In vivo characterization of *rad1* alleles in DNA repair pathways

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Saccharomyces cerevisiae Rad1-Rad10 is involved in a variety of DNA repair pathways essential for maintaining genome stability; including nucleotide excision repair (NER), interstrand cross-link repair (ICLR) and double strand break repair (DSBR) by homologous recombination. Rad1-Rad10 is a structure specific endonuclease, with specificity for double-strand/single-strand junctions with 3' single-strand DNA. In some DSBR pathways, such as single strand annealing, 3' non-homologous single-stranded DNA is present, and must be removed in order to prime DNA synthesis. Rad1-Rad10 is critical for processing these 3' non-homologous tails. Other proteins, Msh2-Msh3, Saw1 and Slx4, are also involved in this process. Similarly, Rad1-Rad10 requires different protein co-factors for its activity in NER and possibly ICLR.

To understand how Rad1-Rad10 coordinates with different partner proteins in these distinct DNA repair pathways, we generated two *rad1* alleles with mutations in a previously uncharacterized region of Rad1. We performed an *in vivo* characterization of these *rad1* alleles in all three repair pathways (NER, ICLR and DSBR). Both alleles exhibited a separation-of-function phenotype, with the most pronounced defects occurring in DSBR. Neither allele was dominant in the presence of the wild-type *RAD1* allele. Further characterization of these mutations will provide important insight into these Rad1-Rad10-dependent DNA repair pathways. (NIH GM087459)

RESULTS: What is the *in vivo* phenotype of the *rad1* alleles in 3'NHTR, NER, and ICLR?

Rad1-Rad10 acts in three distinct DNA repair pathways

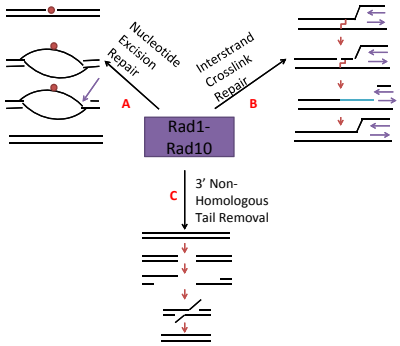

Figure 1: Rad1-Rad10 is a structure specific endonuclease, with specificity for double-strand/single-strand junctions with 3' single-strand DNA. *In vivo*, Rad1-Rad10 is localized to different lesions by distinct protein partners.
A. In Nucleotide Excision Repair (NER), the DNA around the lesion (red ball) is unwound to form a bubble. Rad1-Rad10 is recruited to the bubble by Rad14 and cleaves on the 5' side of the bubble (purple arrow). Rad2 cleaves the other side of the bubble, thereby removing the damaged DNA to allow resynthesis.
B. In some double-stand DNA break repair (DSBR) pathways, a recombination intermediate is formed that contains a 3' non-homologous tail, which must be removed in order to prime DNA synthesis to complete repair. Rad1-Rad10 is required to cleave the tail, but must be recruited to the substrate by Saw1. Msh2- Msh3 may also be involved in recruitment.
C. In interstrand cross-link repair (ICLR) a covalent bond is formed across the double helix, which blocks DNA replication and transcription. Rad1-Rad10 is thought to be involved at multiple steps in this pathway, perhaps interacting with different partners in each case.

rad1 alleles in uncharacterized region

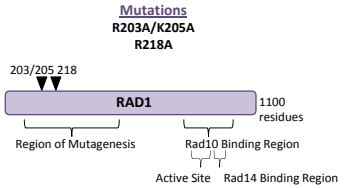

Figure 2: To distinguish the role of Rad1-Rad10 in these distinct repair pathways, we created 10 alleles in an uncharacterized region of *RAD1*, away from the endonuclease active site and the regions that interact with Rad10 and Rad14. These alleles changed charged residues to alanine. Two of these alleles, *rad1R203A, K205A* and *rad1R218A*, exhibited *in vivo* phenotypes.

Two *rad1* mutants are defective in DSBR

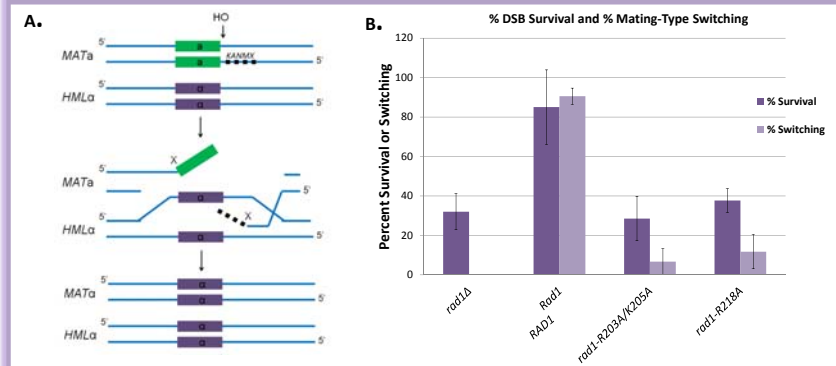

Figure 3. *RAD1*, under the control of its own promoter, complements a *rad1Δ* in both DSBR and NER. The *rad1* alleles were cloned into this plasmid, and we tested the ability of our *rad1* mutants to complement DSBR activity in a *rad1Δ* strain. **A.** A DSB is induced at the *MAT* locus with a galactose-inducible HO endonuclease. Only cells that are able to repair the break will survive. We also tested for mating-type switching to determine whether a DSB had been generated. Cells that did not suffer a break will not switch mating-type. **B.** *rad1-R203A, K205A* and *rad1-R218A* are defective for DSBR in this assay.

rad1 alleles are not dominant negative for DSBR

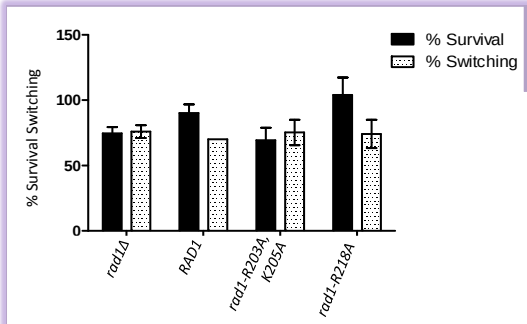

Figure 4: We performed the DSBR assay in the presence of the chromosomal *RAD1* allele. The same high copy number plasmids (empty vector, *RAD1* or *rad1* alleles) used in Fig. 3 were introduced into the *RAD1* background. Galactose was used to induce the DSB and survival and mating-type switching were analyzed. Neither allele caused a defect in DSBR in this constant, i.e. they did not compete with wild-type Rad1-Rad10 for DSBR activity.

Both *rad1* alleles retain ICLR activity.

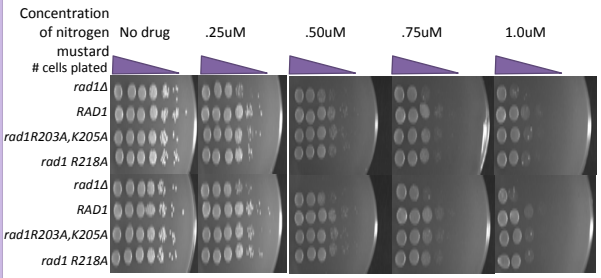

Figure 5: Nitrogen mustard is a chemotherapeutic drug that induces interstrand crosslinking. Cells that are impaired for ICLR would have increased sensitivity to nitrogen mustard, relative to the wild-type. Serial dilutions of saturated cultures for each strain were spotted onto plates containing increasing concentrations of nitrogen mustard. Previous work with another interstrand crosslinking drug, cisplatin, demonstrated similar results.

Both *rad1* alleles retain NER activity.

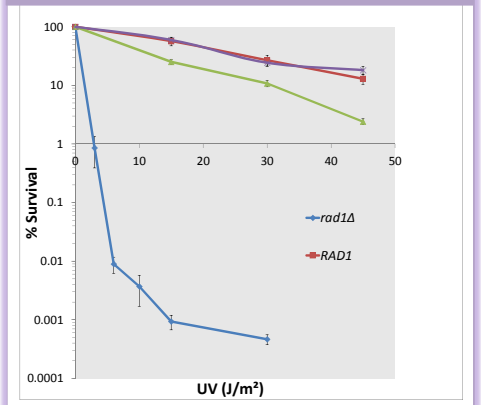

Figure 6: The ability of cells containing different *rad1* alleles to survive increasing UV doses was determined and compared to survival in the presence of *RAD1* or in its absence (*rad1Δ*). Percent survival (log scale) was plotted versus UV exposure (J/m^2), which is calculated based on exposure time. Following UV irradiation, the plates were wrapped in foil to prevent light-activated repair by photolyase so that we were only testing NER activity. *rad1R203A, K205A* exhibited somewhat reduced NER activity compared to *RAD1*, but still retained significant activity relative to the *rad1Δ*. *rad1R218A* NER activity is indistinguishable from that of *RAD1*.

Summary of *in vivo* phenotypes of *rad1* alleles

	3'NHTR		NER	ICLR
	GC	SSA		
RAD1	++	++	++	++
rad1R203A, K205A	-	ND	+	++
rad1R218A	-	-	++	++

Figure 7: This chart shows a summary of the repair activity of our *rad1* alleles. Single-strand annealing (SSA) is another homology-direct DSBR pathway that involves 3' non-homologous tail removal. (++) indicates wild-type activity; (+) indicates mild deficiency; (-) indicates no activity. ND = not determined.

Conclusions:

- Both *rad1* alleles are separation-of-function mutants. They are deficient in DSBR but retain NER and ICLR activity. Therefore there are distinct requirements for Rad1-Rad10 in DSBR compared to the other pathways. One possibility is that interactions with partner proteins specific to DSBR (Saw1 and/or Msh2-Msh3) may be compromised.
- These alleles are powerful tools to dissect these pathways both *in vivo* and *in vitro*.