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The Role of RAD1-RAD10 and Saw 1 in Interstrand Crosslink Repair

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Abstract

Genomic stability is critical in all organisms and multiple pathways are required to prevent increased mutation rates and chromosomal rearrangements. *Saccharomyces cerevisiae* Rad1-Rad10 is a structure-specific endonuclease that plays a role in three distinct DNA repair pathways: nucleotide excision repair, interstrand crosslink repair (ICLR) and a specialized form of DNA double-strand break repair (DSBR) that involves the removal of 3' non-homologous tails. In each of these pathways, Rad1-Rad10 requires protein co-factors for localization to the DNA lesion. We previously demonstrated that Rad1-Rad10 forms a stable complex with Saw1, which is essential for its activity in DSBR; Saw1 recruits Rad1-Rad10 to the DNA and stimulates its endonuclease activity. *In vivo*, the Rad1-Rad10/ Saw1 complex has been implicated in ICLR, a pathway that removes covalent bonds formed across the DNA helix. In the absence of ICLR, DNA replication is blocked. Our goal is to determine the step at which Rad1-Rad10/ Saw1 functions in ICLR *in vitro*. We have purified the wild-type and mutant forms of the complex. We are performing endonuclease assays with these complexes, using synthetic substrates that mimic distinct steps in ICLR. A mechanistic understanding of ICLR would allow new drug target discovery to prevent resistance to interstrand crosslinking agents.

Rad1-Rad10 in DNA Repair

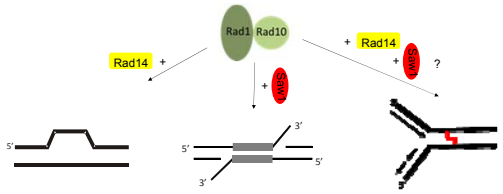


Figure 1:
Rad1-Rad10, an endonuclease that cleaves at double strand single strand junctions, is active in multiple repair pathways, acting on different DNA substrates. Rad1-Rad10 requires distinct protein partners in each pathway. In nucleotide excision repair (NER) Rad1-Rad 10 is recruited by Rad14 and cleaves on the 5' side of damaged DNA. In some types of double-strand DNA break repair (DSBR), recombination intermediates form with 3' non-homologous single-stranded DNA tails. Rad1-Rad10 is recruited by Saw1 and cleaves the 3' single-strand DNA tails in a process termed 3' non-homologous tail removal (3' NHTR). Rad1-Rad10 is also involved in interstrand cross-link repair I(CLR), but the step at which it functions is unclear as is the identity of recruiting protein partners.

The Role of Rad1-Rad10 and Saw1 in 3'NHTR

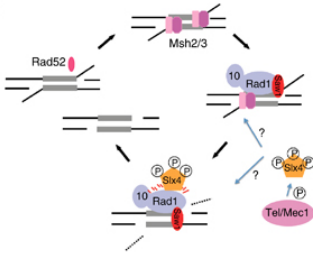
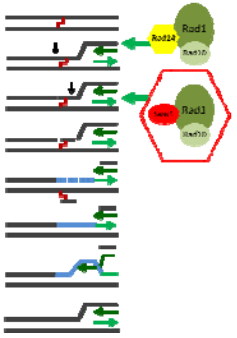


Figure 2:
We have previously shown that Saw1 is necessary to localize Rad1-Rad10 to the 3' flap substrate of 3'NHTR. Msh2-Msh3 also plays a role in recruiting Rad1-Rad10, although the mechanistic details are unclear. Slx4 is thought to act after Rad1-Rad10 has been recruited to the recombination intermediate. We propose that Saw1 may modulate Rad1-Rad10 activity in ICLR, as well. (figure from Li et al 2013)

Interstrand Crosslink Repair Pathway



Cory Holland, Univ of Texas, San Anotonio

Figure 3: Model for activity of Rad1-Rad10 in ICLR. The ICLR pathway repairs is a multistep process, several steps of which are not well defined. When the replisome encounters a crosslink, the replication machinery stalls and the replication fork is modified so that the lesion is a recognizable substrate for repair proteins. After that, a nick is made in the DNA on either side of the crosslink and the covalently bound bases are swung to the outside of the molecule; this is called unhooking of the interstrand cross link. A translesion DNA polymerase fills in the gap across from the unbound lesion. To re-form the replication fork a process including strand invasion is undergone. Our focus is on understanding what step (or steps) Rad1-Rad10 functions in. We propose that Rad1-Rad10 first cleaves the DNA adjacent to the cross-link to facilitate "unhooking" and then cleaves at the modified replication fork to promote homologous recombination to regenerate the replication fork.

Saw1 Forms a Stable Complex with Rad1-Rad10

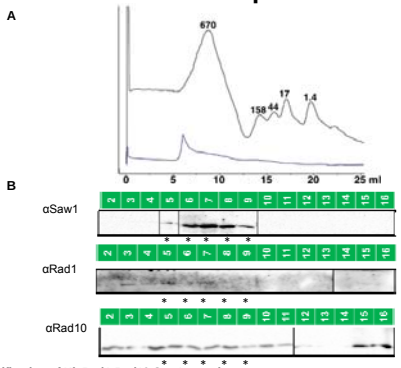


Figure 4: Purification of HisRad1-Rad10-Saw1 complex.
Histidine-tagged Rad1 and untagged Rad10 and Saw1 were co-overexpressed in *Escherichia coli.*, harvested and lysed. The soluble protein is separated from cellular debris by centrifugation and incubated with a cobalt resin. The cobalt resin chelates the histidine-tag of Rad1. Any Rad10 and Saw1 that interacts with HisRad1 will also be retained on the cobalt column. The HisRad1-Rad10-Saw1 complex can then be eluted with imidazole, which competes with histidine for binding to the cobalt resin. The eluate is then applied to a size exclusion chromatography column in which large molecules elute faster while small molecules elute slower because they travel through all pore sizes. **A.** The grey chromatogram) is the elution profile of molecular weight standards through the gel filtration column. The blue line is the elution profile of the cobalt eluate. (figure from Li et al 2013 Rad1 is a 126kD protein, Rad10 is a 24kD protein and Saw1 is a 29kD protein. **B.** Western blots to identify fractions that contain HisRad1, Rad10 and Saw1. It should be noted that the Rad1 antibody is not very sensitive, but silver stained gels of various preparations indicated that Rad1 is also in fractions 10-12. Fractions that contain all three proteins are indicated by an asterisk.

Rad1-Rad10 Cleavage at Modified Replication Fork Structures

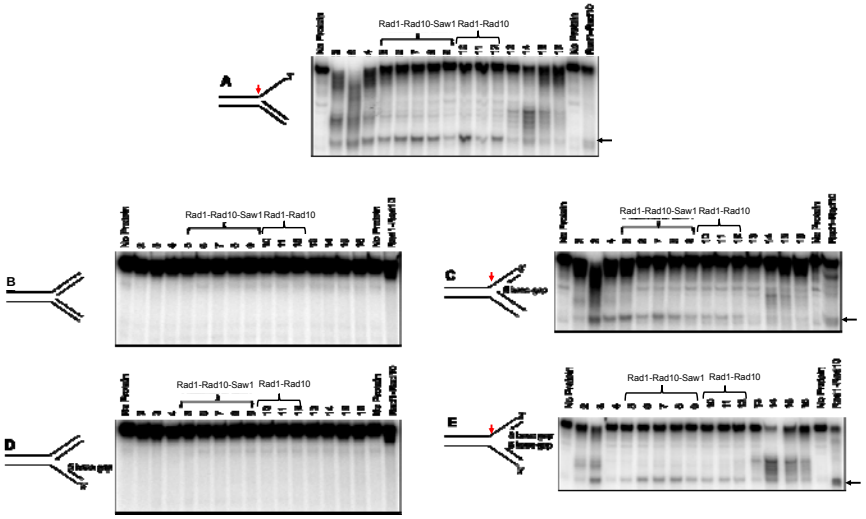


Figure 5:
Fractions from the gel filtration column were tested for the ability to cleave radiolabeled synthetic DNA substrates that represent different possible replication fork structures that are predicted to form in the later steps of ICLR. Red arrows signify where we think Rad1-Rad10 is cleaving. Black arrows signify the cleavage product for these substrates. **A.** This 3' flap substrate is an intermediate in 3' NHTR and is a substrate for Rad1-Rad10. The presence of Saw1 enhances it's endonuclease activity. **B.** This substrate without a double strand single strand junction could represent a stalled replication fork with no gaps. Neither Rad1-Rad10 nor Rad1-Rad10-Saw1 cleaves this substrate. **C.** This substrate has a 8 base gap on leading strand, creating a similar structure to the 3' flap substrate. Rad1-Rad10 cleaves this substrate and cleavage appears enhanced by Saw1. **D.** This substrate has a 5 base gap on the lagging strand. Rad1-Rad10 exhibits no activity on this substrate, with or without Saw1. **E.** This substrate has a gap on both the leading and lagging strands. Rad1-Rad10 cleaves this substrate and Saw1 appears to enhance this activity

Conclusions

- Rad1-Rad10-Saw1 forms a stable complex that can survive to purification steps
- Rad1-Rad 10 preferentially cleaves DNA substrate with a gap on the 3' splayed end, i.e. the leading strand of the synthetic replication fork junction. A gap in the lagging strand does not affect Rad1-Rad10 endonuclease activity.
- The presence of Saw1 enhances Rad1-Rad10 endonuclease activity on these substrates.

Future Directions

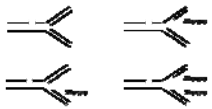


Figure 6:
Substrates we are planning to synthesize and test that are representative of the ICLR pathway after unhooking of the ICL.

- Repeat endonuclease assays
- Treatment with DNaseI to explore the role of DNA in Rad1-Rad10 and Saw1 complex formation and/or maintenance.
- Explore additional DNA substrates to further dissect the role of Rad1-Rad10 and Saw1 in interstrand crosslink repair

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References

Li F, Dong J, Eichmiller R, Holland C, Minca E, Prakash R, et al. Role of Saw1 in Rad1/Rad10 complex assembly at recombination intermediates in budding yeast. The EMBO journal. 2013;32(3):461-72.