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## Opposite roles of Rad5 in DNA damage tolerance: playing in both error-free and mutagenic lesion bypass

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### ABSTRACT

DNA Damage Tolerance (DDT) functions to bypass replication-blocking lesions and is divided into two distinct pathways: error-prone Translesion Synthesis (TLS) and error-free Damage Avoidance (DA).

Rad5 is a multifunctional protein that is involved in these DDT processes. Rad5 contains three well defined domains: a RING domain that promotes PCNA polyubiquitination, a ssDNA-dependent ATPase/helicase domain, and a Rev1-binding domain. Both the RING domain and the ATPase/helicase domain are conserved in human Rad5 ortholog HLF.

In this study we used domain-specific mutants to address the contribution of each of *Saccharomyces cerevisiae* Rad5 functions to lesion tolerance. Using an assay based on the insertion of a single lesion into a defined locus in the genome of a living yeast cell, we demonstrate that Rad5 plays two opposite roles in lesion tolerance: i) Rad5 favors error-free lesion bypass by activating template switching through polyubiquitination of PCNA; ii) Rad5 is required for TLS by recruiting the TLS polymerase Rev1. We also show that the helicase activity does not play any role in lesion tolerance.

### INTRODUCTION

The DNA of every living cell is constantly threatened by various damaging agents. Despite the efficient action of DNA repair mechanisms, some damage may persist long enough to be present during replication, blocking the replicative polymerases, which threatens genome stability [1]. Therefore, to complete replication, cells need to tolerate the encountered DNA damage. There are two distinct DNA Damage Tolerance (DDT) mechanisms: i) error-prone Translesion Synthesis (TLS), employing specialized low-fidelity DNA polymerases able to insert a few nucleotides opposite the lesion [2]; ii) Damage Avoidance (DA), a generally error-free pathway that relies on homologous recombination (HR) to retrieve the genetic information from the non-damaged sister chromatid [3] (also reviewed in [4,5]).

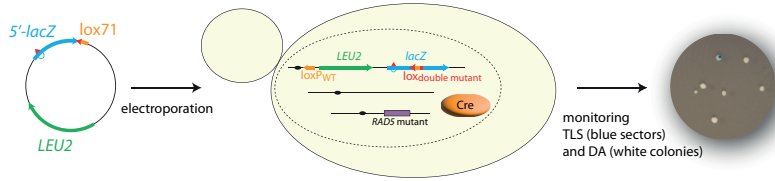
The balance between error-prone TLS and error-free DA defines the level of mutagenesis during lesion bypass. However, the current understanding of the precise molecular mechanisms regulating the process of DNA Damage Tolerance is far from complete.

In eukaryotes, lesion tolerance is controlled by the ubiquitination of proliferating cell nuclear antigen (PCNA) (reviewed in [6,7]). PCNA monoubiquitination by Rad6 and Rad18 promotes the recruitment of TLS polymerases. Extending this modification to polyubiquitination by Mms2/Ubc13 and Rad5, enables the recombination-mediated mechanisms [8].

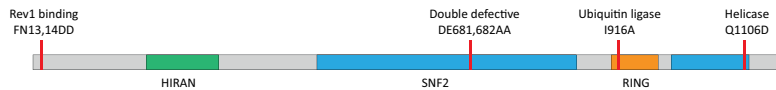
Rad5 is a large multifunctional protein that contains both ubiquitin ligase and ssDNA-dependent ATPase activities [9]. These domains and functions are shared with its human orthologs HLF and SHPRH [10,11]. As E3 ubiquitin ligase, Rad5 catalyzes PCNA polyubiquitination by bridging PCNA with the E2 (Mms2-Ubc13) and accelerates ubiquitin transfer from the E2 to Ubi-PCNA. It also acts as a bridging factor to bring Ubc13 and Mms2 into contact with the Rad6/Rad18 complex, thereby providing a means to coordinate the distinct ubiquitin-conjugating activities of Rad6 and Ubc13/Mms2 [12]. As a DNA-dependent ATPase, Rad5 is a member of the DEAD box family of helicases. *In vitro* it has the capacity to catalyze the reversal of replication fork-like structures [13–15], although in yeast fork reversal has been consistently interpreted as a pathological transaction at replication forks that have lost their replication capacity [16]. It has been suggested that Rad5 ATPase activity is important rather for DSB repair [17]. It has been demonstrated that the Rad5 ATPase/helicase activity is not required for PCNA polyubiquitination [18]. Incidentally, the relevant catalytic domains of Rad5 overlap. The RING E3 ligase domain responsible for E2 interaction resides within the helicase domain, inserted between the conserved helicase motifs III and IV [12,19] (Figure 1B).

Rad5 also plays a structural role in the recruitment of TLS polymerases through physical interaction with Rev1 via its N-terminus [20]. The fact that Rad5 is involved in both branches of DDT implies that it may play a role in the pathway choice and balance within DDT. However,

### A. monitoring TLS and DA in *RAD5* mutants



### B. *RAD5* domains and mutations



**Figure 1 A:** outline of the integration system: A non-replicative plasmid containing a single lesion is integrated into one of the yeast chromosomes using Cre/lox site-specific recombination. The integrative vector carrying a selection marker (LEU2) and the 5'-end of the lacZ reporter gene containing a single lesion is introduced into a specific locus of the chromosome with the 3'-end of lacZ. The precise integration of the plasmid DNA into the chromosome restores a functional lacZ gene, enabling the phenotypical detection of TLS and DA events (as blue and white colonies on X-gal indicator media). **B:** Schematic of *RAD5* gene highlighting the helicase domain as well as the RING ubiquitin E3 domain. Mutations used in this study are indicated. Mutation FN13,14DD affects the Rev1 binding site. Mutation DE681,682AA inactivates both the helicase and ubiquitin ligase activity. Mutation I916A inactivates the ubiquitin ligase activity. Mutation Q1106D inactivates the helicase activity.

we do not know precisely how the three domains of Rad5 (ubiquitin ligase, Rev1-binding, and helicase) affect the choice of the DNA Damage Tolerance pathway.

In this study we used domain-specific mutants to address the contribution of each of the Rad5 domains to the lesion tolerance. We demonstrate that the two critical functions of Rad5 during DNA damage tolerance are the activation of template switching through polyubiquitination of PCNA and the recruitment of TLS. We also show that the helicase activity does not play any role in lesion tolerance.

## RESULTS & DISCUSSION

### Rad5 is involved in Damage Avoidance through its ubiquitin ligase domain

Our group has recently developed an assay based on the insertion of a single lesion into a specific locus in the genome of a living yeast cell, which allows a phenotypical detection of TLS and DA events (as blue and white colonies on X-gal indicator media) (Figure 1A) [21]. In the present work, we have used this assay to determine the role played by the three domains of Rad5 in the balance between TLS and DA.

Using this method, we have introduced a (6-4)TT photoproduct lesion (thymine-thymine

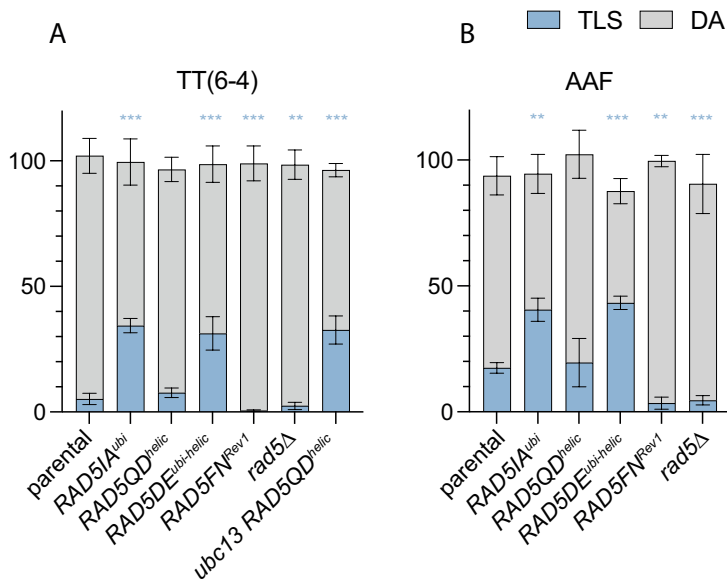
pyrimidine(6-4)pyrimidone photoproduct), or a N2dG-AAF (N2-dG-Acetylaminofluorene) adduct in the genome of cells carrying mutations affecting different domains of the Rad5 protein (Figure 1B): i) an allele simultaneously deficient in Ubc13-binding and ATPase/helicase activity (DE681,682AA) named *RAD5DE<sup>ubi-helic</sup>* [13]; ii) the Ubc13-binding RING domain (I916A) named *RAD5IA<sup>ubi</sup>* [19]; iii) the helicase domain (Q1106D) named *RAD5QD<sup>helic</sup>* [18]; iv) and the Rev1-binding domain (FN13,14AA) named *RAD5FN<sup>Rev1</sup>* [20]; v) and a complete deletion of *rad5* gene. The results were compared to the parental strain expressing wild-type *RAD5* gene. In all strains we inactivated *rad14* to avoid repair of the lesion and focus on lesion tolerance mechanisms, and *msh2* to avoid repair of the strand marker that allows to distinguish TLS from DA events.

The mutation affecting Ubc13-binding (*RAD5IA<sup>ubi</sup>*) led to a strong increase in TLS at both (6-4)TT photoproduct and N2dG-AAF lesions (Figure 2A and B). This increase in TLS is similar to the one previously observed in the absence of *ubc13* [21,22]. As shown in Figure 4, *RAD5IA<sup>ubi</sup>* shows a high sensitivity to a more global genotoxic stress such as 4NQO (4-Nitroquinoline-1-oxide) treatment, similar to the *ubc13Δ* strain.

We have previously described a competition between TLS and DA: in the absence of polyubiquitination of PCNA, DA is inhibited favoring TLS. We can note here that the loss of DA in the absence of PCNA polyubiquitination is only partially compensated by an increase in TLS. There is still a proportion of cells surviving using a recombination pathway that is independent of PCNA ubiquitination and that has previously been described as the salvage recombination pathway [23].

### Rad5 helicase function is not involved in damage tolerance

The mutation affecting solely the ATPase/helicase domain (Q1106D) of Rad5 (*RAD5QD<sup>helic</sup>*) did not show any effect on the level of TLS and DA at the (6-4)TT photoproduct and N2dG-AAF lesions (Figure 2 A and B). It seems therefore that this function is not involved in the bypass of the tested lesions. One could wonder if



**Figure 2:** Partitioning of DDT pathways through (6-4)TT and N2dG-AAF lesions in domain-specific Rad5 mutants. Tolerance events represent the percentage of cells able to survive in presence of the integrated lesion compared to the lesion-free control. The data represent the average and standard deviation of at least three independent experiments. Unpaired t-test was performed to compare TLS values from the different mutants to the parental strain (\* $p < 0.05$ ; \*\* $p < 0.005$ ; \*\*\* $p < 0.0005$ ).

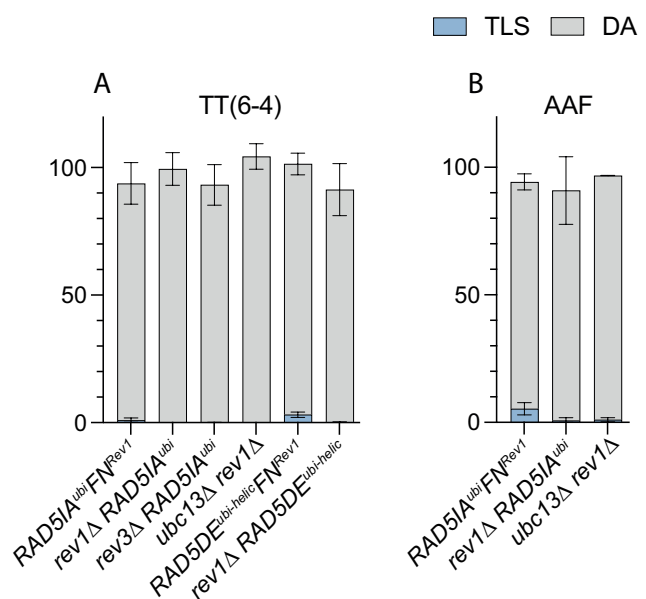
the helicase function could act as a backup in the absence of DA. The levels of TLS and DA are the same in the mutant deficient for both helicase and ubiquitin ligase (*RAD5DE<sup>ubi-helic</sup>*) as for the mutant deficient solely for the ubiquitin ligase (*RAD5IA<sup>ubi</sup>*). Similarly, there is no difference between *ubc13Δ* and *ubc13Δ RAD5QD<sup>helic</sup>* strains. These observations indicate that even in the absence of genuine DA that is dependent on PCNA polyubiquitination (*ubc13Δ* or *RAD5IA<sup>ubi</sup>*), the helicase has no function in damage tolerance. These results are compatible with previous observations from Gallo *et al.* [24] who showed that the helicase mutant had no effect on mutagenesis or survival to HU treatment. The absence of sensitivity of the *RAD5QD<sup>helic</sup>* mutant to 4NQO treatment (Figure 4) confirms that the helicase domain is not involved in lesion tolerance. Previous reports from Chen *et al.* [17] have shown the involvement of the helicase function of Rad5 in double-strand break repair, a role that is independent from its ubiquitin ligase function. *In vitro* experiments have shown the involvement of the helicase domain in fork regression [13], a structure that could favor error-free lesion bypass. *In vitro* experiments have also suggested that Rad5 can facilitate strand invasion-dependent mechanisms in addition to fork regression for the template switching in a Rad51-independent [25]. It appears from our *in vivo* data and others [17,24] that this is not a major pathway *in vivo*, at least for the tested lesions.

It is important to note that previous studies have considered the DE mutant as an ATPase defective strain and evidenced a role for the helicase function in DDT

[26]. However, it has since been shown that the DE mutation affects both the helicase and ubiquitin ligase domains [27], therefore the sensitivity that was observed for this mutant was most likely due to the polyubiquitination defect and not the helicase defect.

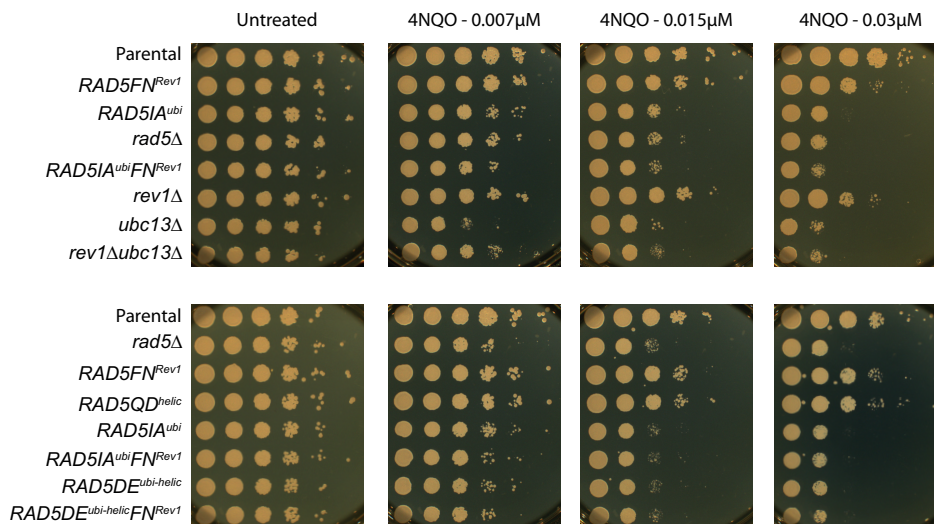
### Rad5 interaction with Rev1 is required for Polζ-TLS

As we have shown previously, TLS bypass of the (6-4)TT photoproduct relies almost exclusively on the TLS polymerases Rev1 and Pol ζ [21,22]. The bypass of N2dG-AAF lesion is mostly dependent on Rev1 and Pol ζ, while a small part can be performed by pol h [22]. The *RAD5* allele unable to bind Rev1 (*RAD5FN<sup>Rev1</sup>*) causes a severe decrease in the level of TLS at both (6-4)TT photoproduct and N2dG-AAF lesions (Figure 2A and B). This indicates that the interaction of Rev1 with Rad5 is critical for its TLS activity *in vivo*. This is in agreement with previous studies which had demonstrated that lack of the Rad5 N-terminal activity severely compromises spontaneous and DNA-damage-induced mutagenesis [20,24]. For the (6-4)TT photoproduct, we confirmed that in the absence of PCNA polyubiquitination in mutants *RAD5DE<sup>ubi-helic</sup>* and *RAD5IA<sup>ubi</sup>*, the strong increase in TLS (to a level >30%) was still exclusively due to Rev1-Pol ζ; as observed in Figure 3, the inactivation of *rev1* in the *ubc13Δ*, *RAD5IA<sup>ubi</sup>*, or *RAD5DE<sup>ubi-helic</sup>* mutants completely abolishes TLS ( $\leq 0.1\%$ ). Similarly, inactivation of Rev3 in the *RAD5IA<sup>ubi</sup>* also abolishes TLS.



**Figure 3:** Role of Rev1 and Rad5-Rev1 interaction in the partitioning of DDT pathways through (6-4)TT and N2dG-AAF lesions. Tolerance events represent the percentage of cells able to survive in presence of the integrated lesion compared to the lesion-free control. The data represent the average and standard deviation of at least three independent experiments.





**Figure 4:** Sensitivity to UV and 4NQO treatment of the different *RAD5* mutants.

When preventing the recruitment of Rev1 by Rad5 (*RAD5FN<sup>Rev1</sup>*) in the same mutants *RAD5IA<sup>ubi</sup>* and *RAD5DE<sup>ubi-helic</sup>*, we also observed a strong decrease of TLS. However, unlike in the *rev1Δ* strains, TLS is not completely abolished when combining default of PCNA polyubiquitination with *RAD5FN<sup>Rev1</sup>*. It appears that when DA is inhibited, Rev1/Pol ζ can access the stalled fork and some TLS can occur despite the absence of recruitment of Rev1 by Rad5.

In response to 4NQO treatment, the *RAD5FN<sup>Rev1</sup>* show the same increased sensitivity as the *rev1Δ* mutant,

confirming the importance of Rad5 in recruiting Rev1. It remains however less sensitive than the mutant affecting PCNA polyubiquitination, reflecting the lesser role of TLS for survival over DA. When DA is abolished in the absence of PCNA polyubiquitination (strains *ubc13Δ*, *RAD5IA<sup>ubi</sup>* or *RAD5DE<sup>ubi-helic</sup>*), the further inactivation of *rev1* either directly (*rev1Δ* strain) or through the lack of recruitment by Rad5 (*RAD5FN<sup>Rev1</sup>*) does not further increase the sensitivity to 4NQO, indicating again the minor role of TLS for survival.

While the complete deletion of *rad5* leads to an increased sensitivity to 4NQO (Figure 4), it does not lead to a drastic phenotype when monitoring the bypass of a single (6-4)TT photoproduct or N2dG-AAF lesions compared to WT *RAD5* (Figure 2A and B). We observed a significant decrease in TLS for the N2dG-AAF lesions, and a very moderate decrease for the TT(6-4) photoproduct compared to the parental strain. It is important to note that in the *rad5Δ* strain, no polyubiquitination of PCNA occurs: we could therefore

expect in these strains a strong increase of TLS as observed in the *ubc13Δ*, *RAD5IA<sup>ubi</sup>*, or *RAD5DE<sup>ubi-helic</sup>* mutants [21,22]. However, due to the absence of Rad5 and its function of recruiting Rev1, TLS does not increase in this strain. Overall, the loss of DA due to the absence of PCNA-ubiquitination could not be compensated by an increase in TLS in the absence of Rev1 recruitment, and is therefore compensated by an increase in the salvage recombination pathway.

| Strain | Relevant Genotype  |
|--------|--|
| SC53   | <i>VI(167260-167265)::(lox66-3'lacZ-MET25/lag)</i>                           |
| SC55   | <i>VI(167260-167265)::(lox66-3'lacZ-MET25/lead)</i>                          |
| SC82   | <i>rev1-Δ VI(167260-167265)::(lox66-3'lacZ-MET25/lag)</i>                    |
| SC83   | <i>rev1-Δ VI(167260-167265)::(lox66-3'lacZ-MET25/lead)</i>                   |
| SC151  | <i>ubc13-Δ VI(167260-167265)::(lox66-3'lacZ-MET25/lag)</i>                   |
| SC152  | <i>ubc13-Δ VI(167260-167265)::(lox66-3'lacZ-MET25/lead)</i>                  |
| SC137  | <i>Rad5(Q1106D) VI(167260-167265)::(lox66-3'lacZ-MET25/lag)</i>              |
| SC138  | <i>Rad5(Q1106D) VI(167260-167265)::(lox66-3'lacZ-MET25/lead)</i>             |
| SC141  | <i>Rad5(DE681,682AA) VI(167260-167265)::(lox66-3'lacZ-MET25/lag)</i>         |
| SC142  | <i>Rad5(DE681,682AA) VI(167260-167265)::(lox66-3'lacZ-MET25/lead)</i>        |
| SC167  | <i>Rad5(I916A) VI(167260-167265)::(lox66-3'lacZ-MET25/lag)</i>               |
| SC168  | <i>Rad5(I916A) VI(167260-167265)::(lox66-3'lacZ-MET25/lead)</i>              |
| SC186  | <i>Rad5(FN13,14AA) VI(167260-167265)::(lox66-3'lacZ-MET25/lag)</i>           |
| SC187  | <i>Rad5(FN13,14AA) VI(167260-167265)::(lox66-3'lacZ-MET25/lead)</i>          |
| SC155  | <i>Rad5-Δ VI(167260-167265)::(lox66-3'lacZ-MET25/lag)</i>                    |
| SC156  | <i>Rad5-Δ VI(167260-167265)::(lox66-3'lacZ-MET25/lead)</i>                   |
| SC240  | <i>rev1-Δ Rad5(DE681,682AA) VI(167260-167265)::(lox66-3'lacZ-MET25/lag)</i>  |
| SC241  | <i>rev1-Δ Rad5(DE681,682AA) VI(167260-167265)::(lox66-3'lacZ-MET25/lead)</i> |
| SC560  | <i>rev3-Δ::hisG Rad5(I916A) VI(167260-167265)::(lox66-3'lacZ-MET25/lag)</i>  |
| SC561  | <i>rev3-Δ::hisG Rad5(I916A) VI(167260-167265)::(lox66-3'lacZ-MET25/lead)</i> |
| SC623  | <i>rev1-Δ Rad5(I916A) VI(167260-167265)::(lox66-3'lacZ-MET25/lag)</i>        |
| SC624  | <i>rev1-Δ Rad5(I916A) VI(167260-167265)::(lox66-3'lacZ-MET25/lead)</i>       |
| SC709  | <i>ubc13-Δ rev1-Δ VI(167260-167265)::(lox66-3'lacZ-MET25/lag)</i>            |
| SC710  | <i>ubc13-Δ rev1-Δ VI(167260-167265)::(lox66-3'lacZ-MET25/lead)</i>           |
| SC723  | <i>Rad5(I916A, FN13,14AA) VI(167260-167265)::(lox66-3'lacZ-MET25/lag)</i>    |
| SC724  | <i>Rad5(I916A, FN13,14AA) VI(167260-167265)::(lox66-3'lacZ-MET25/lead)</i>   |

**Table 1:** Strains used in the study. All strains are: MATa his3-Δ1 leu2-3,112 trp1-Δ ura3-Δ met25-Δ rad14-Δ phr1-Δ msh2Δ::hisG.

## CONCLUSION

From these data, we can draw the following model: Rad5 is recruited to the replication fork through its interaction with both Rad18 and PCNA [12], where it recruits Rev1 to allow TLS [20] and polyubiquitinates PCNA to allow DA. If Rad5 is unable to polyubiquitinate PCNA (*RAD5DE<sup>ubi-helic</sup>* or *RAD5IA<sup>ubi</sup>* mutant), it will recruit Rev1 and permits a high level of TLS as DA is inhibited. If Rad5 is unable to interact with Rev1 (*RAD5FN<sup>Rev1</sup>* mutant), then Rev1-Polζ-TLS will

be strongly reduced. A Rad5 protein defective for its two main functions (*RAD5IA<sup>ubi</sup>*, *FN<sup>Rev1</sup>*) has a phenotype similar to a complete deletion of Rad5: it shows the same level of TLS and DA at the (6-4)TT photoproduct (compare Figure 2B and Figure 3), and similar sensitivity to 4NQO (Figure 4). In the absence of Rad5, Rev1 can access the replication fork and perform TLS, but with a lower efficiency as it is not actively recruited by Rad5. In conclusion, we have shown that Rad5 plays two critical and opposite roles in lesion tolerance: i) through its ubiquitin ligase activity, Rad5 promotes error-free lesion bypass by damage avoidance, and ii) through its interaction with Rev1, it promotes Rev1/Pol  $\zeta$ -dependent error-prone TLS. The helicase activity that has been suggested to favor error-free bypass by promoting fork regression does not seem to play a key role in the tolerance of isolated lesions [17].

## MATERIAL AND METHODS

### Strains and media

All strains used in the present study are derivative of strain EMY74.7 [28] (MATa his3- $\Delta$ 1 leu2-3,112 trp1- $\Delta$  ura3- $\Delta$  met25- $\Delta$  phr1- $\Delta$  rad14- $\Delta$  msh2 $\Delta$ ::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 [29] or URABlaster [30] techniques. Rad5 point mutations were created using the delitto perfetto method [31]. All strains used in the study are listed in Table 1.

### Integration system

Integration of plasmids carrying 6-4 (TT) / N2dG-AAF lesions (or control plasmids without lesion) and result analysis was performed as previously described [21]. 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  $\pm$  s.d.

### Spotting assay

Overnight cultures of strains carrying Rad5 point mutations in YPD were adjusted to an OD<sub>600</sub> value of 1. Volume of 10  $\mu$ l from 10-fold serial dilutions of OD<sub>600</sub>-adjusted cultures were spotted on YPD agar plates containing different concentrations of 4-NQO (0  $\mu$ M, 0,015  $\mu$ M, 0,03  $\mu$ M).

### Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Author contribution

V.P. and K.M. conceptualization; V.P. and K.M. data curation; V.P. and K.M. formal analysis; K.M. investigation; V.P. supervision; V.P. and K.M. funding acquisition; V.P. and K.M. writing-original draft

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