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

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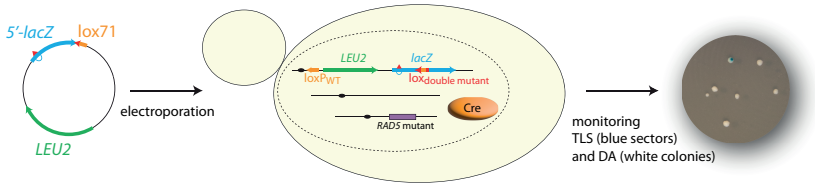
Abstract:
DNA Damage Tolerance (DDT) functions to bypass replication-blocking lesions and is divided into two distinct pathways: error-prone Translesion Synthesis (TLS) and error-free Damage Avoidance (DA). Rad5 is an important player in these processes. Indeed, *Saccharomyces cerevisiae* Rad5 is a large multifunctional protein that contains three well defined domains: a RING domain that promotes PCNA polyubiquitination and a ssDNA-dependent ATPase/helicase domain, that are both conserved in Rad5 human ortholog HLTF. Yeast Rad5 also contains a Rev1-binding domain.
In this study we used domain-specific mutants to address the contribution of each of the 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 opposite roles in lesion tolerance: i) Rad5 favors error-free lesion bypass by activating template switching through polyubiquitination of PCNA; ii) Rad5 is also 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), an 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 TLS and DA is very important since it 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]). PCNA mono-ubiquitination by Rad6 and Rad18 promotes the recruitment of TLS polymerases. Extending this modification to poly-ubiquitination by Mms2/Ubc13 and Rad5, enables the recombination-mediated mechanisms [7].
Rad5 is a a large multifunctional protein that contains both ubiquitin ligase and ssDNA-dependent ATPase activities [8]. As E3 ubiquitin ligase, Rad5 catalyzes PCNA poly-ubiquitination 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 [9]. 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 [10–12], although in yeast fork reversal has been consistently interpreted as a pathological transaction at replication forks that have lost their replication capacity [13]. It has been suggested that Rad5 ATPase activity is important rather for DSB repair [14]. It has been demonstrated that the Rad5 ATPase/helicase activity is not required for PCNA polyubiquitination [15]. 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 [9,16] see figure 1B.
Rad5 also plays a structural role in the recruitment of TLS polymerases through physical interaction with Rev1 via its N-terminus [17]. The fact that Rad5 is required in both branches of DDT implies that it may play a role in the pathway choice and balance within DDT.

A. monitoring TLS and DA in *RAD5* mutants



B. *RAD5* domains and mutations

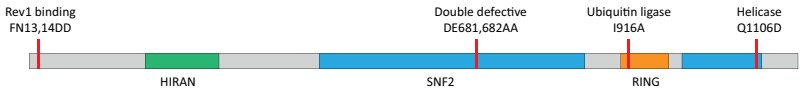


Figure 1 A: outline of the integration system. B: Schematic of *RAD5* highlighting the helicase domain as well as the RING ubiquitin E3 domain. Mutations used it this study are indicated. Mutation FN13,14DD affects the Rev1 binding site. Mutation DE681,682AA inactivates both the helicase and ubiquitin ligase activity. Mutation I916A inactivates the ubiquitin ligase activity. Mutation Q1106D inactivates the helicase activity.

In this study we used domain-specific mutants to address the contribution of each of the Rad5 functions to the lesion tolerance. We demonstrate that Rad5 is critical for both the activation of template switching through polyubiquitination of PCNA and the recruitment of TLS polymerases during DNA damage tolerance. 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) [18]. In the present work, we have used this assay to determine the role played by the different 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 1): i) an allele simultaneously deficient in Ubc13-binding and ATPase/helicase activity (DE681,682AA) named *RAD5DE*$^{ubi\text{-}helic}$ [10]; ii) the Ubc13-binding RING domain (I916A) named *RAD5IA*^{ubi} [16]; iii) the helicase domain (Q1106D) named *RAD5QD*^{helic} [15]; iv) and the Rev1-binding domain (FN13,14AA) named *RAD5FN*^{Rev1} [17]; v) and a complete deletion of *rad5* gene. The results were compared to the parental strain expressing wild-type *RAD5* gene, and where *rad14* was inactivated to avoid repair of the lesion and focus on lesion tolerance mechanisms. We also inactivated *msh2* in these strains to avoid repair of the strand marker that allows to distinguish TLS from DA events.

The mutation affecting both Ubc13-binding and the helicase activity (*RAD5DE*$^{ubi\text{-}helic}$) led to a strong increase in TLS at both (6-4)TT photoproduct and N2dG-AAF lesions (Figure 2A and B). The same increase was observed for the mutation affecting only the ubiquitin ligase activity (*RAD5IA*^{ubi}), indicating that it is the lack of PCNA poly-ubiquitination in the two tested mutants that is responsible for the increase in TLS. This increase is similar to the one previously observed in the absence of *ubc13* [18,19]. 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 poly-ubiquitination 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

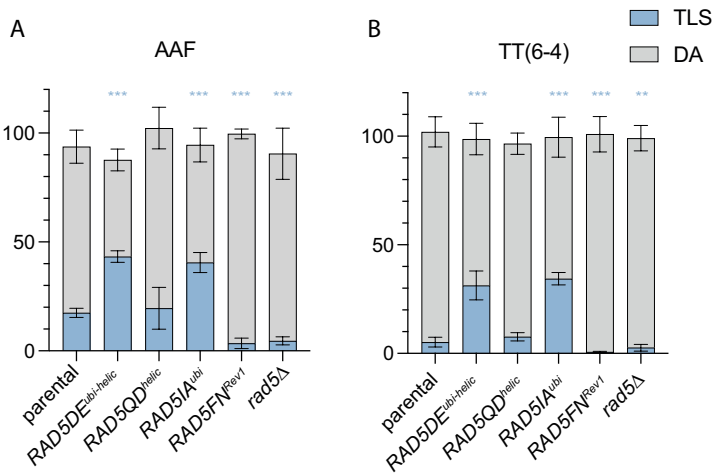


Figure 2: Partitioning of DDT pathways through N2dG-AAF and (6-4)TT lesions in domain-specific Rad5 mutants. Tolerance events represent the percentage of cells able to survive in presence of the integrated lesion compared to the lesion-free control. The data represent the average and standard deviation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

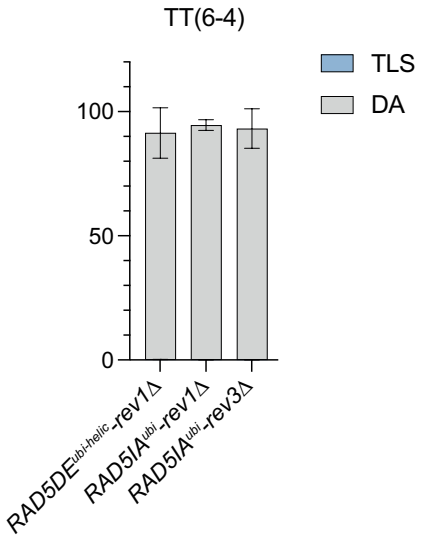


Figure 3: Partitioning of DDT pathways through (6-4)TT lesion in domain-specific *RAD5* mutants combined with *rev1* or *rev3* deletions.

pathway [20]. As shown in Figure 4, both mutants *RAD5DE*$^{ubi-helic}$ and *RAD5IA*^{ubi} show a high sensibility to a more global genotoxic stress such as UV irradiation or 4NQO treatment. Indeed, these two mutants show a sensitivity similar to the *rad5*Δ strain.

• Rad5 helicase function is not involved in damage tolerance

On the other hand, the mutation affecting solely the ATPase/helicase domain (Q1106D) of Rad5 (*RAD5QD*^{helic}) 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 the helicase function could act as a backup in the absence of DA. Since the level of TLS and DA were the same in the mutant deficient for both helicase and ubiquitin ligase (*rad5DE*$^{ubi-helic}$) that for the mutant deficient solely for the ubiquitin ligase (*rad5IA*^{ubi}), it indicates that even in the absence of genuine DA that is dependent on PCNA poly-ubiquitination, the helicase has no function in damage tolerance. These results are compatible with previous observations from Gallo *et al.* [21] who showed that the helicase mutant had no effect on mutagenesis or survival to HU treatment. The absence of increase sensitivity of the *RAD5QD*^{helic} mutant to UV and 4NQO (Figure 4) confirms that the helicase domain in not involved in lesion tolerance. However, at the highest doses of UV or 4NQO, the *RAD5QD*^{helic} mutant shows a slight increased sensitivity compared to the *RAD5* strain. We suggest that at such high doses for a nucleotide excision repair deficient strain, a high number of unrepaired lesions might lead to fork collapse that in turn will lead to double-strand breaks. The helicase function might then be required to repair these double-strand breaks. Indeed, previous reports from Chen *et al*. [14] have shown the involvement of the helicase function of Rad5 in double-strand break repair, a role that is independent from it ubiquitin ligase function. *In vitro* experiments have shown the involvement of the helicase domain in fork regression [10], 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 [22]. It appears from our *in vivo* data and others [14,21] 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 and evidenced a role for the helicase function in DDT [23]. However, it has since been showed that the DE mutation affects both helicase and Ub ligase domain [24].

• Rad5 interaction with Rev1 is required for Polζ-TLS

For both (6-4)TT photoproduct and N2dG-AAF lesions, the *RAD5* allele unable to bind Rev1 (*RAD5FN*^{Rev1}) causes a very strong decrease in the level of TLS through those lesions (Figure 2A and B). As we have shown previously [18,19], TLS bypass of those lesions relies

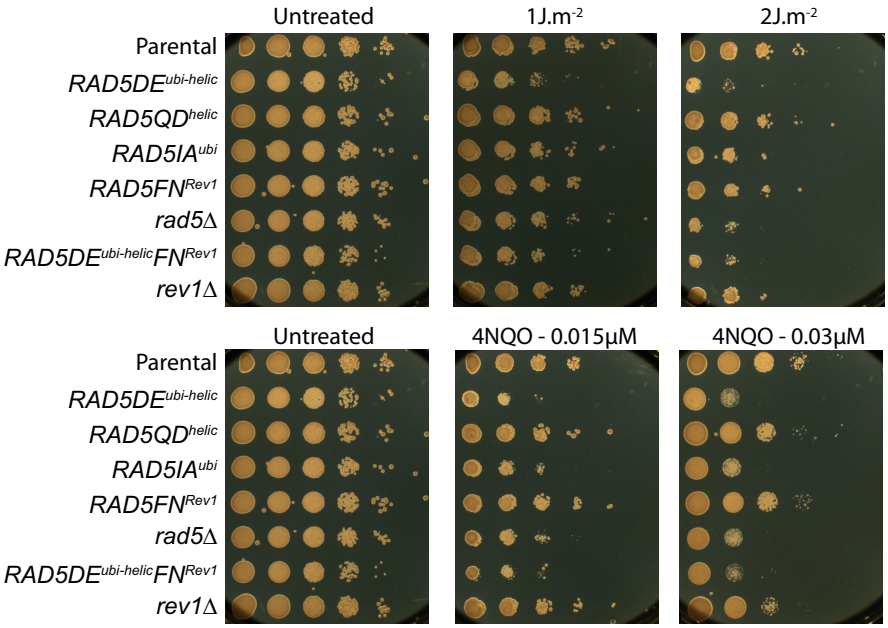


Figure 4: Sensitivity to UV and 4NQO treatment of the different *RAD5* mutants.

almost exclusively on the TLS polymerases Rev1 and Pol ζ. 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 [17]. The *RAD5FN*^{Rev1} mutant shows similar sensitivity to UV and 4NQO to the *rev1*Δ mutant at lower doses (Figure 4). At higher dose the *rev1*Δ strain is only slightly more sensitive. This could again be attributed to the formation of double-strand breaks and the involvement of Rev1 in their repair as previously evidenced [25]. For the (6-4)TT photoproduct, we confirmed that in the absence of PCNA ubiquitination in mutants *RAD5DE*$^{ubi\text{-}helic}$ and *RAD5IA*^{ubi}, 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 *RAD5DE*$^{ubi\text{-}helic}$ mutant or the inactivation of *rev1* or *rev3* in *RAD5IA*^{ubi} mutant completely abolishes TLS.

While the deletion of *rad5* leads to an increased sensitivity to UV and 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 slight decrease in TLS for the N2dG-AAF lesions, and a very moderate decrease for the the TT(6-4) photoproduct compared to the parental strain. It is important to note that in the *rad5*Δ strain, no polyubiquitination of PCNA occurs: we could therefore expect in these strains a strong increase of TLS as observed in the *RAD5DE*$^{ubi\text{-}helic}$ or *RAD5IA*^{ubi} mutants or in the *ubc13*Δ mutant [18,19]. 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.

CONCLUSION

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 ζ 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. However, in the presence of a strong genotoxic stress, we can hypothesize that lesion proximity can generate fork collapse leading to double strand breaks where the helicase domain of Rad5 can then play a role [14].

MATERIAL AND METHODS

Strains and media

All strains used in the present study are derivative of strain EMY74.7 [26] (MATa his3-Δ1 leu2-3,112 trp1-Δ 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 [27] or URAblaster [28] techniques. Rad5 point mutations were created using the delitto perfetto method [29]. 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 [18].
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.

Spotting assay

Overnight cultures of strains carrying Rad5 point mutations in YPD were adjusted to an OD_{600} value of 1. Volume of 10 µl from 10-fold serial dilutions of OD_{600}-adjusted cultures were spotted on YPD agar plates and irradiated with different doses of UV (0J, 1J, 2J), or on

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

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.

YPD agar plates containing different concentrations of 4-NQO (0 µM, 0,015 µM, 0,03 µM).

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