdp1* mutant cells in the fission yeast, *S. pombe*, in vegetative and quiescent states. We further analysed the impact of CPT, oxidative stress and cellular respiration on the viability of the *tdp1Δ* and *pnk1Δ* mutant strains in the physiological quiescent conditions, a state relevant for SCAN1. We also considered the role of the Rad3/ATR and Tel1/ATM DNA damage checkpoint kinases in the phenotypes observed. This study shows that *tdp1Δ* mutant cells progressively accumulate unrepaired oxidative DNA damage, which ultimately induces an ATM/Tel1-dependent apoptotic-like programme in G0.

Results

Tdp1 mutant cells die in the quiescent state with an apoptotic-like phenotype

To evaluate the role of Tdp1 in DNA repair in fission yeast, we constructed a *tdp1Δ*-null mutant and analysed chronic sensitivity to CPT. We found that *tdp1Δ* cells grow as fast as wild-type cells but are sensitive to CPT (Figure 1A). Then, we compared the CPT sensitivity of *tdp1Δ* mutants in the presence or absence of the *top1* gene. The CPT resistance of the *top1Δ* and *tdp1Δ top1Δ* mutant strains confirmed the importance of Tdp1 in the repair of Top1-induced lesions (Figure 1A) (Pommier, 2006).

Next, we attempted to understand, why in humans, TDP1 dysfunction specifically affects non-dividing neuronal cells

and allows normal development. Notably, it was recently shown that SCAN1 lymphoblastoid cells blocked in S phase, to mimic the postmitotic state, are also impaired in SSB repair following CPT treatments, supporting the idea that Tdp1 has an important role in SSB repair, independently of DNA replication (El-Khamisy *et al*, 2005; Miao *et al*, 2006). To evaluate the contribution of Tdp1 in non-dividing *S. pombe* cells, we grew cells in nitrogen starvation medium, which arrests cells in the G0/quiescent state (Su *et al*, 1996). In this state, wild-type cells are metabolically active, remain fully viable for several weeks and are able to respond and efficiently repair DNA damage (Mochida and Yanagida, 2006; Shimanuki *et al*, 2007).

We assessed cell viability by counting the number of colonies as a function of time in nitrogen-starved conditions. Only about 60% of the *tdp1Δ* mutant cells remained viable after 8 days in G0, whereas nearly all wild-type cells survived (Figure 1B). Microscopic observation of wild-type and *tdp1Δ* cells starved for 8 days and then re-seeded on rich media confirmed that about 40% of the *tdp1Δ* cells could not form colonies and remained as single cells after 3 days of incubation. Among the non-dividing *tdp1Δ* cells, about one-half were enlarged, indicating an attempt to re-enter into the vegetative cycle, whereas the other half did not change their appearance, suggesting that they died during the G0 period (Figure 1C). Because Tdp1 is involved in SSB repair, we analysed the nuclear DNA by fluorescence microscopy of

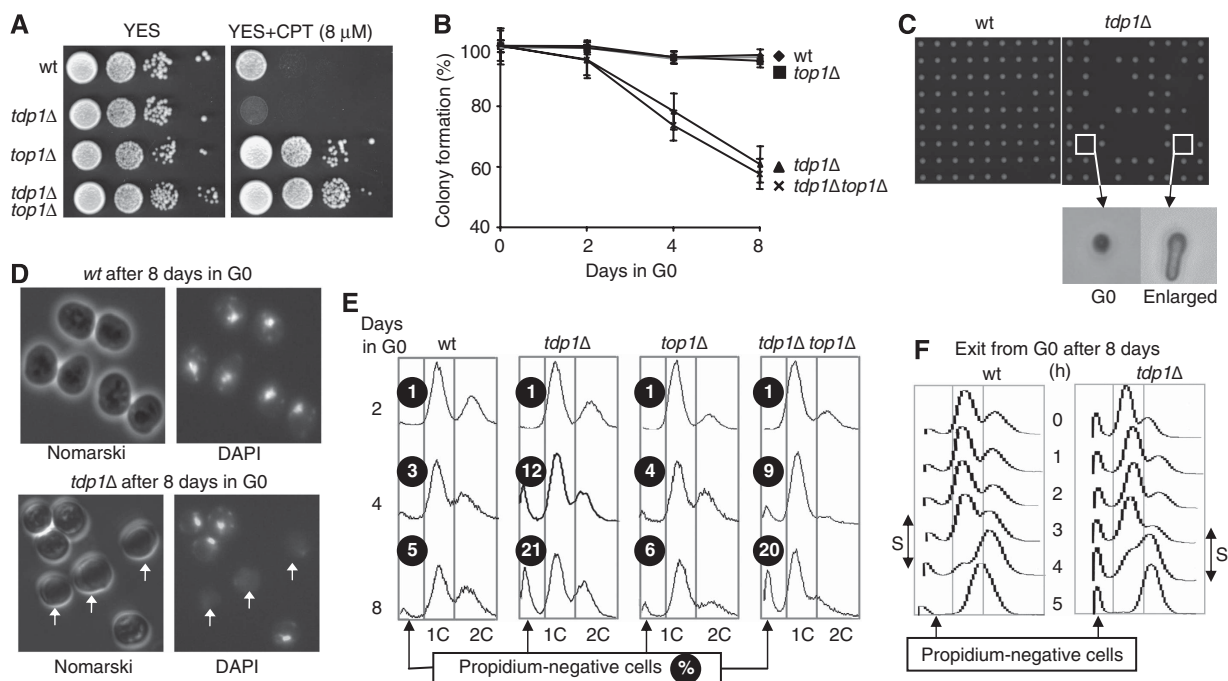


Figure 1 Mortality of *tdp1Δ* cells in the quiescent state. (A) Spot assay showing that the *tdp1Δ* strain is sensitive to camptothecin in a topoisomerase I-dependent manner. Ten-fold serial dilutions of wild-type, *tdp1Δ*, *top1Δ* and *tdp1Δ top1Δ* cells grown on YES (rich) media and spotted on YES and YES + 8 μM CPT agar plates. (B) Loss of viability of the *tdp1Δ* strain; in the quiescent state is topoisomerase I independent. Plating efficiencies of quiescent wild-type, *tdp1Δ*, *top1Δ* and *tdp1Δ top1Δ* strains (more than 500 colonies are counted for each strain, $n > 3$ independent experiments). (C) The 8-day starved wild-type and *tdp1Δ* cells were re-seeded on YES plates by micromanipulation and allowed to grow for 3 days at 32°C. Microscopic images show, that among the *tdp1Δ* cells unable to form colonies, about one-half are dead and the other half are enlarged but never divide. (D) DAPI staining of the 8-day quiescent wild-type and *tdp1Δ* cells (white arrows indicate the DAPI-negative *tdp1Δ* cells). (E) FACS profiles of wild-type, *tdp1Δ*, *top1Δ* and *tdp1Δ top1Δ* strains as a function of time in G0 (2-, 4- and 8-day starved cells) and quantification of the accumulation of propidium iodide-negative cells. The percentage (%) is indicated. (F) FACS profiles of wild-type and *tdp1Δ* strains, during the five first hours of vegetative cycle re-entry, show that the *tdp1Δ* cells enter into the first S phase. Note that the percentage of propidium iodide-negative cells remains constant during this period.

the wild-type and *tdp1Δ* cells by 4',6'-diamino-2-phenylindole (DAPI) staining and show that DAPI-negative cells accumulate in the *tdp1Δ* population (Figure 1D). To quantify this observation, we examined the cellular DNA content by FACS. After 2 days of nitrogen starvation, the FACS profile of the wild-type and *tdp1Δ* strains is not different (Figure 1E). However, increasing the incubation time in G0 (4 and 8 days), which does not affect the wild-type FACS profiles, revealed a new population of propidium iodide-negative *tdp1Δ* cells that reaches 21% after 8 days in G0 (Figure 1E).

The loss of viability in G0 is independent of Top I

To assess the role of Top I in fission yeast death, we analysed the viability and FACS profile of the starved *top1Δ* and *tdp1Δ top1Δ* mutant strains (Figure 1B and E). We show that in the presence or absence of Top I, the *tdp1Δ* strain accumulates dead cells and propidium iodide-negative cells in G0, whereas the single *top1Δ* mutant strain remains viable. Altogether, these results demonstrate that Top I is not responsible for the loss of viability observed in the quiescent *tdp1Δ* fission yeast mutant strain. Following nutrient addition and regardless of the anucleated cell fraction, the FACS profiles of the 8-day starved wild-type and *tdp1Δ* cells show that both strains were able to replicate their DNA (Figure 1F).

Tdp1 mutant cells accumulate DNA damage in G0

Given that Tdp1 is involved in SSB repair and that the HR process functions as a backup pathway during DNA replication in the absence of Tdp1 (Pouliot *et al*, 2001; Vance and Wilson, 2002; El-Khamisy and Caldecott, 2007), we next analysed the re-entry into the vegetative phase of starved wild-type and *tdp1Δ* cells for spontaneous formation of Rad22-YFP (the homologue of RAD52 in *S. pombe*) foci, a marker for the HR repair process (Lisby *et al*, 2001; Meister *et al*, 2003). Following addition of rich medium, the G0 cells re-entered into the vegetative cycle synchronously (Su *et al*, 1996) and the number of Rad22-YFP foci per cell as a function of time was determined by microscopic analysis (Figure 2A). The first DNA replication is readily assessed by FACS analysis and the second DNA replication coincides with the formation of the first septum of division, prior to the first cell division (data not shown). For the wild-type strain, extending the G0 incubation period had little effect on the proportion of nuclei containing Rad22-YFP foci during the first and the second DNA replication periods. However, the *tdp1Δ* strain exhibited a progressive and dramatic increase in the proportion of nuclei containing Rad22-YFP foci during the first DNA replication (Figure 2A–D). During the second DNA replication (time point 6 and 7 h in Figure 2D), the percentage of *tdp1Δ* cells with Rad22-YFP foci consistently decreased, although it did not reach the level observed in wild-type cells, indicating that many, but not all of the lesions, are repaired between the first and second DNA replication periods. The pivotal role of the HR process in DNA repair in the *tdp1* mutant was confirmed by the rapid and extensive death of the *tdp1Δ rhp51Δ* double mutant strain (Figure 2E). Because one Rad22-YFP focus can recruit more than one DSB (Lisby *et al*, 2001), the number of foci per cell underestimates the real level of unrepaired DNA lesions, which might overload the capacity of the HR machinery to repair all of the damage. However, the lack of cancer predisposition in SCAN1 (Takashima *et al*, 2002) suggests

that most of the lesions are repaired without gross genetic alterations.

DNA damage checkpoints control the apoptotic-like cellular response

In an attempt to determine whether the nuclear DNA degradation observed for *tdp1Δ* cells is genetically controlled, we designed experiments to test the role of the DNA damage checkpoints. Wild-type and *tdp1Δ* strains have been treated with 2 mM caffeine in G0, a known inhibitor of the phosphoinositide 3-kinase-like kinases involved in cell cycle arrest, DNA repair and apoptosis in higher eukaryotes. Both the wild-type and *tdp1Δ* strains, when treated with 2 mM caffeine, exhibit a similar decrease in viability in the G0 state (Figure 3A and data not shown). When the DNA content of these strains is analysed by FACS, after 8 days in G0, we observed that caffeine abolished DNA degradation of quiescent *tdp1Δ* cells (Figure 3B).

As the DNA damage checkpoints (*ATR/rad3* and *ATM/tel1*) are sensitive to caffeine, we directly addressed the role of the two checkpoint kinases with and without Tdp1 individually. We observed that the *tel1Δ* and *tdp1Δ tel1Δ* mutants are slightly more sensitive in G0 than the *rad3Δ* and *tdp1Δ rad3Δ* mutants, respectively (Figure 3A and C). We then followed by microscopic observation, the return to growth of the 8-day-starved mutant cells (Figure 3C). For the *rad3Δ* and *tdp1Δ rad3Δ* mutants, a new population of cells is able to form microcolonies (2–100 cells) and the individual cells from these colonies are not able to grow further, suggesting that they accumulate lethal chromosomal aberrations. These results are consistent with an important function of ATR/Rad3 in the S and G2/M DNA damage checkpoints, arresting the cells after S phase and before their first divisions (Figures 1F and 3C). For the *tel1Δ* mutant, compared with wild type, we observed a slight increase in the proportion of enlarged cells, which further increases in the *tdp1Δ tel1Δ* mutant concomitant with the decrease of the G0 dead cells (Figure 3C).

FACS analyses of these checkpoint mutants after 8 days in G0 indicate that both *rad3Δ* and *tel1Δ* mutants accumulate propidium iodide negative cells (Figure 3B). In contrast, this feature is abrogated in the *rad3Δ tel1Δ* double mutant strain or after caffeine treatment (Figure 3A and B). This result indicates that both kinases control nuclear DNA degradation in G0, and participate in the DNA repair of physiological lesions. This dual role is consistent with the reduced proportion of propidium negative *tdp1Δ* cells in either checkpoint mutant background (Figure 3B). However, the more pronounced decrease in propidium iodide signals found for *tdp1Δ tel1Δ* than for *tdp1Δ rad3Δ* mutant strains, indicates that Tel1 is having a predominant role in the G0 death response. Therefore, this new cell death programme appears genetically determined and functional during the G0 state.

Role of Tdp1 and Pnk1 in the oxidative DNA damage repair pathway

Given the limitation of our approach to directly identify the DNA lesion(s) accumulating in the absence of Tdp1, we have compared the relative contribution of Tdp1 and Pnk1, the DNA kinase/phosphatase of fission yeast (Meijer *et al*, 2002), in the quiescent state and after treatments inducing DNA breaks. We show that the viabilities of the *tdp1Δ*, *pnk1Δ* single mutants and *tdp1Δ pnk1Δ* double mutant in G0 are

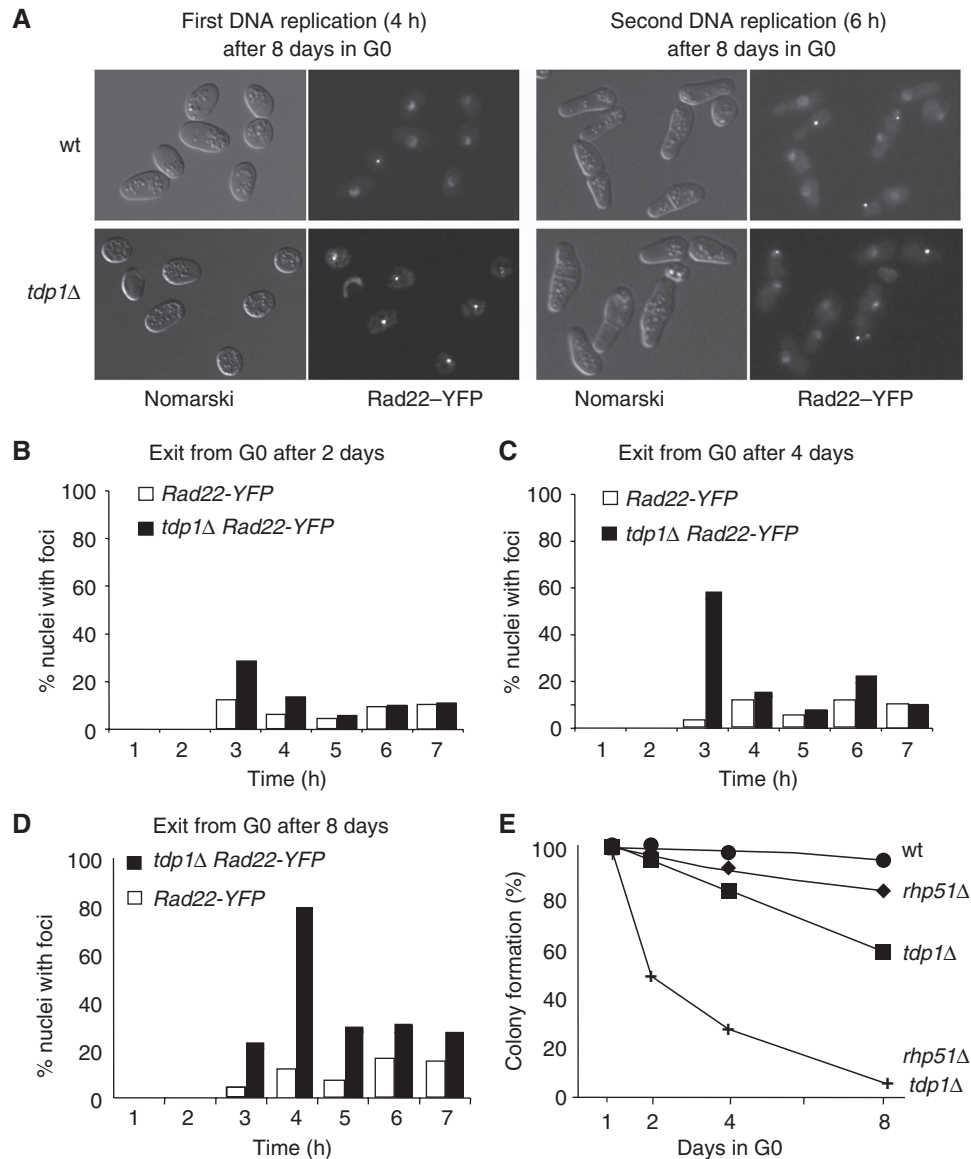


Figure 2 *tdp1Δ* cells in the quiescent state accumulate unrepaired DNA lesions. (A) Rad22^{RAD52}-YFP foci of *tdp1Δ* cells dramatically increases during the first replication of cell cycle re-entry. Fluorescence microscopy of 8-day-starved wild-type and *tdp1Δ* cells during the first and second DNA replication periods after cell cycle re-entry. (B–D) Quantification of the Rad22-YFP foci of 2-, 4- and 8-day-starved wild-type and *tdp1Δ* cells during cell cycle re-entry (more than 500 cells are counted for each time point; $n=2$ independent experiments). (E) The DNA damage accumulating in quiescent *tdp1Δ* cells is repaired by the HR machinery. Plating efficiencies of quiescent wild-type, *rhp51Δ*, *tdp1Δ* and *rhp51Δ tdp1Δ* strains (more than 200 colonies are counted for each strain; $n>2$ independent experiments).

very similar, consistent with both enzymes sequentially processing the same type of DNA lesions (see Introduction). We then compared the cellular viabilities of these mutant strains following treatments with agents inducing SSBs, such as CPT, hydrogen peroxide (H₂O₂), menadione sodium bisulphite (MSB a generator of superoxide) or with BLM, a radiomimetic drug inducing DSBs. As mentioned above, CPT induces 3'-Top I termini, whereas H₂O₂ or MSB produces SSBs with 3'-end DNA lesions, including 3'-phosphoglycolates, 3'-abasic sites and 3'-phosphates. A similar relative sensitivity was found in G0 (Figure 4A) and following CPT, H₂O₂ and MSB treatments, for the single and the double mutants, indicating that the physiological and induced DNA lesions are similarly processed by both enzymes (Figure 4B). When the same mutants are treated with BLM, known to

produce DSBs with 3'-phosphoglycolate ends (Povirk, 1996; Demple and DeMott, 2002), such as ionizing radiation (Strumberg *et al*, 2000), we found that the *tdp1Δ* mutant is less sensitive than the *pnk1Δ* and *tdp1Δ pnk1Δ* mutants (Figure 4B). This result indicates a predominant processing role for Pnk1 in such breaks.

Oxidative DNA damages are chemically complex, as most of the atoms comprising DNA are subject to attack, which can undergo secondary chemical rearrangements. The end products are commonly described as 3'-phosphoglycolates, 3'-abasic sites and 3'-phosphates. Tdp1 is described as a general 3'-DNA phosphodiesterase and was shown to remove a number of the naturally occurring 3'-mononucleosides, but not 3'-phosphates (Interthal *et al*, 2005a; Dexheimer *et al*, 2008). Our data show that *tdp1* and *pnk1* single and double

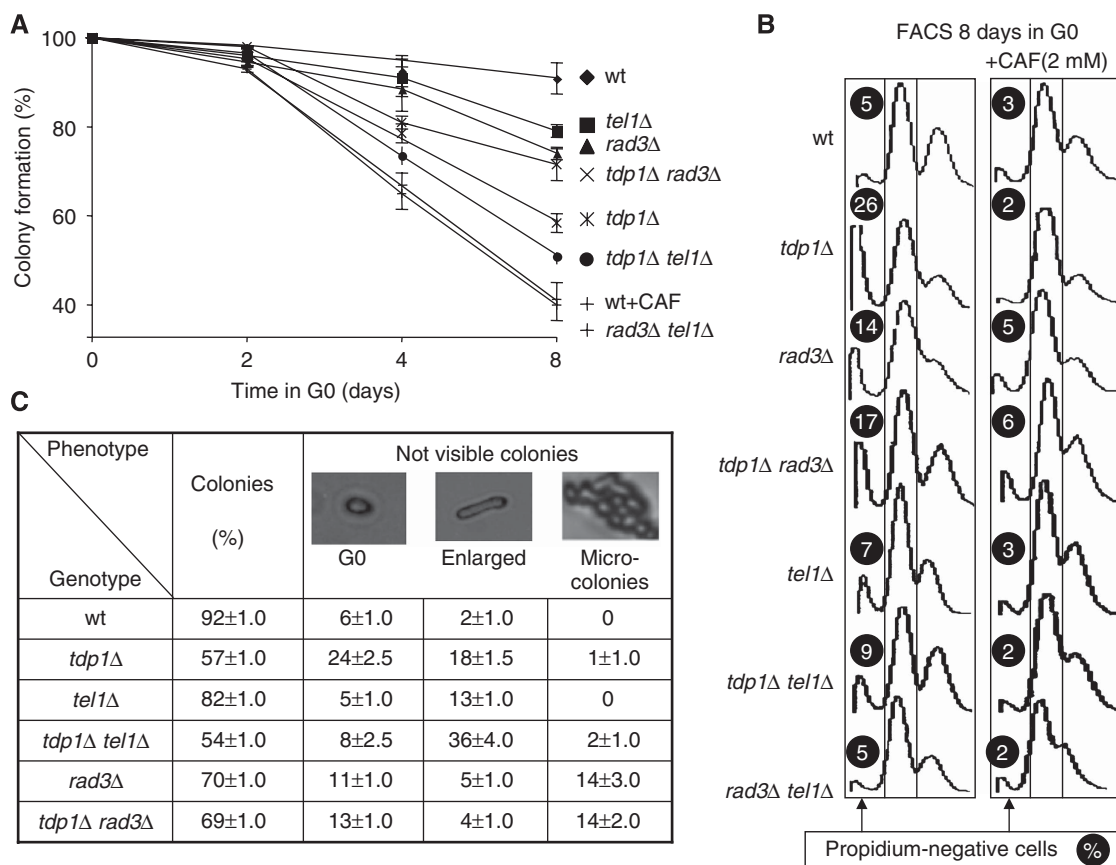


Figure 3 Tel1 and Rad3 regulate nuclear DNA degradation. (A) Viability of the DNA damage checkpoints *rad3Δ* and *tel1Δ* in the presence or absence of *tdp1Δ*. More than 200 G0 cells from each strain are plated in rich media after 2, 4 and 8 days in G0 conditions and counted after 3 days at 33°C. The wild-type strain is also incubated with 2 mM caffeine (wt + CAF). (B) Tel1 and Rad3 control the nuclear DNA degradation in G0. The DNA content for each mutant after 8 days in G0, with and without caffeine (2 mM CAF) is analysed by FACS. The proportion of propidium iodide-negative cells is shown for each mutant, this experiment was repeated two times with similar results. (C) The 8-day-starved mutant cells, analysed in (B), were re-seeded on YES (rich) plates by micromanipulation and allowed to grow for 3 days at 32°C. Microscopic images show the proportion (%) of cells forming colonies, blocked in G0, enlarged or forming micro-colonies (containing less than 100 cells). More than 90 cells are analysed for each mutant; $n > 2$ independent experiments.

mutants are equally sensitive in G0 and exhibit the same sensitivity to H₂O₂ (and MSB). Altogether, we propose that the 3'-phosphate lesion is proportionally a minor physiological or H₂O₂-induced oxidative DNA damage and that Tdp1 and Pnk1 are sequentially processing 3'-mononucleosides at SSBs.

Tdp1 protects against physiological oxidative DNA damage in G0

The central role of oxygen in metabolism, together with the high level of oxygen consumption of brain cells and their post-mitotic nature, has led to the hypothesis that cumulative oxidative DNA lesions might be responsible for neuronal cell death, as observed in SCAN1 patients (El-Khamisy *et al.*, 2005; El-Khamisy and Caldecott, 2007; Rass *et al.*, 2007). Great importance has been attributed to vitamins as antioxidants in the prevention and treatment of conditions associated with oxidative stress for many years. We found that 100 μM of vitamin B6 derivative, pyridoxal 5'-phosphate (PLP) protected the 8-day-starved *tdp1Δ* mutant cells, more efficiently than vitamin C or N-acetyl-L-cysteine antioxidants (Figure 5A). When the cells were simultaneously treated, in the same conditions as above, with an equimolar ratio of

MSB to PLP, viability was fully maintained in both strains (Figure 5B). Because PLP does not react with MSB in liquid medium at pH 5.5 or 7.0 (Chumnantana *et al.*, 2005), we conclude that the antioxidant effect of PLP is intracellular.

As mitochondrial respiration by default generates endogenous ROS, we assessed the contribution of mitochondria for the death of cells lacking Tdp1 by using the *tsf1Δ* strain (Chiron *et al.*, 2005). The *tsf1* gene encodes the mitochondrial translational nucleotide exchange EF-Ts protein. *S. pombe* is a petite-negative yeast and loss of its mitochondrial DNA or translational machinery is lethal. However, the absence of Tsf1 allows residual protein synthesis, low respiration, healthy growing cells in glucose and no flocculation phenotypes. In addition, *tsf1Δ* cells contain a wild-type amount of mitochondrial DNA (Chiron *et al.*, 2005). Plating experiments show that the *tsf1Δ* strain, and more importantly, the *tdp1Δ tsf1Δ* double-mutant strain, maintains almost full viability and do not accumulate propidium iodide-negative cells in G0 (Figure 5C and D). This result clearly shows that low cellular respiration levels protect the *tdp1Δ* mutant cells and indicates that the production of endogenous ROS by-products, generated by mitochondria, is a major cause for the accumulation of DNA lesions in the absence of Tdp1 (Zhou *et al.*, 2005; Interthal *et al.*, 2005b).

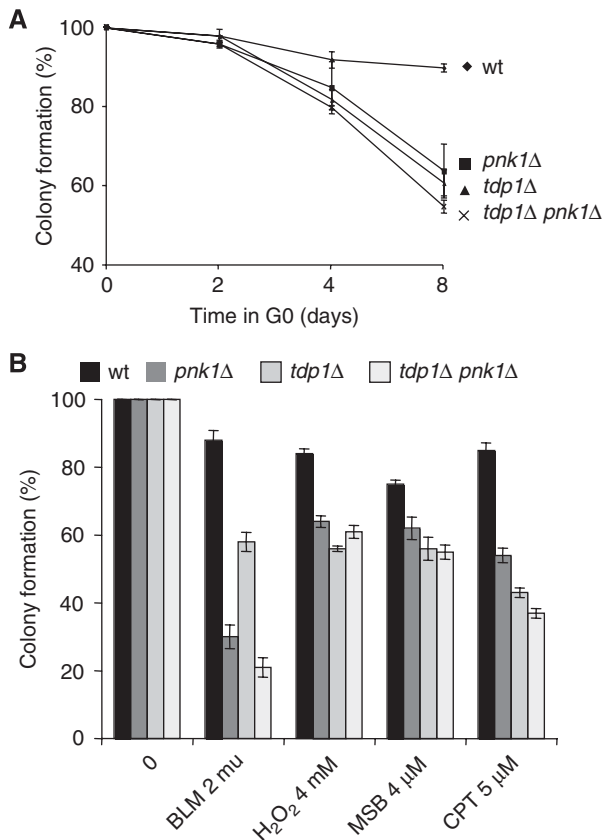


Figure 4 Quiescent *tdp1Δ* cells accumulate SSBs with 3'-phosphoglycolate termini. (A) Viability of *tdp1Δ* and *pnk1Δ* single and double mutants in G0. More than 200 G0 cells from each strain are plated in rich media after 2, 4 and 8 days in G0 conditions and counted after 3 days at 33°C. (B) Sensibility of *tdp1Δ* and *pnk1Δ* single and double mutants following treatment with bleomycin (BLM, 2mU), hydrogen peroxide (H₂O₂, 4 mM), menadione sodium bisulphite (MSB, 4 mM) or camptothecin (CPT, 5 mM). Each drug concentration sensitized the wild-type strain similarly. The strains are incubated for 24 h in G0 and then treated for 24 h before being plated on rich media for 3 days at 32°C and counted colonies are reported in percentage. More than 200 colonies are counted for each condition and the experiments have been repeated two times independently.

Discussion

Because only few unrepaired DNA lesions are sufficient to kill *S. pombe*, it becomes very difficult to directly identify the physiological substrate for Tdp1. We indirectly asked this question by comparing the relative sensitivity of *tdp1Δ* and *pnk1Δ* single and double mutants for known DNA lesions. Our results indicate a similar sensibility of *tdp1Δ*, *pnk1Δ* and *tdp1Δ pnk1Δ* mutants after prolonged incubation in G0 or after H₂O₂, MSB or CPT treatments, but not after BLM treatment. However, Top I is clearly not involved in the physiological death observed after several days in G0, despite the fact that CPT treatment sensitized *tdp1Δ* mutant cells during vegetative and quiescent states. Consistent with this, two recent reports have shown the hypersensitivity to CPT and BLM of Tdp1^{-/-} mice, but they could not reproduce the ataxia phenotype observed in SCAN1 patients, and observed only a modest age-dependent decrease in cerebellar size, with several interpretations proposed therein (Hirano *et al*, 2007; Katyal *et al*, 2007). Our model system allowed us to geneti-

cally test the impact of Top I in combination with Tdp1 in G0, ruling out its putative role, at least in *S. pombe*. Moreover, we were able to reveal a specific sensitivity in G0 in the absence of Tdp1, in physiological conditions, during a 1-week period, instead of 2 years in mouse and 15 years in humans (Takashima *et al*, 2002). This is probably due to a high respiration metabolism and compact haploid genome (Wood *et al*, 2002), in which each essential gene is present in one copy and is coded by less than 3 Mb in this yeast. Even though the main DNA repair enzymes are well conserved between yeast and mammals, some important factors involved in DNA end processing and SSB repair, such as PARP1, XRCC1, Polβ and ligase 3, are not found in simple eukaryotes. This might indicate the need to increase the rate of SSB repair to cope with the increasing size of the genomic message and the complexity of cellular metabolism, organization and developmental fate arising during evolution (Caldecott, 2008).

It is well recognized that ATM/Tel1 checkpoint responses are triggered by DSBs, thus maintaining the integrity of the genome, minimizing the risk of cancer and neurodegeneration (Lavin, 2008) and consequently one concern is whether SSBs or DSBs are accumulating in G0 in the absence of Tdp1. The spectrum of drug sensitivities discussed above for the *tdp1Δ* mutant argues that the initial lesions are SSBs, but we cannot exclude that such SSBs are slowly transformed into gaps and ultimately into DSBs, by some unknown process. However, there is growing evidence that the initial stimulus for ATM activation correlates with relaxation of chromatin structure. For examples, low doses of irradiation, oxidative stress or agents altering the chromatin structure, not known to introduce DSBs, activate ATM and stabilize the tumour suppressor p53, but fail to activate other substrates, including SMC1 and NBS1, involved in DSB repair (Lavin, 2008). Moreover, it was recently reported that oxidative damage activates the ATM/Tel1 pathway independently of DSB formation (Bencokova *et al*, 2009; Chou *et al*, 2008), a phenomenon still poorly understood in higher eukaryotes (Goodarzi *et al*, 2008; Soutoglou and Misteli, 2008).

The nuclear DNA degradation results observed in the individual (*rad3Δ* or *tel1Δ*) checkpoint mutants, suggested that each checkpoint mutant accumulates lesions, which are detected by the remaining checkpoint. In the absence of both checkpoints, the number of lesions presumably increases, as observed by the loss of viability, but without a nuclear degradation response. In the absence of Tdp1, the unrepaired lesions accumulated in an age-dependent manner and both checkpoints trigger DNA degradation. When either checkpoint is absent, in *tdp1Δ* strain, the amplitude of DNA degradation diminished correspondingly, and reached a baseline level in the presence of caffeine. The cellular aspect of the cells during their re-entry in the vegetative state provides some additional information. When we compared the *tdp1Δ* and *tdp1Δ tel1Δ* mutants, we observed that the double mutant decreases the proportion of small G0 cells, concomitantly increasing the proportion of enlarged cells (Figure 3C). This result indicates that the *tdp1Δ tel1Δ* cells are not dead and are still able to re-enter into the vegetative cycle, albeit not successfully, despite the proficient ATR/Rad3 checkpoint and HR processes. Taken together, these results suggest that the ATR and ATM checkpoints are partially redundant and that the ATM/Tel1 checkpoint is the main candidate to

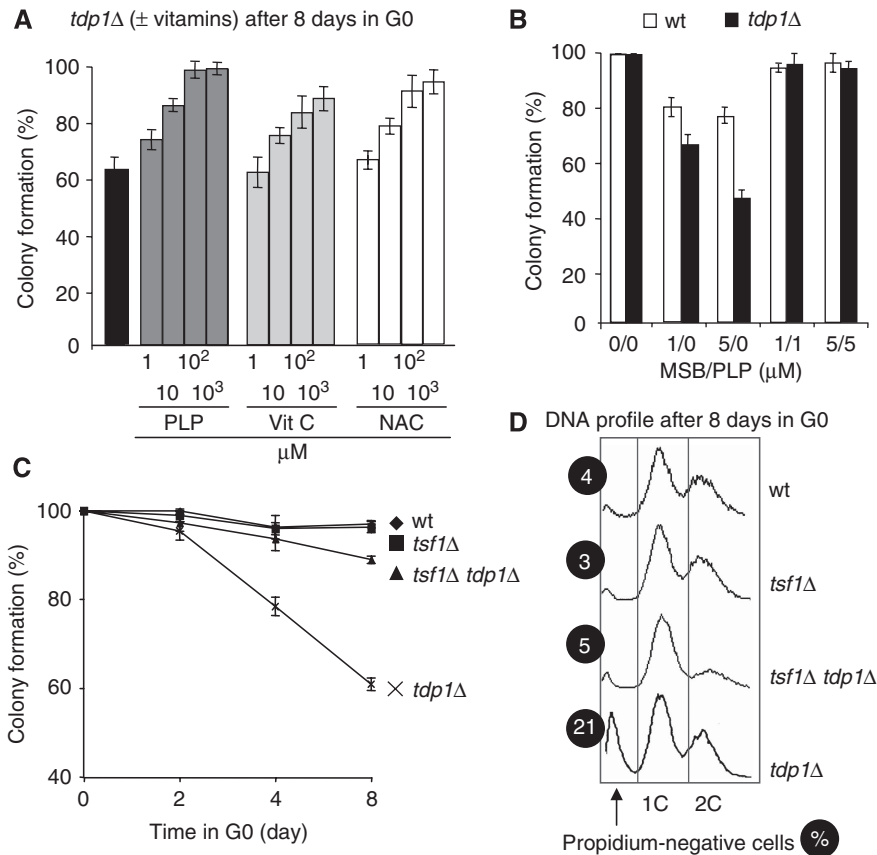


Figure 5 Mitochondrial-generated ROS induce the death of *tdp1*Δ cells. (A) Vitamin B6 (or PLP), vitamin C (vit C) and *N*-acetyl-L-cysteine (NAC) protect the quiescent *tdp1*Δ cells. The strains are incubated for 24 h in G0 and then treated for 24 h with pyridoxal 5'-phosphate (PLP), vitamin C or *N*-acetyl-L-cysteine before being plated and incubated for 3 days. (B) PLP counteracts the deleterious effects of the superoxide generator MSB. PLP was introduced before MSB in the culture. The procedure is the same as above (for (A, B), more than 200 colonies are counted for each condition and the experiments have been repeated three times independently). (C) The low respiration rate observed in the *tsf1*Δ mutant protects the quiescent *tdp1*Δ cells. Plating efficiencies after 2, 4 and 8 days in G0 of quiescent wild-type, *tdp1*Δ, *tsf1*Δ and *tsf1*Δ *tdp1*Δ strains (more than 500 colonies are counted for each strain, $n=3$ independent experiments). (D) FACS analyses of the 8-day-starved *tsf1*Δ and *tsf1*Δ *tdp1*Δ mutant strains showing the lack of propidium iodide-negative cells. The percentage (%) of propidium iodide-negative cells is indicated for each strain.

monitor the threshold and severity of oxidative DNA damage, to trigger cell death by nuclear DNA degradation during quiescence. How the repair complexes sense damaged DNA and activate the ATM/Tel1 checkpoint response are poorly understood and several models have been proposed, which include the DNA lesions by themselves, a change in the chromatin structure or transcription abortion (Ljungman and Lane, 2004; Soutoglou and Misteli, 2008). In addition, although the ATR/Rad3 pathways during S and G2 are quite well understood in *S. pombe*, involving the Chk1 and Cds1 kinases, the specific downstream effectors of ATM/Tel1, with the exception of the Nbs1 protein, are still unknown (Subramanian *et al*, 2008). The absence of the tumour suppressor p53 protein in simple eukaryotes, and particularly in *S. pombe*, reveals a novel response to DNA damage and further analysis of the downstream targets of ATM/Tel1 will hopefully shed light on this intriguing cell death programme (Shimanuki *et al*, 2007).

In conclusion, the natural hypersensitivity of the *tdp1*Δ fission yeast cells in quiescent conditions is due to the accumulation of oxidative unrepaired SSBs, and lowering respiration or treatment with the vitamin B6 derivative, PLP, abrogates this sensitivity. We consistently observed

that vitamin B6 showed a better protective effect in the absence of Tdp1 than the other antioxidant molecules used in this study and a similar relative effect was reported during vegetative growth upon exogenous oxidant treatments (Chumantana *et al*, 2005). We do not know the reason for this, but we propose that PLP might be specifically imported inside the cell to function directly as an antioxidant. Moreover, PLP is built from ribose 5-phosphate, a precursor common with other important pathways and is the main cofactor of enzymes involved in amino-acid metabolism, a critical function in G0 (Amadasi *et al*, 2007).

This study may impact the prevention or treatment of human autosomal recessive ataxias, such as the SCAN1 syndrome (Rass *et al*, 2007) and other neurodegenerative diseases linked to defects in genes influencing physiological levels of ROS (Bohr *et al*, 2007; Navarro and Boveris, 2007). We also showed that the *S. pombe* quiescent state (Su *et al*, 1996) provides a model to revisit the roles of the different DNA repair pathways in non-dividing physiological conditions, a poorly understood physiological state, and to establish a new hierarchy among them. Relevant to cancer, Tdp1 overexpression is commonly observed in primary non-small cell lung cancers (Liu *et al*, 2007; Rosell *et al*, 2007),

indicating an increased DNA repair requirement, potentially due to high oxidative stress in these frequent solid tumours (Dexheimer *et al*, 2008).

Materials and methods

Strains and growth conditions

Wild-type and mutant haploid heterothallic strains are all isogenic and prototrophic. The *tdp1Δ* mutant was constructed by substituting the *tdp1* gene by the *kanamycin* gene by one-step homologous insertion. The strains are grown in minimal well-defined medium (MM) (Moreno *et al*, 1991) containing 3 or 5% glucose. Yeast cells are grown in MM to a density of 6×10^6 cells/ml at 32°C, harvested by centrifugation (3000 r.p.m./5 min) washed in MM-N (without nitrogen) and re-suspended in MM-N at a density of 10^6 cells/ml at 32°C, reaching after two rounds of cell division 4×10^6 cells/ml. This density of cell bodies is stable for several weeks and was used as a source of G0 cells. The re-entry into the vegetative cycle is performed by replacing the MM-N by one volume of fresh rich medium (YES). Samples were taken before or after the addition of YES at various incubation times for assays. Vitamins (B6, PLP and C) and drugs (BLM, H₂O₂, CPT and MSB) were obtained from Sigma-Aldrich and added to yeast cultures in the dark for the indicated time periods.

Cell viability, FACS analysis and fluorescence microscopy

Cell density was determined by the particle counter Z1 (Beckman). Cell viability was determined by plating on rich (YES) plates, and after 3 days of incubation at 32°C, colonies were counted. Viability

is expressed as a fraction (%) of colonies divided by particles plated. Haploid *S. pombe* cells spend more time in G2 in growing conditions and the DNA content of a cell is 2C. In nitrogen-starved conditions, cells accumulate in G1 after two cell divisions with a 1C DNA content. For flow cytometry analysis (FACS), 4×10^6 cells (1 ml) were harvested and resuspended in 1 ml of cold 70% ethanol overnight at 4°C. To improve the propidium iodide staining, after removal of the ethanol, the cells are resuspended in 1 ml of 0.1 N HCl containing 2 mg/ml pepsin for 1 h at room temperature, washed in 1 ml of 50 mM sodium citrate (pH 7.0) and resuspended in 0.5 ml of 50 mM sodium citrate buffer, containing 0.1 mg/ml RNAase A for 1 h at 37°C. Next, we added 0.5 ml of 50 mM sodium citrate, containing 4 μg/ml propidium iodide followed by moderate sonication before FACS analysis. For the Rad22–YFP foci analysis, following replacement in rich medium, samples were taken at various time intervals and directly observed by fluorescence microscopy. For DAPI staining, the cells are fixed with 70% cold ethanol, washed in 1 ml of 50 mM sodium citrate (pH 7.0), resuspended in 0.5 ml of 50 mM sodium citrate buffer containing DAPI and observed with a fluorescence microscope using a UV filter. All of the assays were independently repeated several times (at least two times).

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