SAW1 is increasingly required to recruit Rad10 as SSA flap-length increases from 20 to 50 bases in single-strand annealing in S. cerevisiae

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ABSTRACT

SAW1 is required by the Rad1-Rad10 nuclease for efficient removal of 3′ non-homologous DNA ends (flaps) formed as intermediates during two modes of double-strand break repair in S. cerevisiae, single-strand annealing (SSA) and synthesis-dependent strand annealing (SDSA). Saw1 was shown in vitro to exhibit increasing affinity for flap DNAs as flap lengths varied from 0 to 40 deoxynucleotides (nt) with almost no binding observed when flaps were shorter than 10 nt. Accordingly, our prior in vivo fluorescence microscopy investigation showed that SAW1 was not required for recruitment of Rad10-YFP to DNA double-strand breaks (DSBs) when flaps were ~10 nt, but it was required when flaps were ~500 nt in G1 phase of the cell cycle. We were curious whether we would also observe an increased requirement of SAW1 for Rad10 recruitment in vivo as flaps varied from ~20 to 50 nt, as was shown in vitro. In this investigation, we utilized SSA substrates that generate 20, 30, and 50 nt flaps in vivo in fluorescence microscopy assays and determined that SAW1 becomes increasingly necessary for SSA starting at about ~20 nt and is completely required at ~50 nt. Quantitative PCR experiments corroborate these results by demonstrating that repair product formation decreases in the absence of SAW1 as flap length increases. Experiments with strains containing fluorescently labeled Saw1 (Saw1-CFP) show that Saw1 localizes with Rad10 at SSA foci and that about half of the foci containing Rad10 at DSBs do not contain Saw1. Colocalization patterns of Saw1-CFP are consistent regardless of the flap length of the substrate and are roughly similar in all phases of the cell cycle. Together, these data show that Saw1 becomes increasingly important for Rad1-Rad10 recruitment and SSA repair in the ~20–50 nt flap range, and Saw1 is present at repair sites even when not required and may depart the repair site ahead of Rad1-Rad10.

1. Introduction

1.1. Steps of DSB repair and role of Rad1-Rad10

DSB repair pathways contain many genes with overlapping functions in multiple subpathways [1]. This complexity is necessary to enable repair in all phases of the cell cycle and in numerous contexts [2]. When DSBs form between DNA repeats, for example, single-strand annealing (SSA) is usually the pathway utilized in S. cerevisiae even though it results in the elimination of DNA lying between the repeats [1,3]. In SSA, DSB ends located between repeats are resected in a 5′→3′ fashion generating 3′ single-stranded ends that become annealed by Rad52 in a process that is generally RAD51-independent [4]. The DNA sequence originally situated between the repeats becomes two overhanging 3′-flaps that are hydrolyzed by the Rad1-Rad10 nuclease before ligation, eliminating the flap sequences and one repeat [5]. In higher eukaryotes, many diseases are associated with DNA repeat instability, including breast cancer and leukemia [6,7].

1.2. Saw1 and role of flap-length in Rad1-Rad10 recruitment

In S. cerevisiae, SSA requires SAW1 and mutation of SAW1 is epistatic to rad1Δ, sdx4Δ, msh2Δ, and rad52A [8]. Chromatin immunoprecipitation experiments showed that SAW1 is needed in order to recruit Rad1 to...
Additionally, it was revealed that Saw1 binds flap and non-synapsed end DNA. Furthermore, it was shown in vitro and in vivo that Saw1 is required for Rad1-Rad10 recruitment during SSA [8, 11]. In strains containing DSB substrates that generate 20 nt, the saw1Δ mutant exhibited a slightly higher SSA efficiency without Saw1. Interestingly, our prior work revealed that despite not being required to recruit Rad1-Rad10 to SSA sites in ~10 nt flaps, Saw1 nonetheless localizes to such substrates [13].

### 1.3. Questions addressed in this study

Since Saw1 does not bind to flaps shorter than ~10 nt and appears to exhibit maximal binding at approximately 30–40 nt, we tested whether the requirement for Saw1 in recruitment of Rad1-Rad10 to chromosomally-situated DSBs in vivo would also manifest in the ~20–50 nt range (the ~10 nt substrate having already been tested in our earlier work and shown not to require Saw1 [13]). We also wanted to determine if any requirement for Saw1 would extend to SSA repair, and whether Saw1 localization at repair sites would correlate with its requirement to recruit Rad1-Rad10.

### 2. Materials and Methods

#### 2.1. Cloning

HIS3 (663 bp) genes were integrated so they flanked the 1-SceI site in YER186C in strain PF025-7A (Table 1) using adapter-mediated PCR.
and gene transplacement \[10,13\]. Transformants were selected on Synthetic Complete agar lacking histidine (SC-his agar), screened by PCR, and sequenced for the HIS3::x-bpf1-SceI-bpf1::HIS3 cassette where “x” was either 10, 20 or 40 deoxynucleotide base pairs giving rise to substrates that would contain 20, 30 or 50 deoxynucleotide flaps, respectively. The resulting strains were crossed to give rise to the strains used in microscopy and qPCR (Table 1).

2.2. Microscopy

Strains contain chromosomally integrated copies of the Tetracycline repressor protein fluorescently labeled with RFP (TetR-RFP) and 224 tandem copies of the Tetracycline operator (tetO) sequence near the I-SceI restriction site at YER186 as described \[14\]. This restriction site is cleaved by exogenous I-SceI and contains 60–70% efficiency in asynchronously growing cells \[14\]. Strains were transformed with plasmid pWJ1320 containing the I-SceI gene under a GAL1 promoter and an ADE2 selection marker \[15\]. Transformants were plated on SC-ade medium containing 2% raffinose for selection and cultured in SC-ade with raffinose liquid medium at 23 °C. Overnight cultures were diluted to 0.1 OD\text{600} followed by incubation (23°C, 3 h). DSBs were induced by adding galactose to a final concentration of 2% (w/v) and incubating the cultures (23°C, 30 min) prior to preparation for microscopy \[10\].

Microscopy was carried out using a Zeiss AxioImager M1 microscope outfitted as previously described except that the Velocity Software package was version 6.3, the objective was a Plan-Apochromat 100×, 1.46 numerical aperture oil immersion lens, and the fluorescence filter sets were ET-EYFP, Chroma #49003 (\(\lambda_{ex}=500/20, \lambda_{dichroic}=515, \lambda_{em}=535/30\), ET-CFP, Chroma #49001 (\(\lambda_{ex}=436/20, \lambda_{dichroic}=455, \lambda_{em}=480/40\) and ET-CY3/TRITC, Chroma #49004, (\(\lambda_{ex}=545/25, \lambda_{dichroic}=565, \lambda_{em}=605/75\)) \[16\]. Integration times were 800 ms (Rad10-YFP) and 400 ms (TetR-RFP) for experiments in which only YFP and RFP were being imaged. Integration times were 400 ms (Rad10-YFP), 200 ms (Saw1-CFP) and 200 ms (TetR-RFP) for experiments in which YFP, CFP, and RFP were all being imaged. In these triple-labeled experiments, only 3 focal planes were imaged per field of cells. In all cases, focal planes were offset by 0.3 \(\mu\)m intervals along the Z-axis (a Z-stack). Colocalization of foci were analyzed by inspecting images from each focal plane of the Z-stack contrast enhanced as described \[13\]. Cells were classified as “G1”, “S/G2” or “M” as previously described \[13\]. Unless noted, at least 100 cells were analyzed per experimental condition in each trial. Graphs report percentages calculated from sums of foci counts from at least 100 cells analyzed per condition. Data from experiments with 20 nt strains show that Rad10-YFP/DSB-RFP foci in fluorescence images (Fig. 1A lower images). Finally, strains were either wild-type in, or deleted of the SAW1, RAD51 or RAD52 genes. Following induction of DSBs and time for repair, fluorescence images were recorded and inspected for the presence of Rad10-YFP/DSB-RFP colocalized foci in fluorescence images (Fig. 1A lower images).}

3. Results

To determine whether Saw1 is required for recruitment of the Rad1-Rad10 complex in SSA when flaps vary between 20 and 50 nt, we prepared a panel of S. cerevisiae strains containing an inducible DSB site installed on Chromosome V between two copies of the HIS3 gene, so that the ~500 bp HIS3 sequences could serve as the flanking repeats to mediate repair by SSA (Fig. 1A). These SSA substrates differ in the spacing between the HIS3 repeats at the inducible DSB site (Fig. 1A) thereby producing flaps of different lengths and are derivatives of strains we have previously used \[13\]. They also contain an adjacent fluorescent label on Chromosome V comprising an array of 224 copies of the TetR binding site that bind fluorescently labeled TetR proteins expressed from another chromosome under the control of a constitutive promoter (DSB-RFP) (Fig. 1A). The strains additionally contain a fluorescently labeled Rad10 (Rad10-YFP) that allows for the detection of Rad1-Rad10 recruitment to induced DSBs (Fig. 1A). Recruitment manifests as the appearance of Rad10-YFP/DSB-RFP colocalized foci in fluorescence images (Fig. 1A lower images). Finally, strains were either wild-type in, or deleted of the SAW1, RAD51 or RAD52 genes. Following induction of DSBs and time for repair, fluorescence images were recorded and inspected for the presence of Rad10-YFP/DSB-RFP colocalized foci. Data from experiments with 20 nt strains show that Rad10-YFP/DSB-RFP foci colocalization is observed in ~19% of the cells in the wild-type strain following DSB induction while uninduced cells show ~2.0–3.0% regardless of the phase of the cell cycle (Fig. 1B). However, in the absence of the SAW1 gene (saw1Δ), the percentages of cells with colocalized foci are only about 8.9–10% following induction while uninduced are similar to uninduced wild-type (Fig. 1B). This ~50% reduction was observed in both dividing and non-dividing cells and is statistically significant (Fig. 1B). These data indicate that Rad1-Rad10 recruitment to repair sites is partially dependent on SAW1 when flaps are ~20 nt, and contrasts with our previously published ~10 nt-flap data in which there was not a statistically significant difference between wild-type and saw1Δ \[13\].

When we tested strains containing 30 or 50 nt flaps, we found an increasing reliance on SAW1 for Rad10-YFP recruitment. Substrates with 30 nt flaps showed a ~66% reduction in induced Rad10-YFP/DSB-RFP foci in saw1Δ S/G2/M cells compared to wild-type S/G2/M (Figs. 1C) and 50 nt flap substrates showed no difference in foci between induced and uninduced saw1Δ samples in any phase of cell cycle (Fig. 1D). Interestingly, although the 30 nt flap substrate exhibited a modest 7.3% induced foci in saw1Δ in S/G2/M cells (vs. 1.9% in uninduced controls), saw1Δ G1 cells did not show any induction of foci (similar to 50 nt flap in G1), possibly indicating a subtle cell cycle dependency for SAW1 with shorter flaps.
We also carried out control experiments in \textit{rad51}Δ and \textit{rad52}Δ strain backgrounds which showed that colocalized focus induction was \textit{RAD51}-independent but \textit{RAD52}-dependent, indicating that repair was proceeding by SSA in all three substrates (Fig. 1B, C and 1D). Together, these data show in vivo that a flap size of about ~20–30 nt is the key length triggering an increasing requirement of \textit{SAW1} for \textit{Rad1-Rad10} recruitment and that this requirement becomes absolute by ~50 nt.

We next asked whether Saw1 would localize to our SSA repair sites since, in our prior work, we observed Saw1 localizing to SSA repair sites in 10 nt as well as 500 nt flap substrates (despite \textit{SAW1} not being required for recruitment of \textit{Rad1-Rad10} in 10 nt-flap substrates) [13].

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We found ample evidence that Saw1 colocalizes at 20, 30 and 50 nt-flap substrates with \textit{Rad10-YFP}; an example colocalized focus is shown (Fig. 2A). Further, results from experiments in which we quantified colocalized \textit{Rad10-YFP/Saw1-CFP/DSB-RFP} foci in cells with 20, 30 and 50 nt-flap substrates were similar to each other and to the prior 10 nt-flap data (Fig. 2, B–D [13]). Specifically, we observed a significant induction of similar percentages of \textit{Rad10-YFP/Saw1-CFP/DSB-RFP} foci in S/G2/M phases in all three substrates (Fig. 2B–D., 11–17%), and in G1 (Fig. 2B–D., 9–10%). Analogously to our prior report on 10 nt-flap substrates [13]. We also observed a significant induction of double foci containing only \textit{Rad10-YFP/DSB-RFP} (~15% in S/G2/M, ~8–14% in G1), but very few double foci containing \textit{Saw1-CFP/DSB-RFP} (~1–4% in all phases of cell cycle). These findings suggest that Saw1 and \textit{Rad1-Rad10} are recruited to the repair site at the same time, but that Saw1 may depart ahead of \textit{Rad1-Rad10} in a manner that is independent of flap length and cell cycle phase. Since Saw1-CFP is a low-abundance protein, photobleaching prevented us from imaging the entire thickness of the cells with an 11-slice Z-stack (as in Fig. 1), so these results reflect a 3-slice Z-stack. Both the presence of Saw1-CFP at all these repair sites
and the lack of flap length-dependence for this presence provide further evidence that Saw1 may be present at SSA repair sites even when it is not required for recruitment of Rad1-Rad10. We also analyzed images for the presence of Saw1-CFP/Rad10-YFP foci that were not colocalized with DSB-RFP foci and, analogously to our prior report, found that there were only background levels in all substrates (Fig. 2B–D) [13].

We next sought to establish whether we would observe repair product formation in the absence of SAW1. We carried out a qPCR assay in which repair product formation was monitored as a function of time in our 20, 30 and 50 nt panel of SSA strains similarly to prior work [13]. In this assay, qPCR was conducted on genomic DNA samples isolated from the same strains used in Fig. 1, following DSB induction under the same conditions. The test region amplifies the DSB site if a repair product is formed (Fig. 3A). Amplification of the test region was normalized to a control region deriving from the gene also located on Chromosome V (Fig. 3A). Consistent with the fluorescence microscopy data in Fig. 1, we observed that there was no statistically significant difference in repair product formation between wild-type and saw1Δ strains (Fig. 3B). However, we saw significantly diminished repair product formation in 50 nt flap substrates (Fig. 3B). Interestingly, some of the charged residues in this region were previously shown to be critical for DNA binding [9]. Since scRPA binds a stretch of approximately 27–30 nt [18], a reasonable hypothesis is that the when the DNA flap is long enough to sufficiently engage RPA (~20–30 nt based on our results), Saw1 is required to dislodge RPA and help position Rad1-Rad10 at the single-strand/double-strand junction.

Interestingly, in 30 nt flaps, we detected a slight difference in the SAW1 requirement; dividing S/G2/M cells showed a ~66% diminishment in Rad1-Rad10 recruitment to DSBs, while nondividing G1 cells showed complete loss. This indicates that Rad1-Rad10 recruitment in SSA is not mediated identically throughout the cell cycle and that SSA is more important in G1. Recruitment in dividing cells may require additional proteins. In triple-labeled experiments in which Saw1 was also fluorescently labeled, we observed Saw1 and Rad1-Rad10 frequently localized to the repair sites at the same time, in all flap substrates. Prior literature provided evidence for a Msh2-Msh3-Saw1-Rad1-Rad10 protein complex and other evidence suggesting that Msh2-Msh3 might stabilize the annealed repair intermediate thereby recruiting Saw1 and Rad1-Rad10 to the DSB site [9,19]. It is possible that Msh2-Msh3 is unable to distinguish between varying flap lengths and recruits Saw1 to all flap substrates regardless of necessity. This uniform recruitment might explain why Saw1 localizes to shorter flaps even when it is not required for Rad1-Rad10 recruitment.

4. Discussion

Herein, we show in vivo evidence that ~20–30 nt is the key flap length that triggers a requirement for SAW1 for recruitment of the Rad1-Rad10 complex in SSA. These data expand on earlier findings that ~10 nt flaps did not require SAW1 but that 500 nt flaps did [13]. They are also consistent with in vitro work showing that the binding of Saw1 to flap DNAs increases gradually as flaps vary from 10 to 50 nt [9]. A possible explanation for the necessity of Saw1 in longer flap substrates is that it aids in displacing Replication Protein A (RPA) from the single-stranded DNA region, thus making the repair site more accessible to Rad1-Rad10. To provide a visual aid, we ran Adaptive Poisson-Boltzmann Solver (APBA) Electrostatic analyses in PyMOL on the AlphaFold-predicted structure of S. cerevisiae Saw1, and on Rfa1 (PDB:6i52) from the scRPA trimer (Supplementary Fig.). We also included multiple sequence alignments to the closest orthologs of each (NCBI BLAST, Supplementary Fig.). The positively charged residues of the AlphaFold of Saw1 form a groove, and many of these residues are highly conserved with the closest scSaw1 orthologs (Supplementary Fig.). Interestingly, some of the charged residues in this region were previously shown to be critical for DNA binding [9]. Since scRPA binds a stretch of approximately 27–30 nt [18], a reasonable hypothesis is that the when the DNA flap is long enough to sufficiently engage RPA (~20–30 nt based on our results), Saw1 is required to dislodge RPA and help position Rad1-Rad10 at the single-strand/double-strand junction.

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A significant percentage of foci were also observed that contained only Rad1-Rad10 at the repair site and not Saw1. However, there were very few foci observed that contained Saw1 at the repair site but not Rad1-Rad10, similar to our previous observations in 10 nt flap substrates [13]. Taken together these results suggest Rad1-Rad10 may be recruited to the repair site as a complex with Saw1 at all flap lengths, but that Saw1 may depart ahead of Rad1-Rad10. It is also a formal possibility that Rad1-Rad10 is sometimes recruited with Saw1 and at other times without it, but we disfavor this latter possibility since it contradicts prior data indicating that the necessity for Saw1 is flap-length dependent. Together our data support a model (Fig. 3F) in which the flap-length dependent repair in SSA. The annealed intermediate formed during SSA produces flaps of a length representing the distance between each repeat and the DSB site (dark blue + pink boxes). As flap length varies from ~20 to 30 nt Saw1 is increasingly required to recruit Rad1-Rad10 to the flaps, which becomes a total requirement by ~50 nt (red arrows). This trend is also observed in formation of SSA repair products (black arrows). Increased thickness of the arrows corresponds to the increasing requirement for Saw1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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Declaration of competing interest

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.101125.

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