Cells limit mutagenesis by dealing with DNA lesions behind the fork

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Abstract

DNA lesions are a threat for genome stability. In order to cope with these lesions, cells have evolved lesion tolerance mechanisms: Translesion Synthesis (TLS) that allows the cell to insert a nucleotide directly opposite to the lesion, with the risk if introducing a mutation. Or error-free Damage Avoidance (DA) that uses homologous recombination to retrieve the genetic information from the sister chromatid.

In this article, we investigate the timing of lesion bypass. We show that TLS can occur at the fork, rapidly after the encounter with the blocking lesion. But TLS can also occur behind the fork, at postreplicative gaps that are generated downstream of the lesion after repriming has occurred. We show that in this latter situation, TLS is reduced because it is in competition with the Damage Avoidance pathway. We also showed that EXO1 seems to modulate the size of the post-replicative gaps, that in turn modulate the balance between TLS and damage avoidance.

Introduction

The DNA of every organism is constantly damaged by several exogenous and endogenous agents. These damages will frequently block the progression of the replicative DNA polymerase, impeding the progression of the replication fork, and in turn threatening genome stability. Cells have evolved lesion tolerance mechanisms that allow them to deal with DNA lesions. Translesion synthesis (TLS) is an error-prone process that involves using specialized DNA polymerases to insert nucleotides directly opposite the damaged site. Damage avoidance (DA) is an error-free process that relies on homologous recombination to bypass the damaged site. The balance between error-prone TLS and error-free DA defines the level of mutagenesis during lesion bypass.

Whereas cells deal with DNA lesion at the fork or post-replicatively has been a long-standing debate. The initial work of Rupp and Howard-Flanders (Rupp and Howard-Flanders, 1968) suggested that repriming could occur behind a lesion, leading to the generation of post-replicative gaps that were dealt with behind the replication fork. Later on, this idea was put aside when the TLS polymerases were discovered: the model was then that TLS Polymerases would transiently replace the replicative DNA polymerase at the fork, with no need for repriming or generation of gaps (Pagès and Fuchs, 2002). This model was then challenged when gaps were directly observed by EM in UV-irradiated *S. cerevisiae* (Lopes et al., 2006), and when restarting of replication downstream a lesion was observed in vitro in *E. coli* (Heller and Marians, 2006). Even more recently the discovery of primpol in mammalian cells (Bianchi et al., 2013; García-Gómez et al., 2013) strongly argues in favor of the repriming model and therefore post replicative lesion bypass.

While repriming is now generally admitted, there are still some debates about what happens at the replication fork. Indeed, TLS could in theory occur both at the replication fork and at a postreplicative gap. Similarly, DA can occur by HR at a postreplicative gap, but some other kind of HR-related strand exchange could also occur directly at the replication fork by the formation of regressed fork also named "chicken-foot" structure. Such structure could allow the use of the sister chromatid as a matrix, without the need to generate post-replicative gaps (Sogo et al., 2002).

In this work, we reconciliated both model showing that some lesions could be bypassed by TLS directly at the replication fork, while for some others, TLS occurs post-replicatively. We also show that when lesions are bypassed behind the fork, TLS and therefore mutagenesis is limited because of the competition with HR-related DA.

Results and Discussion

We have recently developed a system by which we are able to insert a single lesion at a specific locus in the genome of *S. cerevisiae*, and to monitor the bypass of this lesion by either TLS or DA (Masłowska et al., 2019). In short, we construct a plasmid that contains the single lesion of interest. We insert this plasmid at a specific locus of the yeast genome by the mean of the Cre recombinase and modified lox sites. Lesion bypass is monitored by counting blue and white colonies as the lesion is inserted in the lacZ reporter gene. For more detail, see (Masłowska et al., 2019).

TLS and DA are in competition

Using this system, we have monitored the bypass of a common UV lesion, the (6-4)TT photoproduct and have shown that TLS is in competition with DA. Indeed, inactivation of *ubc13* prevents PCNA poly-ubiquitination (the signal activating the DA pathway) led to a decrease in the use of DA and a concomitant 10-fold increase in the level of TLS. Similarly, inactivation of *rad51* led to the same decrease in DA and increase in TLS.

In the *ubc13* or *rad51* strains, some DA still persists as we still observed a significant number of white colonies in our assay. These colonies could arise from RAD51-independent template switching mechanisms that rely on RAD52 (Gangavarapu et al., 2007).

The (6-4)TT photoproduct is bypassed by the combination of TLS polymerases Pol ζ-Rev1. We have repeated the experiment using the G-AAF lesion that is also bypassed mostly by the same two DNA polymerases, even though a small fraction of the bypass at this lesion could be done by Pol η (Masłowska et al., 2022; Pagès et al., 2008). As shown in Figure 1A, we found similar results: inactivation of either *ubc13* or *rad51* led to a decrease in DA and an increase in TLS. It appears therefore that these two pathways are in competition.

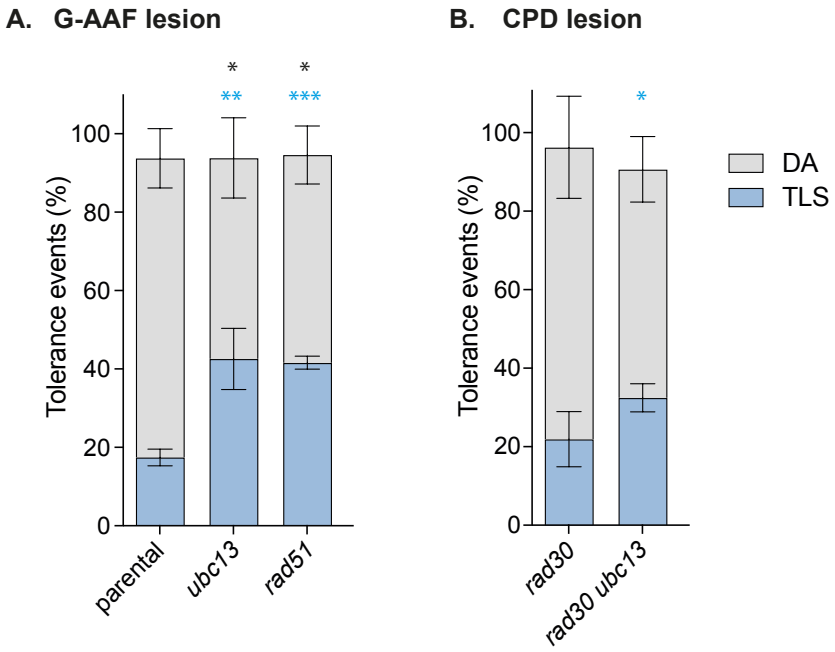

Figure 1: Partitioning of DNA damage tolerance pathways A: at a G-AAF lesion. B: at a CPD lesion

However, when using the TT-CPD lesion that is bypassed essentially by DNA Polymerase η, but also by the combination of Pol ζ-Rev1, we did not observe any increase in TLS when *ubc13* or *rad51* were inactivated (Masłowska et al., 2019). However, when monitoring the bypass of the same lesion in the absence of Pol η, we did observe an increase in TLS when ubc13 was inactivated (Figure 1B).

Model: competition between TLS and DA occurs behind the fork at post-replicative gaps.

As we did not observe a competition between DA and TLS for this lesion when it is bypass by Pol η, we drew the following model:

We hypothesized that the competition between DA and TLS observed (increase of TLS in the *ubc13* strain), occurs behind the fork, during a gap filling reaction: following the encounter with a blocking lesion, a repriming event generates a single-strand DNA gap that will be filled post-replicatively by TLS and DA, putting the two pathways in competition. As Rev1 expression level is much higher in G2/M than in S phase (Waters and Walker, 2006), it implies that it acts mostly post-replicatively rather than at the fork.

On the other hand, Pol η is expressed constantly along the cell cycle (Waters and Walker, 2006), it is therefore able to bypass lesions both at the fork and post-replicatively.

In our model, the majority of CPD lesions that is efficiently bypassed by Pol η, would be bypassed at the fork without the need for repriming, alleviating the competition with DA.

If this model is valid, forcing Pol η to bypass CPD lesion post-replicatively (instead of at the fork) should induce a competition of TLS with DA for this lesion (as observed for the 2 other lesions tested).

In order to force Pol η action to occur only post-replicatively, we restricted its expression to the G2/M phase of the cell cycle. By being absent during S phase, Pol η cannot play at the replication fork. Being present only in G2/M allows it to play post-replicatively.
We used two different strategies for this purpose: i) we expressed *RAD30* from the CLB2 promoter including the cyclin degron that allows expression of the protein only during the G2/M phase of the cell cycle; ii) we used the auxin-inducible degron (AID) system so we were able to induce the degradation of Pol η in G1 and S phase, forcing its expression only in G2/M (Figure 2).

We synchronized our cells by blocking them in G1 using alpha-factor, and while monitoring the cell cycle by FACS, we monitored the level of expression of Pol η by Western blot.
As shown in Figure 2, the Clb2-controlled RAD30 construction allows peak expression of Pol η in G2/M, but while the level is very low in G1 and at the beginning of S phase, it already starts increasing in mid to late S phase. The Auxin-controlled *RAD30* construction shows a better restriction of expression in G1/S, and a strong level of expression in G2/M. Another advantage of this latter construction is that it allows a physiological level of expression of Pol η as it is controlled by its natural promoter.

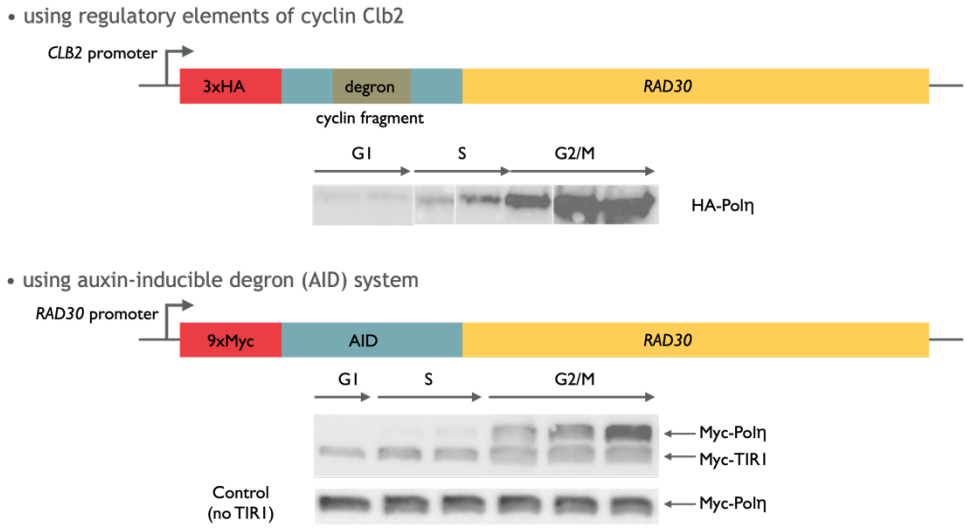

Figure 2: construction to restrict the expression of Pol η in G2/M, using the CLB2 promoter (upper panel), or the Auxin-inducible degron (lower panel). Western blots show the level of expression of HA-Pol η or Myc-Pol η at different phases of the cell cycle.

Using these two strains, we introduced a single CPD lesion in the cell genome during the S phase of the cell cycle, and measured the level of TLS and DA. As shown on Figure 3A, compared to the parental strain, the level of TLS at the CPD lesion significantly decreases when Pol η is expressed only in G2/M, and the level of DA concomitantly increases. The level of TLS is not as low as in the absence of Pol η (Figure 1B): this could be due to the fact that either some Pol η is still present in S phase and participates to TLS, or that it still participates to TLS in G2/M, showing a higher level than in the complete absence of the polymerase. According to our hypothesis, this decrease in TLS would be due to the fact that Pol η acting post-replicatively (in G2/M), TLS is now in competition with DA.

To confirm this hypothesis, we repeated the experiment in a strain where *ubc13* was inactivated in order to prevent DA (Figure 3B). The inactivation of *ubc13* did not lead to an increase in TLS in the parental strain where Pol η is expressed all along the cell cycle as previously shown (Masłowska et al., 2019). However, when Pol η expression was restricted to G2/M, and that TLS was potentially in competition with DA, the inactivation of *ubc13* did lead to an increase in TLS. Inactivation of *ubc13* in the two strains expressing Pol η in G2/M actually

restored TLS to levels similar to the parental strain where Pol η is expressed constantly along the cell cycle.

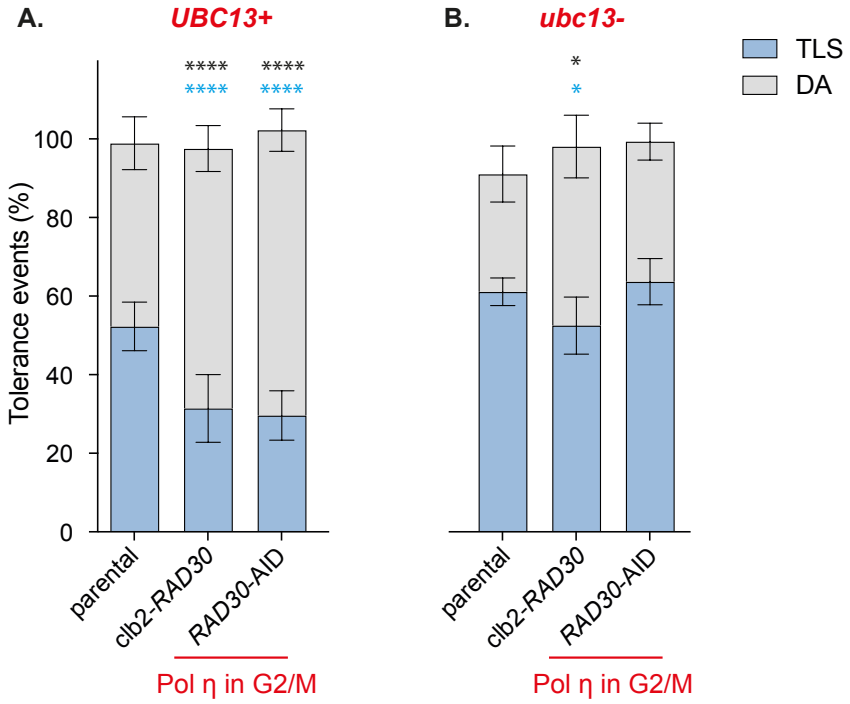

Figure 3: Partitioning of DDT pathways at a single CPD lesion in strains expressing Pol η only in G2/M, A. in cells procificent for DA (in the presence of *UBC13*), B. in cells deficient for DA (in the absence of *ubc13*Δ).

These experiments validate our model. It appears therefore that when a lesion that is bypassed at the replication fork (such as the TT-CPD bypassed by Pol η), TLS is not in competition with DA, allowing a high level of TLS. When the same lesion is bypassed post-replicatively, it is then in competition with DA and the level of TLS is reduced.

It is interesting to note that we obtained similar results with both constructions that express Pol η in G2/M. However, as stated earlier, the CLB2 construction allows some level of expression of Pol η already in mid to late S-phase. Our lesion being located close to an early replication origin (see description of the system in (Masłowska et al., 2019)), we can expect it to be encountered by the replication fork early in the S-phase, when Pol η is still absent or at least very weakly expressed in our strain. Whether Pol η reappears in mid to late S-phase (CLB2 construction) or in G2/M (AID construction), the replication fork has already bypassed the lesion and the gap is formed implying competition with DA. This suggests that DA is not

restricted to G2/M phase, but can occur in early S-phase, as soon as post-replicative gaps are formed.

This also suggests that TLS has priority over DA. Indeed, if DA can occur in early S-phase when Pol η is absent, in the normal conditions where Pol η is present, the level of TLS is higher, implying that TLS won the competition over DA. This priority given to TLS seems to have been conserved through evolution as we have previously shown that TLS had also priority over DA in the bacteria *E. coli* (Naiman et al., 2014).

Gap extension is key to favor DA and reduce TLS

It appears from our results that post-replicative gaps are key players in the regulation of DNA damage tolerance. In order to further investigate these findings, we looked at the role of *EXO1* in DNA damage tolerance. *EXO1* encodes a 5'->3' exonuclease that has been mostly described for its role in recombination at double strand breaks (during meiosis initially, but also in mitotic cells), and in telomere maintenance (for a review, see (Tran et al., 2004)). It has also been shown that Exo1 was able to extend ssDNA gaps during Nucleotide Excision Repair (Giannattasio et al., 2010). We have recently shown in bacteria that the extension of ssDNA gaps was crucial for DA to occur efficiently. Indeed, in the absence of the 5'->3' exonuclease RecJ, we have observed a reduction of DA and a concomitant increase in TLS (Chrabaszcz et al., 2018; Laureti et al., 2022). The role of yeast Exo1 in postreplication repair has been previously proposed (Tran et al., 2007), suggesting that the same role in gap extension is required for DA.

We have inactivated *exo1* and monitored the bypass of 3 different DNA lesions. As reported Figure 4, we observed a strong increase in TLS for the TT(6-4) (>9 fold) and for the G-AAF (>3 fold) lesions in the absence of Exo1. This confirms that TLS at these two lesions occurs at the post-replicative gap: in the absence of gap extension by Exo1, homologous recombination with the sister chromatid (DA) is less efficient and TLS can occur at a higher rate.

On the other hand, we did not observe any significant increase in TLS at the CPD lesion.

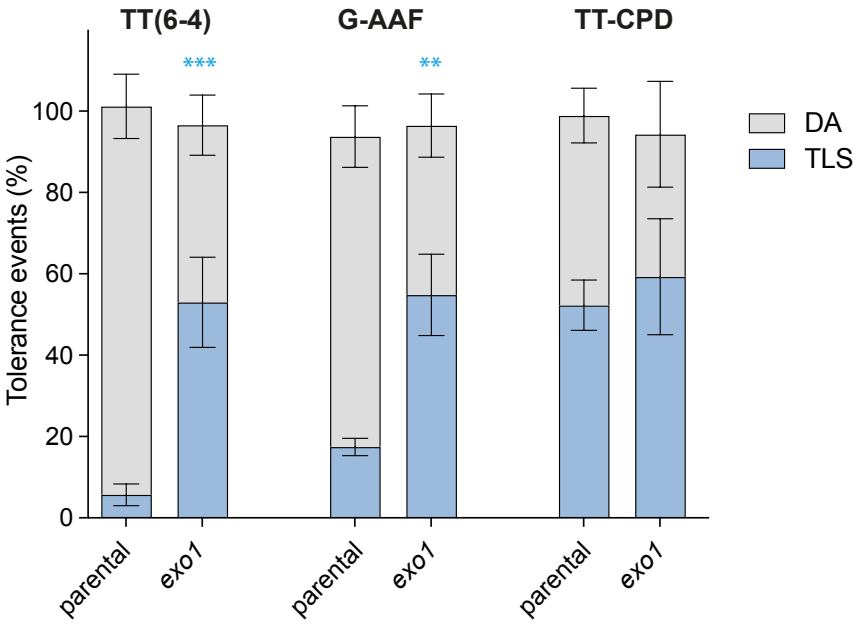

Figure 4: Partitioning of DDT pathways at 3 different DNA lesion in the presence (parental) and absence of Exo 1.

Conclusions

In conclusion, we have shown that TLS could occur both at the fork and behind the fork. When it occurs at the fork, it has priority over DA. On the other hand, while when occurring post-replicatively, TLS is reduced because it is then in competition with DA (Figure 5).
By promoting the extension of postreplicative gaps, Exo1 favors Homologous recombination which favors DA and in turn decreases TLS when the two pathways are in competition.

It is the timing of expression of the TLS polymerase that dictates when TLS occurs: Rev1 being expressed mostly in G2/M, it limits its action and the one of Pol ζ with which it interacts (Acharya et al., 2006) to post-replicative gaps. This leads to a reduce level of TLS by these two polymerases when DA in functional. On the other hand, Pol η being expressed constantly along the cell cycle, it could achieve TLS at the fork in S phase.
It would be interested to test if constant expression of Rev1 along the cell cycle would be able to increase the level of TLS by alleviating the competition with DA. However, the control of Rev1 appears not to be transcriptional (Waters and Walker, 2006) which makes it difficult to modulate and therefore to investigate.

Since TLS by Rev1-Pol ζ at several lesions is much more mutagenic than TLS by Pol η at CPD lesions, over evolution, cells might have restricted the expression of Rev1 to G2/M giving them the advantage to tune down this mutagenic pathway by allowing competition with DA.

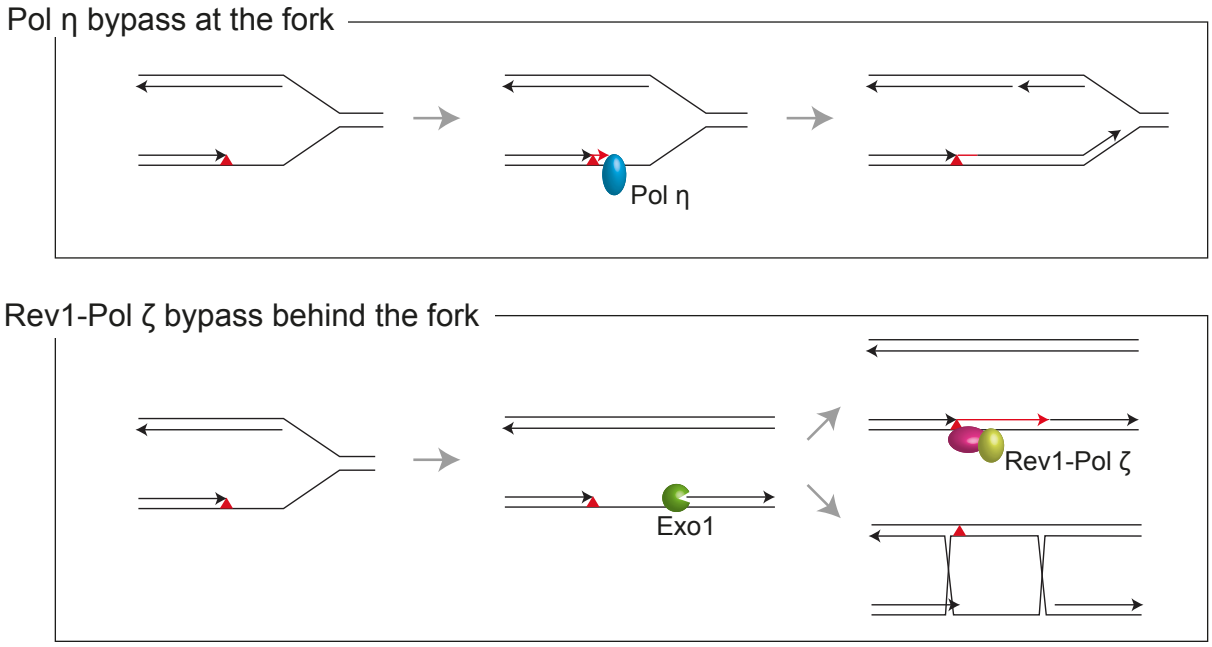

Figure 5: model for lesion bypass at the fork or behind the fork. The bypass of CPD lesion by Pol η occurs at the fork and is not in competition with DA. Lesions bypassed by Rev1-Pol ζ occurs behind the fork at a single-stranded DNA gap. This gap is extended by Exo1 which favors DA. DA is in competition with Rev1-Pol ζ TLS.

Materials and methods

Yeast strains

All strains used in the present study are derivative of strain EMY74.7 (Johnson et al., 1998) (MATa his3-Δ1 leu2-3,112 trp1Δ ura3-Δ met25-Δ phr1-Δ rad14-Δ msh2Δ:hisG). In order to study tolerance events, all strains are deficient in repair mechanisms: nucleotide excision repair (*rad14*), photolyase (*phr1*), and mismatch repair system (*msh2*). Gene disruptions were achieved using PCR-mediated seamless gene deletion (Akada et al., 2006) or URAblaster (Alani et al., 1987) techniques.
Strains carrying polymerase η (*RAD30*) under control of auxin-inducible degron (AID) were created by inserting the pKAN-PRAD30-9myc-AID*(N) cassette into the Rad30 native locus. Strains carrying polymerase η (*RAD30*) under control of regulatory elements of cyclins Clb2 (from pGIK43) or Clb5 (from pKM101) were created by inserting cyclin cassettes into native Rad30 locus.
TIR1 strains were created by integration of osTIR1 cassette from pNHK53 (encoding OsTIR1 under control of the ADH1 promoter) into yeast chromosome VII.
Plasmid pNHK53 was obtained from the National BioResource Project–Yeast (Nishimura et al., 2009), plasmid pKAN-PCUP1-9myc-AID*(N) was from from Addgene (Morawska and Ulrich, 2013), and plasmid pGIK43 from Georgios Karras.

Integration

Integration of plasmids carrying (6-4)TT/N2dG-AAF lesions (or control plasmids without lesion) and result analysis was performed as previously described (Masłowska et al., 2019). For experiments involving cell-cycle restricted polymerase η cells were synchronized in G0 phase using alpha-factor. After synchronization, wash, conditioning and electroporation steps were carried out directly. For strains with AID degrons auxin was added at 1mM during incubation with alpha-factor.
Lesion tolerance rates were calculated as the relative integration efficiencies of damaged vs. non-damaged vectors normalized by the transformation efficiency of a control plasmid (pRS413) in the same experiment. DA events are calculated by subtracting TLS events from the total lesion tolerance events. All experiments were performed at least in triplicate. Graphs and statistical analysis were done using GraphPad Prism applying unpaired t-test. Bars represent the mean value ± s.d.

Synchronisation

Yeast cultures were grown till OD600=0.8. Alpha-factor (GeneScript) was then added at the concentration 10 µg/ml, and cells were further incubated for 90 min. Synchronisation was verified by microscopy, and further by flow cytometry.

Detection of proteins

Total lysates of synchronized yeast cultures were prepared by quick trichloroacetic acid (TCA) extraction: cells (pelleted 10 ml of culture) were resuspended in 250 µl of 20% TCA and vortexed with glass beads for 30s. After centrifugation at 3000 × g for 10 min, the supernatant was removed and the pellet resuspended in LDS loading buffer and incubated at 75 °C for 10 min. Proteins were analysed by SDS–PAGE/Western blotting using monoclonal antibodies anti c-Myc 9E10 or anti HA 12CA5 (Thermo).

Flow cytometry

Cells were fixed in 70% ethanol overnight and washed with 50 mM TE, pH 7.5. After incubation with 0.1 mg/ml DNase-free RNAse A for 4 h at 42°C, and 0.5 mg/ml Proteinase K for 30 min at 50°C, DNA was stained with SYTOX green. Cells were analyzed by flow cytometry, using a Accuri C6 Plus flow cytometer (BD Biosciences).

Acknowledgements

We thank Luisa Laureti for critical reading of the manuscript.

Funding

This work was supported by Fondation pour la Recherche Médicale [Equipe FRM-EQU201903007797] https://www.frm.org (VP). KM was supported by Fondation de France.

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