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The Rad5 helicase activity is dispensable for error-free DNA post-replication repair

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ABSTRACT

DNA post-replication repair (PRR) functions to bypass replication-blocking lesions and is subdivided into two parallel pathways: error-prone translesion DNA synthesis and error-free PRR. While both pathways are dependent on the ubiquitination of PCNA, error-free PRR utilizes noncanonical K63-linked polyubiquitinated PCNA to signal lesion bypass through template switch, a process thought to be dependent on Mms2-Ubc13 and a RING finger motif of the Rad5 ubiquitin ligase. Previous *in vitro* studies demonstrated the ability of Rad5 to promote replication fork regression, a function dependent on its helicase activity. To investigate the genetic and mechanistic relationship between fork regression *in vitro* and template switch *in vivo*, we created and characterized site-specific mutations defective in the Rad5 RING or helicase activity. Our results indicate that both the Rad5 ubiquitin ligase and the helicase activities are exclusively involved in the same error-free PRR pathway. Surprisingly, the Rad5 helicase mutation abolishes its physical interaction with Ubc13 and the K63-linked PCNA polyubiquitin chain assembly. Indeed, physical fusions of Rad5 with Ubc13 bypass the requirement for either the helicase or the RING finger domain. Since the helicase domain overlaps with the SWI/SNF chromatin-remodelling domain, our findings suggest a structural role of this domain and that the Rad5 helicase activity is dispensable for error-free lesion bypass.

1. Introduction

In addition to mechanisms that cells possess to remove DNA adducts, they also possess the ability to bypass replication-blocking lesions. In *Saccharomyces cerevisiae* this mechanism is termed postreplication repair (PRR), which is mediated by the *RAD6* epistasis group genes. PRR can be divided into two parallel pathways; the error-prone translesion synthesis (TLS) pathway and the error-free PRR pathway. The stable complex formed between Rad6 and Rad18 [1] is known to monoubiquitinate the *POL30* gene product, proliferating cell nuclear antigen (PCNA), at the K164 residue [2]. Monoubiquitinated PCNA allows for TLS lesion bypass that requires the Y-family polymerase Rev1 and the B-family polymerase Polζ, consisting of Rev3 and Rev7 [3,4]. Inactivation of the TLS pathway abolishes DNA damage-induced mutagenesis and results in a moderate increase in sensitivity to DNA damaging agents [5].

Genetic characterization of the *mms2* null mutant demonstrated the existence of the error-free PRR branch, as *mms2* displays strong synergistic interaction with *rev3* and a *REV3*-dependent increase in spontaneous mutagenesis [6,7]. Although Mms2 encodes a Ubc-like protein it does not contain the active-site Cys residue essential for a ubiquitin conjugating enzyme (Ubc or E2). It turned out that Mms2 forms a stable complex with Ubc13 to promote non-canonical K63-linked polyubiquitination [8] and that error-free PRR is dependent on the K63-linked polyubiquitination of PCNA by Mms2-Ubc13 and the E3 ubiquitin ligase Rad5 [2]. Although *ubc13* is epistatic to *mms2* and they share all characteristic phenotypes [9], Rad5 appears to have additional activities.

Rad5 is a multifunctional protein known to prevent non-homologous end-joining [10], promote instability of simple repetitive sequences [11] and the repair of DNA double-strand breaks [12]. Rad5 is also implicated in the repair of DNA minor groove adducts in association with nucleotide excision repair [13] and has a potential role in TLS [14]. Indeed, the *rad5* mutant is much more sensitive to killing by DNA-damaging agents than

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mms2/ubc13 mutants, particularly to ionizing radiations [15], and demonstrates elevated rates of spontaneous mitotic recombination as well as gross chromosomal rearrangement [16,17]. The current working model suggests that Rad5 interacts with Ubc13 [18] through its RING finger motif [19] and recruits Mms2-Ubc13 in close proximity to monoubiquitinated PCNA through its association with PCNA [2] and Rad18 [18], which facilitates the sequential polyubiquitination of PCNA. In addition to the E3 ubiquitin-ligase activity, Rad5 also contains a conserved helicase-like domain of the SWI/SNF family of ATPases [11,20].

Despite great efforts made to clarify our understanding of the PRR pathway in the past decade, little is known about the detailed molecular events of error-free PRR. There are two possible competing modes for error-free lesion bypass, namely replication fork regression and template switch, both of which have recent experimental support [21,22]. Previous *in vitro* experiments demonstrated the ability of Rad5 to promote replication fork regression, a function dependent on its helicase activity [22]. It remains plausible that fork regression followed by sister chromatid invasion and resolution may allow for error-free bypass [23]. Alternatively, error-free PRR may employ two parallel modes of lesion bypass. Furthermore, it is unclear whether the Rad5 helicase activity promotes one or both error-prone and error-free pathways of lesion bypass *in vivo*. In order to address the above questions, we created and characterized *rad5* mutants defective in either RING finger or the DNA helicase activity. Genetic analyses clearly demonstrate that both the Rad5 E3 and helicase activities are exclusively involved in error-free PRR and that their mutations are epistatic, suggesting that the two activities function in the same pathway. Surprisingly, lack of the Rad5 helicase function abolishes the PCNA polyubiquitin chain formation and the physical interaction with Ubc13, indicating that the Rad5 helicase domain also plays a structural role in the recruitment of Ubc13-Mms2. Furthermore, fusion of Ubc13 with Rad5 bypasses the requirement of the Rad5 helicase activity, indicating that either the Rad5-promoted fork regression does not operate *in vivo* or its helicase activity is dispensable for error-free PRR.

2. Materials and methods

2.1. Yeast strains and cell culture

The yeast strains used in this study are listed in Supplementary Table S1. All of the strains are isogenic derivatives of DBY747 or HK578. HK578 is a derivative of W303 corrected for the *RAD5* gene by Dr. H. Klein (New York University).

Yeast gene disruption cassettes used in this study and their application have been described previously [24,25]. To create *RAD5* site-specific mutants, a 3.5-kb *Pml*I-*Sal*I yeast genomic fragment containing *RAD5* ORF but missing the N-terminal 100 aa coding region was cloned into plasmid YIplac211 (*URA3*) [26] to form YIpU-Rad5ΔN, which was used to create *rad5-I916A* and *rad5-AA* (D681A + E682A) mutations. Site-specific mutagenesis was performed by a mega-primer approach [27] using mutagenic primers that also create diagnostic restriction sites. The cloned site-specific mutations were screened by restriction analysis and further confirmed by sequencing. The resulting YIp plasmids were linearized by *Nru*I cleavage prior to yeast transformation and the confirmed transformants were subjected to 5-fluoro-orotic acid selection for the appropriate genomic pop-outs that only contain the desired point mutation(s) at the *RAD5* locus.

Yeast cells used in this study were cultured at 30 °C in either rich YPD medium or an SD medium supplemented with essential nutrients as required [28] unless otherwise specified. Yeast cells were transformed *via* a modified lithium acetate method [29]. Newly created strains were confirmed *via* phenotypic change when possible, and by PCR of genomic DNA.

2.2. Plasmid construction

For the yeast two-hybrid analysis and functional complementation assays, the full-length *RAD5* ORF was cloned into pGAD424Bg (a frameshift derivative of pGAD424). *UBC13* and *REV1* ORFs were cloned into pGBT9Bg (a frameshift derivative of pGBT9). The *RAD5* point mutation and truncation derivatives were created by either PCR or restriction fragment isolation followed by cloning into pGAD424Bg, or by step-wise deletions within the plasmid. For the *UBC13/MMS2-RAD5* fusions, the *UBC13* or *MMS2* ORF without the stop codon was PCR-amplified as a *Bam*HI fragment and then cloned into the *Bam*HI site of pGAD424Bg-Rad5 so that *UBC13* or *MMS2* is fused to *RAD5* or its mutant derivatives at the N-terminus. All the point mutation, deletion and fusion constructs were confirmed by restriction analysis and sequencing.

2.3. Testing for cellular sensitivity to DNA-damaging agents

The gradient plate and tenfold serial dilution assays were used as a semi-quantitative measurement of relative MMS sensitivity, while a liquid killing experiment was performed as a quantitative assessment of MMS sensitivity as previously described [30]. The serial dilution assay was performed as described [30] to assess UV sensitivity.

2.4. Spontaneous mutagenesis

The spontaneous mutation rate was measured by monitoring the Trp^+ reversion of the *trp1-289* allele in the DBY747 strain *via* a modified Luria and Delbruck fluctuation test, as previously described [6].

2.5. Yeast two-hybrid analysis of protein–protein interactions

Yeast two-hybrid analysis [31] was performed by transforming the yeast strain PJ69-4a with various combinations of the Gal_{BD} and Gal_{AD} plasmids containing relevant genes before being screened for growth on selective media with or without the indicated amount of 3-aminotriazole (3AT). Plates were incubated for 3 days at 30 °C before being photographed.

2.6. Detection of PCNA ubiquitination

Detection of ubiquitinated PCNA was adapted from a previous report [32]. Briefly, cells grown overnight in YPAD (YPD + 20 mg/ml Ade) were diluted to 0.3×10^7 cells/ml in 100 ml of YPAD and allowed to grow for an additional 2 h. Cultures were then split and one was treated with 0.05% MMS for 90 min. Cells were harvested and immediately frozen in liquid nitrogen for 10 min. After step-wise N-ethylmaleimide (NEM) treatment plus phenylmethylsulfonyl fluoride (PMSF), NaOH plus 7.5% β-mercaptoethanol incubation and trichloracetic acid precipitation, the pellet was then resuspended in a modified HU buffer (8 M urea, 5% SDS, 200 mM Tris–HCL pH 6.8, 1 mM EDTA, 0.025% bromophenol blue, 1.5% DTT, 25 mM NEM, 1 mM PMSF, and 0.5% triton-X-100) prior to protein heat denaturation. Samples were then added to Bio-Rad laemmli sample buffer, frozen overnight and analyzed by SDS-PAGE and Western blotting. Anti-Pol30 monoclonal antibodies were raised and characterized in house.

3. Results

3.1. Experimental design and rationale

While molecular mechanisms of TLS have been well characterized in recent years, little is known about the detailed molecular events leading to error-free PRR in eukaryotes, despite the fact that two models of error-free lesion bypass, namely template switch and replication fork regression, were proposed many years ago [33]. Recently, we provided experimental evidence suggesting that the homologous recombination machinery acts downstream of PCNA polyubiquitination to mediate error-free PRR [21], which favours the template switch model. Meanwhile, it was reported that Rad5 possesses a helicase activity required for fork reversal *in vitro* [22], which supports the fork regression model of error-free PRR.

It has been well established that the E2 complex Ubc13-Mms2 and the E3 protein Rad5 are required for K63-linked polyubiquitination of PCNA, which leads to the error-free mode of PRR [2]. However, unlike Ubc13 and Mms2, which are exclusively involved in the above E2 activity and error-free PRR, Rad5 has been implicated in several DNA repair/damage tolerance pathways. Indeed, although *rad5* is epistatic to *ubc13/mms2* [18], the *rad5* mutant is much more sensitive to DNA-damaging agents than the *ubc13* or *mms2* mutant [24]. Rad5 is a rather large protein and contains several putative functional domains (Fig. 1A). We reasoned that if the Rad5 RING finger E3 activity is required for PCNA polyubiquitination together with Ubc13-Mms2, this activity will be involved in signalling for the template switch. Meanwhile, if the Rad5 helicase activity acts in the same pathway as PCNA polyubiquitination, the helicase- and RING- (or *mms2/ubc13*) mutations will be epistatic. Alternatively, if Rad5-mediated fork regression and PCNA polyubiquitination-mediated template switch constitute two means of lesion bypass, the above mutations will be additive or synergistic with respect to killing by DNA-damaging

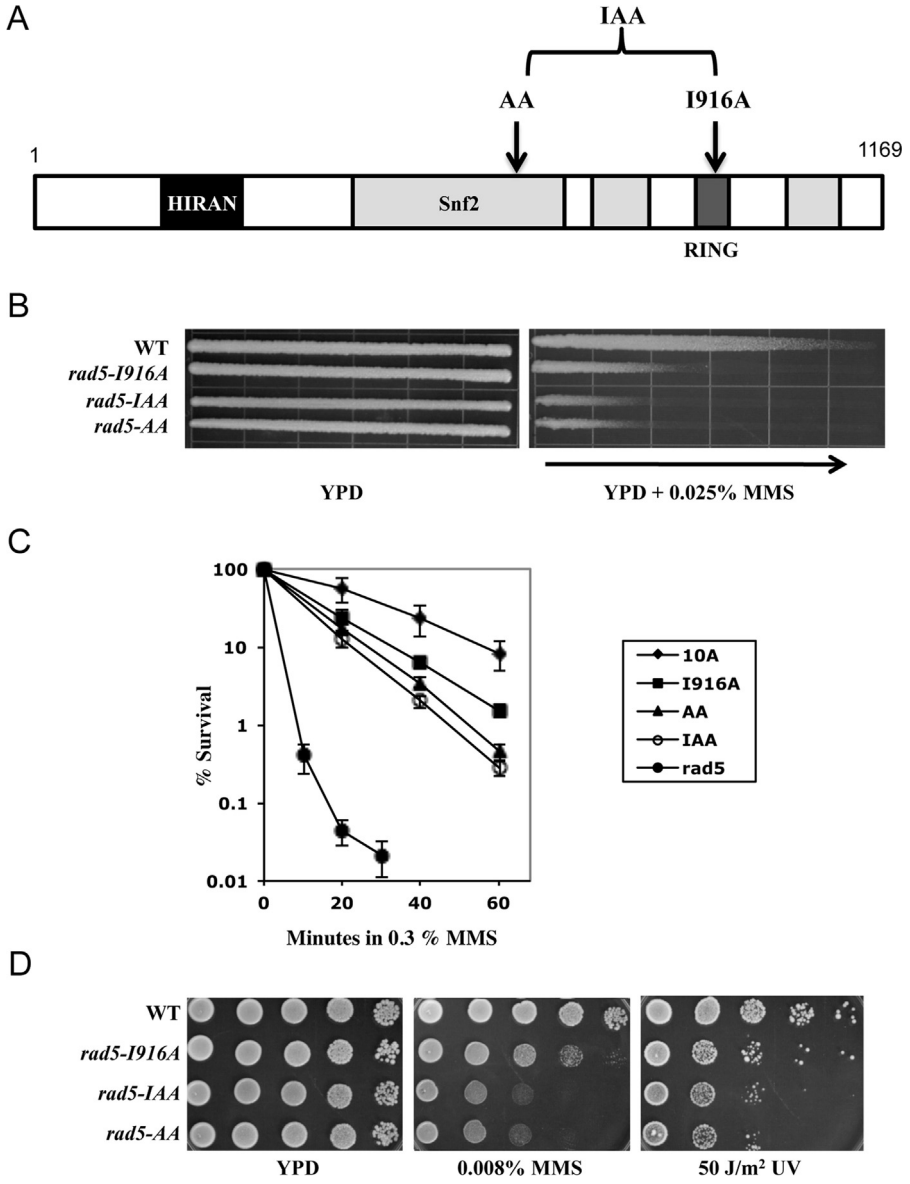

Fig. 1. The *rad5-AA* mutation is epistatic to *rad5-I916A*. (A) A schematic representation of known and suspected Rad5 domains in budding yeast *S. cerevisiae*. Downward arrows indicate the location of point mutations used in this study. (B) A gradient plate assay demonstrating epistasis relationship between *rad5-AA* and *rad5-I916A*. (C) A liquid killing experiment for the relative sensitivity to MMS. Results are the average of four independent experiments with standard deviations shown as error-bars. (D) A serial dilution assay to compare the relative sensitivity of wild-type and *rad5* mutants to MMS and UV irradiation. All strains are isogenic to HK578-10A (WT): HK578-2C (*rad5*Δ), WXY3001 (*rad5-I916A*), WXY2981 (*rad5-AA*) and WXY2982 (*rad5-IAA*).

agents. Experiments were designed to critically test the above two hypotheses.

3.2. The Rad5 helicase mutation is epistatic to its RING finger mutation

To ask whether the Rad5 E3 activity is mediated by its RING finger motif, we initially created a *rad5-C914S* mutation known to inactivate its RING finger activity. Unfortunately, this mutant is much more sensitive to killing by MMS than *mms2* or *ubc13*, although it is epistatic to *mms2* (data not shown), suggesting that the *rad5-C914S* mutation affects other Rad5 functions, most likely by interfering with its proper folding and/or stability. It was reported [19] that an adjacent *rad5-I916A* mutation abolished its interaction with Ubc13 and that this mutation is epistatic to and indistinguishable from *ubc13*. Hence we created the same mutation and examined its genetic interaction with the *rad5-AA* mutation which causes a defect in the helicase activity [22].

A gradient plate assay (Fig. 1B) indicates that the *rad5-AA* mutant is more sensitive to MMS than the *rad5-I916A* mutant; the *rad5-IAA* double mutant is no more sensitive to MMS than the *rad5-AA* single mutant, suggesting that the *rad5-AA* helicase mutation is epistatic to the *rad5-I916A* RING finger mutation with respect to MMS sensitivity. This observation was further confirmed by a quantitative killing experiment (Fig. 1C), in which the *rad5-IAA* double mutant is as sensitive to MMS as the *rad5-AA* single mutant, which clearly demonstrates the epistatic relationship between the two mutations. Furthermore, the *rad5-IAA* double mutant is no more sensitive than the *rad5-AA* single mutant to killing induced by UV irradiation (Fig. 1D), confirming that the two Rad5 activities function in the same pathway.

3.3. The Rad5 helicase mutation is defective in error-free PRR

During the course of the above studies, we noticed that the *rad5-AA* mutant is slightly more sensitive to MMS- and UV-induced killing than the *rad5-I916A* mutant (Fig. 1), which can be explained by one of two possibilities: either the *rad5-I916A* mutation does not completely abolish its RING finger activity or the Rad5 helicase has functions beyond that of error-free PRR. To address the first possibility, we examined genetic interactions between *rad5-I916A* and *mms2*. As seen in Fig. 2A, the *rad5-I916A* mutant is noticeably less sensitive to killing by MMS than the *mms2* mutant. Since Mms2 and Ubc13 are absolutely required for PCNA polyubiquitination and error-free PRR, and this function requires the Rad5 RING finger activity, we infer that the *rad5-I916A* point mutation may not completely abolish its RING finger E3 function. Nevertheless, the *mms2 rad5-I916A* double mutant is as sensitive as the *mms2* single mutant, and the *rev3 rad5-I916A* double mutant displays an extreme sensitivity to MMS and UV (Fig. 2A and Supplementary Fig. S1A). These results reaffirm the exclusive role of the Rad5 RING finger domain in error-free PRR.

To ask whether the Rad5 helicase activity is exclusively involved in error-free PRR, we examined genetic interactions between the Rad5 helicase function and both branches of PRR. It has been previously reported that the *rad5-AA* mutant is comparable to the *mms2* null mutant with respect to killing by UV [34]. As seen in Fig. 2B and Supplementary Fig. S1B, the *rad5-AA* mutant is as sensitive to MMS and UV as the *mms2* mutant, and more importantly the corresponding double mutant has the same sensitivity to MMS and UV as the *rad5-AA* or *mms2* single mutant, indicating that the two mutations are epistatic to each other. Quantitative analysis in a liquid killing experiment (Fig. 2C) indicates that the difference in MMS sensitivity between the *mms2 rad5-AA* double mutant and the *rad5-AA* single mutant is statistically insignificant. In sharp contrast, the *rad5-AA rev3* double mutant is extremely sensitive to MMS, and the genetic interaction is clearly synergistic, reminiscent of the *rad5-I916A rev3* (Fig. 2A) or *mms2 rev3* [6] double mutant. The above observations suggest that the Rad5 helicase activity functions only in the error-free branch of PRR and in the same pathway as Mms2.

A hallmark of error-free PRR defect is elevated spontaneous mutagenesis [6]. To ask whether the Rad5 helicase and RING finger activities confer all error-free PRR functions within Rad5, we compared the spontaneous mutation rates of the above site-specific mutants with that of the *rad5* null mutant. This *trp1-289* reversion assay is particularly sensitive to error-free PRR defect, which channels replication-blocking lesions to Polζ-mediated translesion synthesis [6,7]. As seen in Table 1, both *rad5-AA* and *rad5-I916A* mutants display strongly elevated spontaneous mutation rates, which are comparable to that of the *rad5* null mutant. Interestingly, the *rad5-IAA* double mutant has an elevated spontaneous mutation rate indistinguishable from that of corresponding *rad5-AA* single mutant, once again confirming that the two activities represented by each mutation function in the same error-free PRR pathway.

3.4. The Rad5 helicase domain is required for PCNA polyubiquitination

After establishing that the *rad5-AA* mutation is specifically defective in error-free PRR, we wished to investigate the molecular mechanism(s) by which this helicase activity is involved in error-free lesion bypass. It is well established that Ubc13, Mms2 and Rad5 are required for PCNA polyubiquitination [2] and the subsequent error-free lesion bypass. The current model predicts that the Rad5 RING finger motif is required for the E3 activity and hence the *rad5-I916A* mutation will affect PCNA polyubiquitination. On the other hand, it is unclear whether the Rad5 helicase activity acts upstream or downstream of PCNA polyubiquitination. To critically address the above issue, we raised polyclonal antibodies against purified Pol30 expressed from bacterial cells, and further screened for monoclonal antibodies. Subsequently we verified our antibody for its ability to detect both mono- and di-ubiquitinated PCNA (supplementary Fig. S2). Furthermore, we were able to verify monoubiquitinated PCNA following MMS treatment in wild-type yeast whole cell extracts without the need for His_n-affinity purification (Fig. 3A, lanes 2 and 3). Notably this modification is absent in a strain containing the *pol30-K164R* point mutation (*cf.* lanes 1 and 2, 3 and 4). The predicted PCNA-Ub band is slightly shifted up in the strain containing the Pol30-His_7 allele compared to the native Pol30 allele (*cf.* lanes 2 and 3), further confirming that this band contains modified PCNA. As PCNA sumoylation and diubiquitination at the K164 residue co-migrate under our experimental conditions we introduced a *siz1* null mutation to our query strains to eliminate this PCNA K164 sumoylation. Siz1 is a SUMO ligase of the SIZ/PIAS family [35] and thus a null mutation of *siz1* abolishes sumoylation of PCNA without affecting its ubiquitination (lane 5) [36]. As expected, a prominent Pol30-K164 dependent and *SIZ1*-independent band is present in the *siz1Δ* mutant (lane 5) but absent in the *rad5Δ siz1Δ*

Table 1
Spontaneous mutation rates of *RAD5* wild-type and mutant strains.

Strain[a]	Key alleles	Rate ($\times 10^{-7}$)[b]	Rate relative to wild-type[c]
DBY747	Wild-type	0.15 ± 0.07	1
WXY731	*rad5Δ*	1.61 ± 0.51	10.7
WXY2900	*rad5-AA*	2.24 ± 0.38	14.9
WXY2974	*rad5-I916A*	1.90 ± 0.15	12.7
WXY2990	*rad5-IAA*	2.09 ± 0.05	13.9

[a] All strains are isogenic derivatives of DBY747.
[b] The spontaneous mutation rates are the average of at least three independent experiments with standard deviations.
[c] Expressed as a multiple of the wild-type mutation rate.

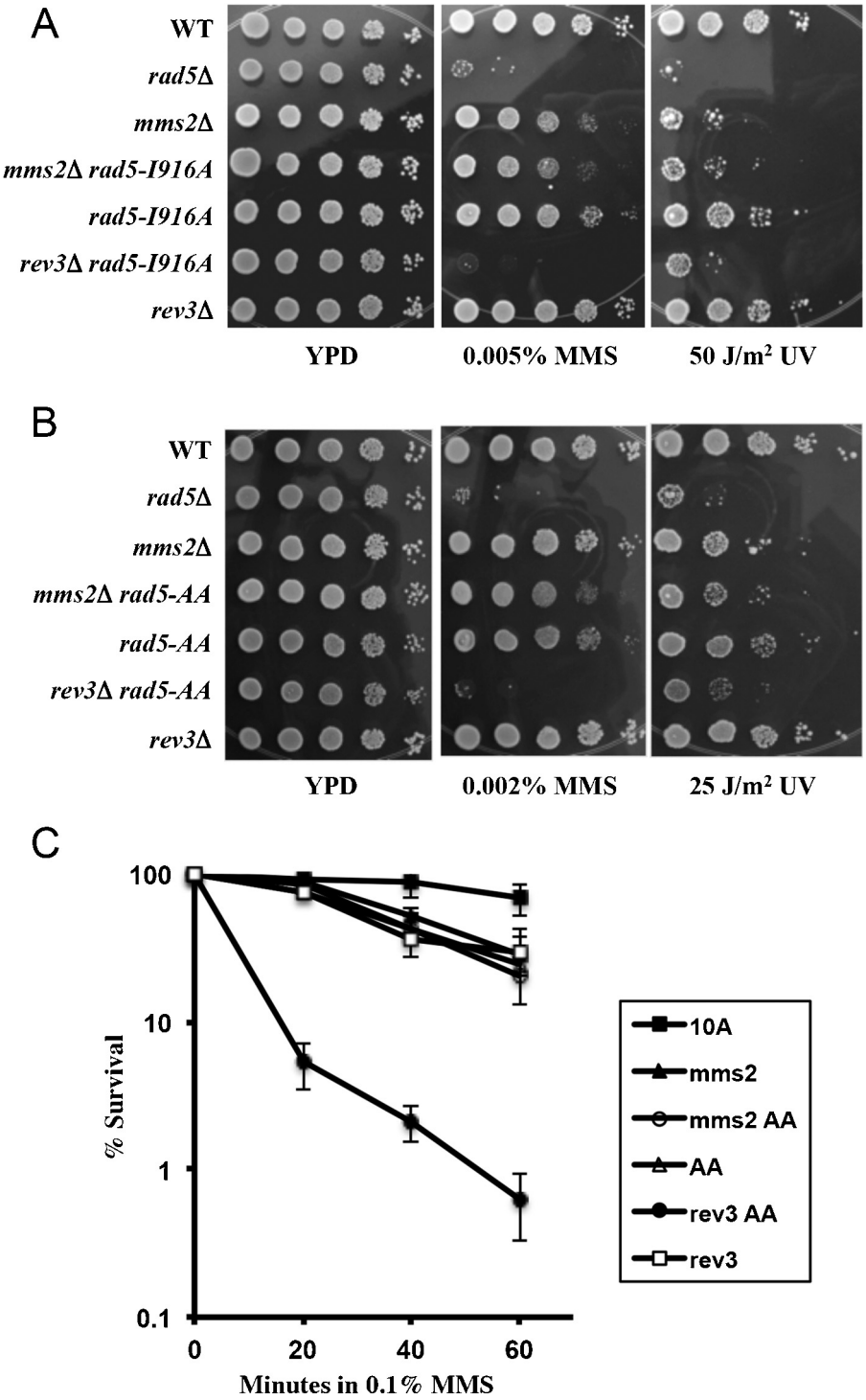

Fig. 2. Genetic interactions between *rad5* mutations and error-free PRR or error-prone TLS mutations. (A and B) Tenfold serial dilution assays. (A) *mms2Δ* is epistatic to the *rad5-I916A* point mutation. (B) The *rad5-AA* point mutation is epistatic to *mms2Δ*. (C) A liquid killing experiment for the relative sensitivity to MMS. Results are the average of at least three independent experiments with standard deviations shown as error-bars. All strains used are isogenic to HK578-10A (WT): HK578-2C (*rad5Δ*), WXY901 (*mms2Δ*), WXY2998 (*rad5-AA mms2Δ*), WXY2981 (*rad5-AA*), WXY2996 (*rad5-AA rev3Δ*), WXY956 (*rev3Δ*), WXY3002 (*rad5-I916A mms2Δ*), and WXY2983 (*rad5-I916A rev3Δ*).

double mutant (lane 6), which is also shifted up in the Pol30-His7 allele (lane 2). This band is deemed to be diubiquitinated PCNA by this and other experimental observations (Supplementary Fig. S2B), which allowed us to examine the role of Rad5 RING finger and helicase motifs in DNA damage-induced PCNA polyubiquitination. As seen in Fig. 3, while *rad5-I916A* (lane 7) and *rad5-AA* (lane 8) point mutations do not affect PCNA monoubiquitination, they both reduce PCNA diubiquitination. These observations indicate that both the RING finger and the helicase motifs are required for MMS-induced PCNA polyubiquitination.

3.5. The rad5-AA mutation abolishes Rad5 interaction with Ubc13

Knowing that, like the *rad5-I916A* E3 ligase mutation, the *rad5-AA* helicase mutation also abolishes PCNA polyubiquitination, we wish to determine at which stage the Rad5 helicase domain is

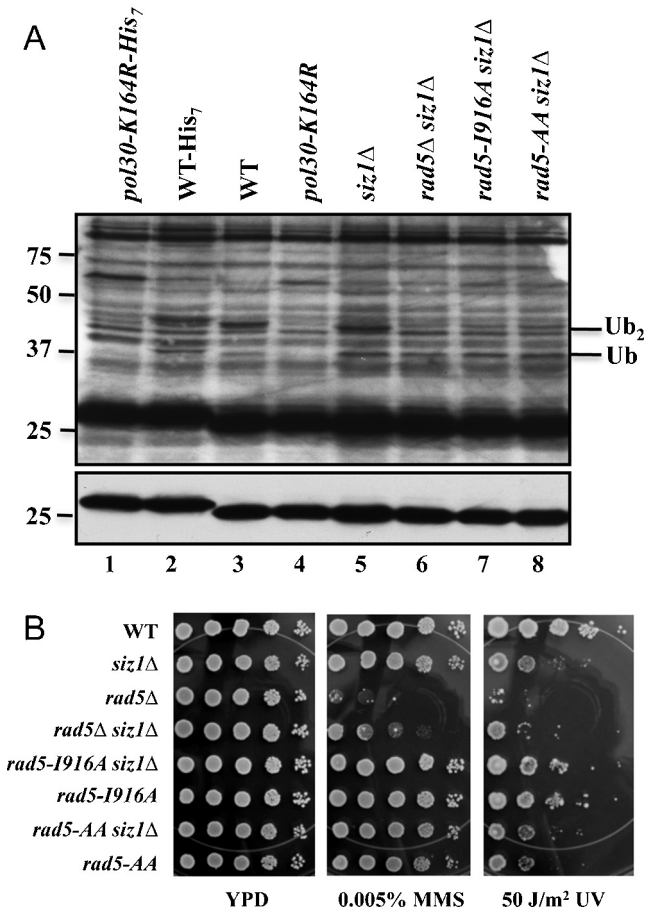

Fig. 3. The effects of *rad5* mutations on PCNA ubiquitination. (A) A PCNA ubiquitination assay showing mono- and di-ubiquitination. Overnight cultures were subcultured and allowed to grow to a cell count of approximately 1×10^7 cells/ml before being treated with or without 0.05% MMS for 90 min followed by harvesting proteins under denaturing conditions. Proteins were separated on an SDS-PAGE gel and PCNA was identified by an anti-PCNA Western blot. Molecular size markers are labelled on the left and mono-ubiquitinated and di-ubiquinated PCNA are labelled on the right as Ub and Ub_2, respectively. A short exposure of the same blot is shown in the lower panel. The blot containing cells extracts from untreated cells and other control blots to identify mono- and di-ubiquitinated PCNA bands are not shown but are available upon request. (B) Relative sensitivity of strains used in panel (A) to killing by MMS or UV in a serial dilution assay. Note that the *siz1* null mutation partially suppresses *rad5*Δ, *rad5-AA* and *rad5-I916A* sensitivity. Strains used were isogenic to HK578-10A (WT), WXY994 (*pol30-K164R*), WXY989 (*pol30-His*$_7$), WXY990 (*pol30-K164R-His*$_7$), WXY2959 (*siz1*Δ), WXY2964 (*rad5*Δ *siz1*Δ), WXY2965 (*rad5-AA siz1*Δ), WXY2966 (*rad5-I916A siz1*Δ), HK578-2C (*rad5*Δ), WXY3001 (*rad5-I916A*), WXY2981 (*rad5-AA*).

required for the PCNA polyubiquitination. Rad5 is known to physically interact with Ubc13 [18], which is presumably required for the assembly of the E2–E3 complex for PCNA polyubiquitination. Under our yeast two-hybrid experimental conditions, Rad5 indeed interacts with Ubc13 and the *rad5-I916A* mutation abolishes this interaction (Fig. 4A). To our surprise, the *rad5-AA* mutation also abolishes the Rad5-Ubc13 interaction (Fig. 4A).

One concern with the yeast two-hybrid analysis is that the *rad5-AA* mutation may affect $Gal4_{AD}$-Rad5 expression, folding or stability, which leads to the loss of Rad5 function(s). To address this concern, we asked whether the same mutated $Gal4_{AD}$-Rad5-AA could interact with another known Rad5 partner. It has been reported that Rad5 is partially required for TLS probably through a Rad5–Rev1 interaction [14,37]. We used the yeast two-hybrid assay to establish the Rad5–Rev1 interaction, and found that neither *rad5-AA* nor *rad5-I916A* point mutation interferes with the Rad5–Rev1 interaction (Fig. 4B). Furthermore, the wild-type and mutant Rad5 yeast two-hybrid constructs complemented the *rad5Δ* null mutant to the characteristic level of MMS resistance (Supplementary Fig. S3), confirming that the *RAD5* fusion genes cloned in the yeast two-hybrid vector fully represent the corresponding alleles.

3.6. The Rad5 RING finger domain alone is insufficient to bind Ubc13

A typical RING finger domain with short flanking regions is sufficient to interact with an E2 [38]. The Rad5-AA mutated residues are quite far away from its RING finger domain (Fig. 1A). To distinguish between two possibilities, that the Rad5-AA mutation interferes with the RING domain folding, or the helicase domain indeed contributes to the recruitment of Ubc13, we cloned various DNA fragments containing the RING finger domain and performed the yeast two-hybrid assay. As shown in Fig. 4C, under conditions that the full-length Rad5 interacts with Ubc13, a typical Rad5-RF fragment fails to interact with Ubc13. In sharp contrast, a RING finger fragment of human Chfr known to interact with human Ubc13 [39] is also capable of interacting with the yeast Ubc13. This observation indicates that, unlike other Ubc13 cognate E3s, the Rad5 RING finger domain alone is insufficient to bind Ubc13, which agrees with a previous report [18].

3.7. Tethering Ubc13 to Rad5 bypasses the requirement for the helicase or RING finger domain

If the role of the Rad5 helicase domain is to recruit Ubc13 to the damage site, it would predict that a Rad5-Ubc13 fusion protein no longer requires this domain. In a *rad5 ubc13* double mutant, transformation of the yeast two-hybrid plasmid carrying *RAD5*, *rad5-AA* or *UBC13* alone results in characteristic levels of sensitivity to DNA-damaging agents. Co-expression of *UBC13* and *rad5-AA* rescues the double mutant to the level indistinguishable from that of *rad5-AA* expression alone, whereas in contrast, expression of the *UBC13-rad5-AA* fusion gene rescues the double mutant to the level comparable to that of *UBC13-RAD5* fusion (Fig. 5A and Supplementary Fig. S4A). The same effect can also be seen in the *rad5Δ* single mutant background, although the *UBC13-rad5-AA* transformant is slightly more sensitive to MMS than the *UBC13-RAD5* transformant (Supplementary Fig. S4B). Hence, once Ubc13 is tethered to Rad5, the helicase activity is no longer required for DNA-damage tolerance. A similar phenomenon is also observed with *RAD5-MMS2* fusions (Fig. 5B). We interpret these data to mean that although Rad5 does not directly recruit Mms2, Mms2 and Ubc13 form a stable complex. Hence the Ubc13-Mms2 complex can be recruited to the damage site through the Rad5-Mms2 fusion protein, again bypassing requirement for the Rad5 helicase domain. To rule out a possible off-target effect of the fusion proteins, we asked whether the expression of the *UBC13-RAD5* fusion bypasses the requirement for the Ubc13-Mms2 complex. As seen in Supplementary Fig. S5, expression of either *UBC13-RAD5* or *UBC13-rad5-AA* cannot rescue MMS sensitivity of the *mms2* mutant, indicating the sole function of the *UBC13-rad5-AA* fusion is to facilitate Rad5-Ubc13-Mms2 complex formation.

The above fusion strategy allows us to further address whether the sole function of Rad5-RING finger domain is also to recruit Ubc13. Fig. 5C and Supplementary Fig. S4C show that the expression of *UBC13-rad5-I916A* fusion gene is as tolerant to DNA damage as the *UBC13-RAD5* fusion, hence completely bypassing the requirement for the RING finger domain.

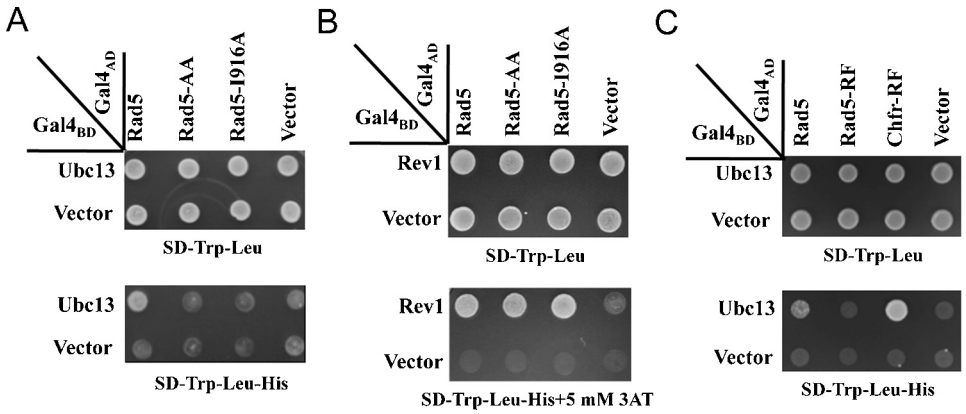

Fig. 4. Yeast two-hybrid analysis to assess physical interactions between Rad5 and Ubc13. (A) Both Rad5-AA and Rad5-I916A fail to interact with Ubc13. (B) Both Rad5-AA and Rad5-I916A are able to interact with Rev1. (C) The Rad5-RF domain alone is insufficient to bind Ubc13. The yeast strain PJ69-4a was co-transformed with various combinations of the Gal_{BD} and Gal_{AD} plasmids before being scored and photographed on selective media after 4 days of incubation at 30 °C. The results represent at least five independent transformants for each treatment.

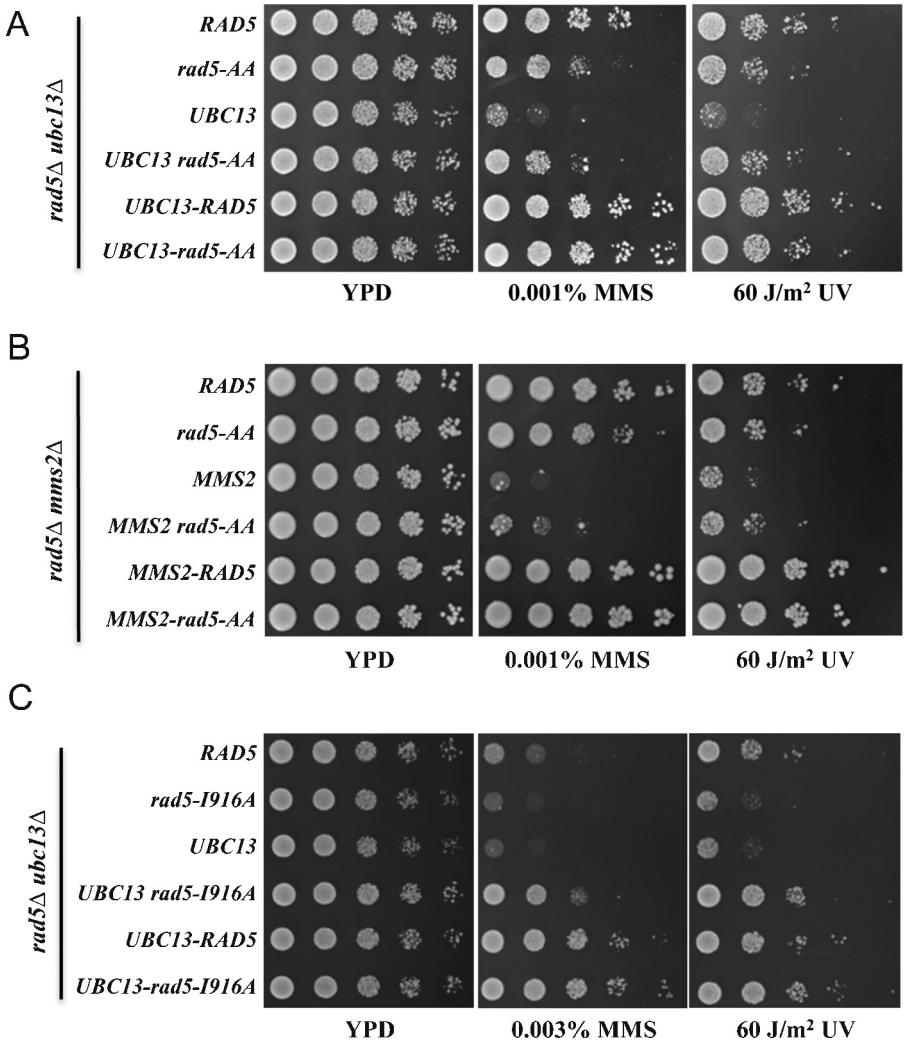

Fig. 5. Tethering Rad5 to Ubc13 or Mms2 bypasses the requirement for the Rad5 helicase/Snf2 and RING domains. (A) Ubc13-Rad5-AA fusion; (B) Mms2-Rad5-AA fusion; (C) Ubc13-Rad5-I916A fusion. In all three cases, fusions of mutated Rad5 with Ubc13 (*UBC13-rad5-AA*) or Mms2 (*MMS2-rad5-AA*) are compared with the two corresponding genes carried by separate plasmids and co-transformed into the same host strain, in response to two representative DNA-damaging agents, UV and MMS. Strains used: WXY922 (*rad5Δ ubc13Δ*) and WXY3138 (*rad5Δ mms2Δ*).

4. Discussion

Although it is well accepted that Rad5-Ubc13-Mms2-mediated PCNA polyubiquitination leads to error-free PRR [2], the exact mechanism for the error-free lesion bypass is unclear. Historically two possible models have been proposed for error-free lesion bypass, namely replication fork regression (the chicken-foot model) and template switch [33,40]. Template switch would involve homologous sister chromatid invasion, high-fidelity DNA synthesis and Holliday junction resolution. Initial support for this model came from visualization of Holliday junctions, which was enhanced in temperature-sensitive DNA Polα and δ mutants held at the restrictive temperature [41]. Recently, template switch was characterized as an X-shaped replication intermediate in a 2-D gel electrophoresis assay [42,43]. In addition, we recently demonstrated that error-free PRR does employ template switch *via* Ubc13-Mms2 and PCNA polyubiquitination, homologous recombination and Sgs1-Top3 resolution to bypass replication blocking lesions that arise during S-phase [21]. Meanwhile, a report by Blastyak et al. demonstrated *in vitro* that Rad5, through its intrinsic ATPase/helicase activity, promotes replication fork regression [22]. In this report, we examined the two competing models based on the *rad5* point mutations that specifically inactivate either the helicase or the RING finger E3 activity. If the two modes of error-free PRR operate separately, the *rad5* RING and helicase double mutant would display an additive or synergistic effect. Alternatively, if the two activities function in the same pathway, the *rad5* double mutant would be as sensitive to DNA damage as one of the single mutants. We found that both *rad5-I916A* and *rad5-AA* mutations exclusively affect error-free PRR and are synergistic to the TLS mutation. Furthermore, they are epistatic to each other and to the *mms2* mutation. Hence, our experimental results collectively support the latter prediction and confirm that the Rad5 helicase activity and PCNA polyubiquitination act together during error-free lesion bypass.

Based on the above observations and their enzymatic activities, the simplest model of error-free PRR would be initiated by PCNA polyubiquitination, which signals for fork regression followed by sister chromatid invasion, synthesis and resolution (Fig. 6). However, this model predicts that the fork regression event promotes but may not be absolutely required for strand invasion, yet the *rad5-AA* mutant is as sensitive to UV [34] and MMS as the *mms2* mutant. It is to our complete surprise that the *rad5-AA* mutation actually reduces PCNA polyubiquitination to the same extent as *mms2* and the *rad5-I916A* mutation, suggesting that this helicase activity is required for PCNA polyubiquitination. Furthermore, like Rad5-I916A, the Rad5-AA protein fails to interact with Ubc13 in a yeast two-hybrid assay, indicating that the *rad5-AA* mutation interferes with E2-E3 assembly. The lack of interaction between Rad5-AA and Ubc13 is unlikely due to altered expression or protein folding, as Rad5-AA is still capable of interacting with Rev1, although we cannot rule out a possibility that Rad5-AA affects a localized folding. An alternative explanation is that Rad5 helicase activity is required for the recruitment of Ubc13-Mms2. However, at least two observations argue against the above possibility. Firstly, the Rad5-Ubc13 interaction is independent of and not enhanced by DNA damage. Secondly, tethering of Rad5 and Ubc13/Mms2 bypasses the requirement for the Rad5 helicase activity. Nevertheless, it remains possible that the Rad5 "helicase motif" plays dual roles within error-free PRR, namely an enzymatic activity for fork regression and a structural role in the E2–E3 complex assembly.

Apparently, the Rad5 RING finger motif differs from some other well-known RING fingers in that a minimum region is insufficient to interact with its cognate E2 (Ubc13). A rather large region including the entire helicase and/or SWI/SNF motif appears to be required for the proper folding of its RING finger domain. The fact that tethering

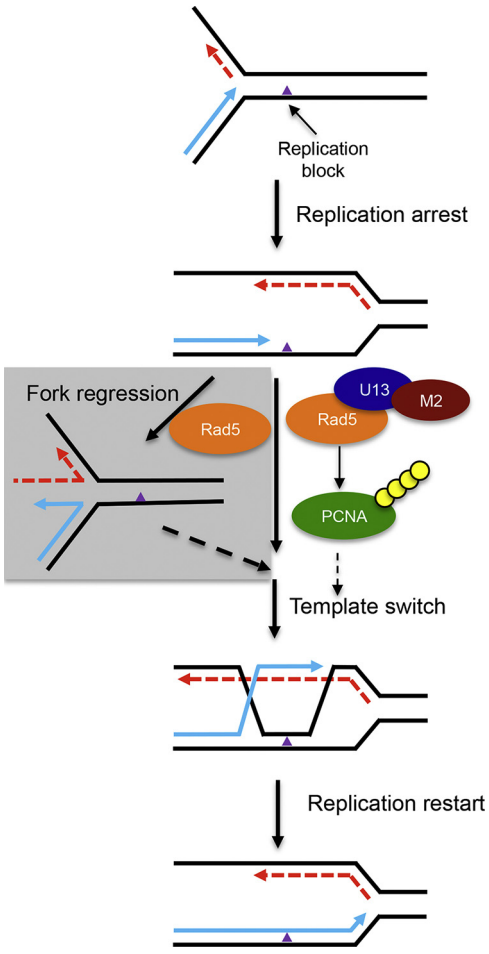

Fig. 6. A working model of budding yeast error-free PRR. When a replication fork meets a replication block, PCNA is monoubiquitinated by Rad6–Rad18, which may promote translesion DNA synthesis (not shown). The monoubiquitinated PCNA may also be polyubiquitinated by the Rad5-Ubc13-Mms2 complex to form a K63-linked ubiquitin chain, which promotes template switch and error-free PRR. Alternatively, Rad5 alone may also mediate fork regression, which eventually leads to template switch. Surprisingly, the *rad5-AA* mutation abolishes both Rad5 functions. Note that the Rad5 fork regression activity may not operate *in vivo*, or at least is dispensable for error-free PRR.

Ubc13 to Rad5 rescues the mutant phenotype of both *rad5-AA* and *rad5-I916A* confirms the structural roles of Rad5 domains defective in the above mutations. Since the Rad5 protein structure is yet unavailable, their exact contributions to the physical interaction remain to be elucidated.

The defect in interaction with Ubc13 caused by *rad5-AA* mutation undermines our investigation into the role of Rad5 helicase in error-free PRR. However, the tethering strategy provides an opportunity for us to re-address the above question. Since the *UBC13-rad5-AA* fusion construct confers DNA-damage tolerance comparable to that of *UBC13-RAD5*, it suggests that the Rad5 helicase activity is dispensable for error-free PRR, as illustrated in Fig. 6. The subtle difference between the *UBC13-RAD5* fusion and *UBC13-rad5-AA* as shown in Supplementary Fig. 5B may reflect the contribution of the Rad5 helicase activity, although the exact role of this activity remains to be determined.

It is noticed that a previous report by Chen et al. [12] concluded that polyubiquitination of PCNA does not require the ATPase activity of Rad5. Furthermore, a recent report by Minca and Kowalski [43] found an additive effect between *rad5* RING finger mutation and the ATPase mutation. In both cases, the authors characterized a *rad5-KT538A,539AA* (GAA) mutation within a presumed "GKT"

ATP-binding motif. Although these point mutations have been previously described to abolish all ATP-binding and hydrolytic activities of Rad5 [44–46], a subsequent report demonstrated that it is the *rad5-DE681,682AA* (AA) mutation that biochemically abolishes the ATPase activity of Rad5 in an *in vitro* assay [34]. The same *rad5-AA* mutation was found to abolish the fork regression helicase activity *in vitro* [22] and was employed in this *in vivo* study. We also characterized a *rad5-K538A* mutation and found that it is indeed additive to the *mms2* or *rad5-I916A* mutation; however, the *rad5-K538A* mutation confers much less sensitivity to DNA-damaging agents than *mms2* or *rad5-AA*, and does not have a clear synergistic effect with TLS mutations (data not shown), indicating that mutations at the Rad5 GKT motif are more complicated than we previously thought, and hence may not represent a true ATPase/helicase dead mutation. Alternatively, the ATPase activity conferred by the Rad5 GKT motif may not be required for its helicase activity as defined by Blastyak et al. [22].

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dnarep.2014.02.016.

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