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Srs2 binding to PCNA and its sumoylation contribute to RPA antagonism during the DNA damage response

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eLife Assessment

This manuscript reports **valuable** findings on the role of the Srs2 protein in turning off the DNA damage signaling response initiated by Mec1 (human ATR) kinase. The data provide **solid** evidence that Srs2 interaction with PCNA and ensuing SUMO modification is required for checkpoint downregulation. However, while the model that Srs2 acts at gaps after camptothecin-induced DNA damage is reasonable, direct experimental evidence for this is currently lacking. The work will be of interest to cell biologists studying genome integrity.

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Abstract

Activation of the DNA damage checkpoint upon genotoxin treatment induces a multitude of cellular changes, such as cell cycle arrest or delay, to cope with genome stress. After prolonged genotoxin treatment, the checkpoint can be downregulated to allow cell cycle and growth resumption. In yeast, downregulation of the DNA damage checkpoint requires the Srs2 DNA helicase, which removes the ssDNA binding complex RPA and the associated Mec1 checkpoint kinase from DNA, thus dampening Mec1-mediated checkpoint. However, it is unclear whether the 'anti-checkpoint' role of Srs2 is temporally and spatially regulated to both allow timely checkpoint termination and to prevent superfluous RPA removal. Here we address this question by examining regulatory elements of Srs2, such as its phosphorylation, sumoylation, and protein-interaction sites. Our genetic analyses and checkpoint level assessment suggest that the RPA countering role of Srs2 is promoted by Srs2 binding to PCNA, which recruits Srs2 to a subset of ssDNA regions. RPA antagonism is further fostered by Srs2 sumoylation, which we found depends on the Srs2-PCNA interaction. Srs2 sumoylation is additionally reliant on Mec1 and peaks after Mec1 activity reaches maximal levels. Based on these data, we propose a two- step model of checkpoint downregulation wherein Srs2 recruitment to PCNA proximal ssDNA- RPA filaments and subsequent sumoylation stimulated upon Mec1 hyperactivation facilitate checkpoint recovery. This model suggests that Srs2

removal of RPA is minimized at ssDNA regions with no proximal PCNA to permit RPA-mediated DNA protection at these sites.

Introduction

The DNA damage response promotes organismal survival of genotoxic stress by eliciting many cellular changes. A critical component of this response is a signaling process termed the DNA damage checkpoint (DDC) (Enoch *et al*, 1992; Hartwell and Weinert, 1989; Lanz *et al*, 2019). Upon encountering stress, apical DDC kinases are recruited to DNA lesion sites by the damage sensing proteins and are then activated by activator proteins (Usui *et al*, 2001; Zou and Elledge, 2003). A universal DDC sensor is the RPA complex that binds with high affinity and specificity to single-strand DNA (ssDNA), whose abundance rises under almost all types of genotoxic stress (Marechal and Zou, 2015). Using the budding yeast system as an example, RPA bound to the ssDNA filament can interact with Ddc2, the co-factor of the Mec1 checkpoint kinase, and recruit the Mec1-Ddc2 complex to DNA lesion sites (Elledge, 1996; Foiani *et al*, 2000). Subsequently, Mec1 is activated by activator proteins, such as the 9-1-1 complex loaded at the 5' DNA ends at ss-dsDNA junctions. Activated Mec1 can then phosphorylate multiple substrates, among which is a key downstream effector kinase Rad53, the phosphorylation of which turns on its own kinase activity and yields further phosphorylation events (Harrison and Haber, 2006).

Collectively, the DDC kinases can modify hundreds of proteins, inducing multitude of pathway changes (Lanz *et al*., 2019). For example, DDC can arrest or delay the cell cycle progression to allow more time for genome repair in promoting cellular survival (Sanchez *et al*, 1999; Wang *et al*, 2001).

While DDC activation is crucial for the cell to cope with genotoxic stress, its timely downregulation is equally vital (Waterman *et al*, 2020). Many changes induced by the DDC kinases need to be reversed to allow continued cell growth and recovery. For example, the DDC-mediated cell cycle arrest has to be lifted to allow proliferation and alternative DNA repair mechanisms permitted at other cell cycle phases (Pellicioli *et al*, 2001; Waterman *et al*., 2020). Thus far, DDC downregulation has been mainly studied using the model organism yeast, with several strategies being identified (Waterman *et al*., 2020). One of these is mediated by the DNA helicase Srs2 that targets the most upstream step of the DDC pathway through removing RPA and the associated Mec1-Ddc2 from chromatin (Dhingra *et al*, 2021). Importantly, the role of Srs2 in DDC dampening (anti-checkpoint) can be separated from its well-studied role in removing the recombinase Rad51 from ssDNA (anti-recombination) that modulates homologous recombination levels and outcomes (Dhingra *et al*., 2021). The former role of Srs2 is critical for cell survival of genotoxins, such as camptothecin (CPT) that traps Top1 on DNA (Dhingra *et al*., 2021). Considering that other organisms also possess DNA helicases capable of removing RPA from ssDNA (Hormeno *et al*, 2022; Jenkins *et al*, 2021; Shorrocks *et al*, 2021), it is plausible that an anti-checkpoint strategy involving RPA antagonism may be present beyond yeast.

Currently, little is known about how checkpoint dampening can be temporally controlled to minimize interference with the initial DDC activation that must occur in the early phase of the DNA damage response. In the case of the Srs2-mediated checkpoint regulation, it is also unclear how the process can minimize superfluous removal of RPA from ssDNA sites that unlikely induce the DDC due to lacking 5' DNA ends at ss-dsDNA junctions for 9-1-1 loading, such as at those formed at R-loops. The main functions of RPA at these sites can be ssDNA protection and repair, rather than DDC induction, thus these sites may benefit from shielding from Srs2-mediated RPA removal. In addressing these questions, we hypothesize that Srs2- based RPA antagonism should be selective in location and timing, and that the regulatory elements within the Srs2 protein may contribute to such selectivity.

To examine the above hypothesis and to define Srs2 features relevant to its RPA antagonism during DDC regulation, we inspected Srs2 protein sites involved in its post-translational modifications and interactions with other proteins. Our genetic analyses of mutations affecting each of these sites suggest that while most of the examined features do not affect RPA antagonism, Srs2 binding to PCNA, which marks 3' DNA ends of ss-dsDNA junctions that flank ssDNA gaps or tails, and Srs2 sumoylation contribute to RPA regulation in the context of DDC. Srs2 binding to PCNA is known to recruit Srs2 to replication perturbation sites, while the roles of Srs2 sumoylation were less clear (Kolesar *et al*, 2012; Moldovan *et al*, 2007; Papouli *et al*, 2005; Pfander *et al*, 2005; Saponaro *et al*, 2010). Unexpectedly, we found that Srs2 binding to PCNA and Srs2 sumoylation are functionally related, since PCNA binding promotes Srs2 sumoylation. Moreover, Srs2 sumoylation level peaks late in the response to genomic stress after the DDC kinases are hyper-activated, and this modification depends on the Mec1 checkpoint kinase. Collectively, our data support a model wherein Srs2 can distinguish between two types of ssDNA regions using PCNA proximity as a guide for subsequent sumoylation, RPA removal, and countering hyper-activated checkpoint.

Results

Srs2 regulatory features and their disabling mutants

To understand which Srs2 attributes are relevant to RPA regulation during the DNA damage response, we examined mutant alleles that perturb known features of the Srs2 protein. Srs2 was reported to contain a helicase domain at its N-terminal region and three protein-protein interaction sites at its C-terminal region (**Fig. 1A**) (Niu and Klein, 2017). We have previously shown that the Srs2 ATPase-dead allele (*srs2-K41A*) fails to downregulate RPA levels on chromatin and Mec1-mediated DDC, indicating a requirement of ATPase-driven stripping of RPA from ssDNA (Dhingra *et al*., 2021). The reported Srs2 protein-bindings sites include a Rad51 binding domain (Rad51-BD), a PCNA interaction motif (PIM), and a SUMO interaction motif (SIM) (**Fig. 1A**) (Colavito *et al*, 2009; Kolesar *et al*, 2016; Kolesar *et al*., 2012). We employed CRISPR-based editing to generate the *srs2-ΔPIM* (Δ1159-1163 amino acids) and the *srs2-SIM^{mut}* ($_{1170}$IIVID$_{1174}$ to $_{1170}$AAAAD$_{1174}$) alleles used in previous studies and examined an established *srs2-ΔRad51BD* allele (Δ875-902 amino acids) (Colavito *et al*., 2009; Kolesar *et al*., 2016; Kolesar *et al*., 2012). We note that despite Srs2 removal of RPA from ssDNA, an interaction between the two proteins has yet to be reported.

We also queried post-translational modifications reported for Srs2 by mutating the corresponding modification sites (**Fig. 1A**). First, seven Cdk1 phosphorylation consensus sites of Srs2 were mutated to produce the *srs2-7AV* (T604V, S698A, S879A, S893A, S938A, S950A, and S965A) allele (Chiolo *et al*, 2005), which was previously shown to affect homologous recombination. Second, three sumoylation sites of Srs2 were mutated to generate *srs2-3KR* (K1081R, K1089R, and K1142R) (Saponaro *et al*., 2010). Finally, we mutated two serine residues (S890 and S933) to alanine to generate *srs2-2SA*, since phosphorylation at these sites was identified in proteomic studies as dependent on both the Mec1 kinase and genotoxic treatments (Faca *et al*, 2020). Because the Srs2 C-terminal end contributes to the protein's functions (Antony *et al*, 2009; Colavito *et al*., 2009), the six mutant alleles described above were not tagged with epitopes at its C-terminus. Using an anti-Srs2 specific antibody, we showed that all six alleles supported wild-type levels of Srs2 protein based on immunoblotting (**Fig. 1B**).

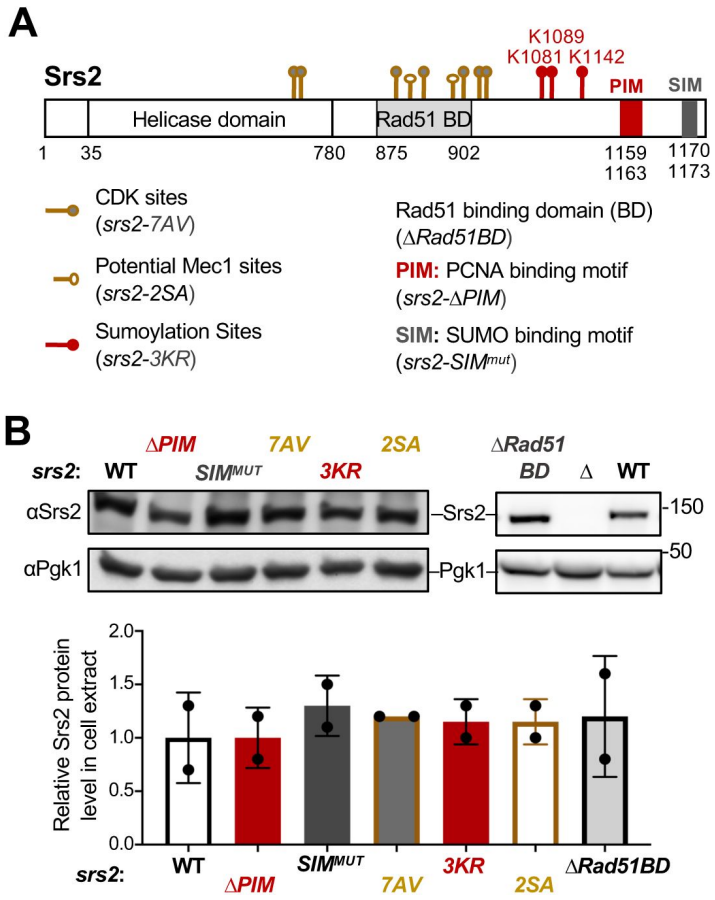

Figure 1.

Regulatory features of Srs2 and corresponding mutants.

(A) Schematic of Srs2 protein domains, regulatory features, and mutations affecting each of its features. Cdk1 phosphorylation sites depicted here include T604, S698, S879, S893, S938, S950, and S965 (Chiolo *et al*., 2005). Potential Mec1 phosphorylation sites include S890 and S933 (Albuquerque *et al*., 2015; Faca *et al*., 2020). Sumoylation sites mapped previously include K1081, K1089, and K1142 (Saponaro *et al*., 2010). Protein-interaction domains or motifs include the Rad51 binding domain (BD), PCNA Interaction Motif (PIM), and SUMO Interaction Motif (SIM) (Colavito *et al*., 2009; Kolesar *et al*., 2016; Kolesar *et al*., 2012). The residues for these domains are indicated. Mutant alleles disabling each of these features are included in the parentheses.

(B) Mutations affecting Srs2 features do not affect Srs2 protein level. Protein extracts were prepared from asynchronous cells. Srs2 was examined using anti-Srs2 antibody by immunoblotting. Srs2 levels in each mutant were first normalized to the Pgk1 loading control and then to those in wild-type cells. Mean of two biological isolates per genotype is graphed with error bars representing standard deviation (SD).

Mutating PCNA binding and sumoylation sites of Srs2 rescues *rfa1-zm2* CPT sensitivity

Srs2 loss leads to persistent DDC and perturbed recombination, both of which contribute to *srs2Δ* sensitivity to genotoxins, including CPT and methyl methanesulfonate (MMS) (**Fig. 2A**) (Niu and Klein, 2017). Significantly, our recent study has shown that RPA mutants that reduce its ssDNA binding affinity rescue the persistent DDC levels in *srs2Δ* cells but not their recombination defects (Dhingra *et al*., 2021). *rfa1-zm2* (N492D, K493R, K494R) is one of the RPA alleles examined that contains mutations at three DNA contacting residues in the Rfa1 subunit of RPA (Fan and Pavletich, 2012). Purified RPA complex containing these mutations exhibited a two-fold increase in dissociation constant using ssDNA as substrate, compared to the wild-type RPA complex (Dhingra *et al*., 2021). In cells, *rfa1-zm2* supports normal Rfa1 protein level, cell growth, DNA replication (Dhingra *et al*., 2021). It also did not affect gene conversion, direct repeat repair or rDNA recombination, nor the hyper-recombination phenotype of *srs2Δ* (Dhingra *et al*., 2021). However, *rfa1-zm2* and *srs2Δ* are mutually suppressive for both their genotoxic sensitivities and their DDC abnormalities (**Fig. 2B**, S1A) (Dhingra *et al*., 2021).

These data suggest that the *srs2* and *rfa1-zm2* genetic relationship can be used as a readout for the antagonism between Srs2 and RPA in DDC and that this functional relationship can be separable for Srs2's anti-recombination role stemming from Rad51 regulation. Based on this rationale, we employed the genetic suppression as an initial readout in querying whether any of the six *srs2* mutant alleles described above are related to RPA regulation. Given that Srs2's role in checkpoint dampening was best examined in CPT (Dhingra *et al*., 2021), which induces the DNA damage checkpoint to delay G2/M progression, but does not induce the DNA replication checkpoint, experiments were mostly carried out in this condition.

We found that on their own, *srs2-SIM^{mut}*, *-ΔPIM*, and *-7AV* showed different levels of sensitivity toward CPT at a concentration ranging from 2 to 8 μg/ml, while *srs2-ΔRad51BD* and *- 3KR* exhibited close to wild-type level of CPT resistance, which are consistent with previous reports (Colavito *et al*., 2009; Saponaro *et al*., 2010) (Fig. S1A). In addition, *srs2-2SA*, which has not been examined previously, did not show CPT sensitivity (Fig. S1A). Since *rfa1-zm2* cells exhibited stronger CPT sensitivity than the tested *srs2* alleles (Fig. S1A), we asked if the latter could suppress *rfa1-zm2* CPT sensitivity. We found that both *srs2-ΔPIM* and *-3KR* improved the CPT resistance of *rfa1-zm2* cells on media containing 2 μg/ml CPT (**Fig. 2C**).

Rescue was also seen at higher CPT concentrations, with *srs2-ΔPIM* exhibiting a stronger effect than *srs2-3KR* (Fig. S1B). In contrast to *srs2-ΔPIM* and *srs2-3KR*, *srs2-ΔRad51BD* and *-2SA* showed no effect on *rfa1-zm2* growth on media containing CPT, whereas *srs2*-7AV *and -SIM^{mut}* worsened it (**Fig. 2D**). The differential effects seen for the *srs2* alleles are consistent with the notion that Srs2 has multiple roles in cellular survival of genotoxic stress (Bronstein *et al*, 2018; Niu and Klein, 2017). The unique suppression seen for mutants affecting Srs2 binding to PCNA and its sumoylation suggests that these features are relevant to RPA regulation during the DNA damage response.

Additional suppressive effects conferred by *srs2-ΔPIM* and *-3KR* toward *rfa1* mutants

We next tested whether the observed *srs2-ΔPIM* and *-3KR* suppression was specific towards *rfa1-zm2* or applicable to other *rfa1* mutants that are also defective in ssDNA binding. While *rfa1-zm2* affects the ssDNA binding domain C (DBD-C) of Rfa1, a widely used *rfa1* allele, *rfa1- t33*, impairs the DBD-B domain (**Fig. 2E**) (Umezu *et al*, 1998). Unlike *rfa1-zm2*, *rfa1-t33* additionally decreases Rfa1 protein level and interferes with DNA replication and repair (Deng *et al*, 2014; Umezu *et al*., 1998). Despite having these additional defects, CPT survival of *rfa1-t33* cells was

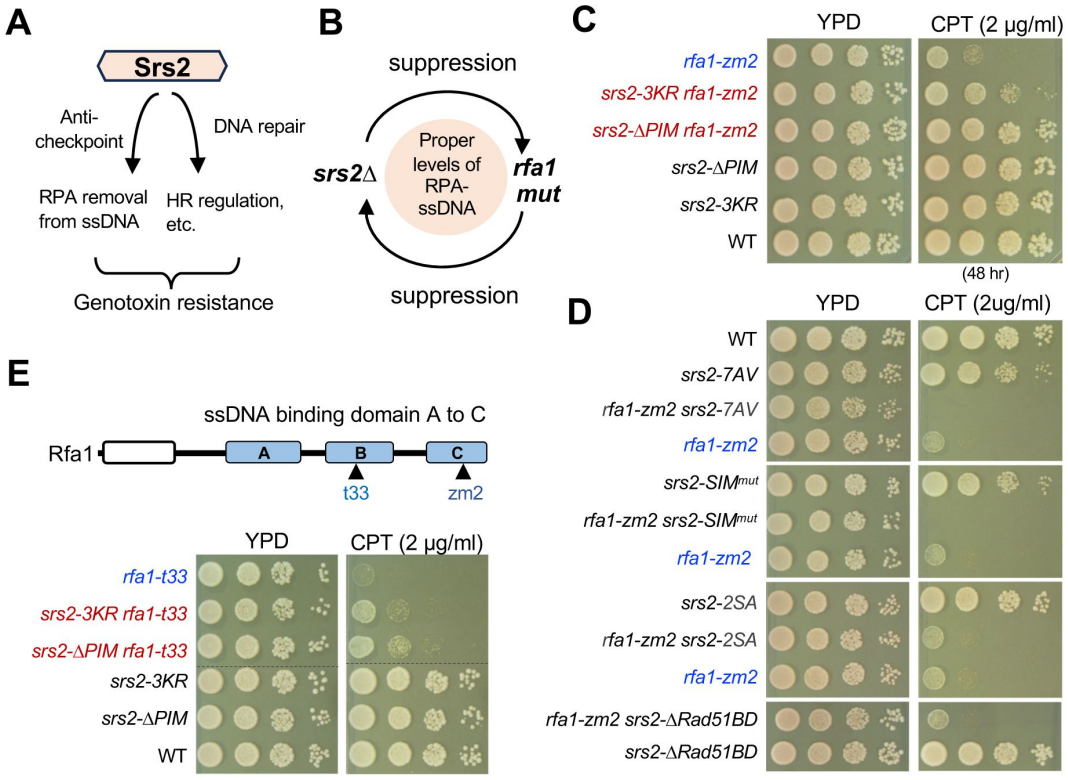

Figure 2.

Srs2 PCNA binding and sumoylation are involved in suppressing *rfa1* mutants.

(A) Schematic to show that Srs2's roles in checkpoint dampening (anti-checkpoint for simplicity) and DNA repair both contribute to genotoxin resistance.
(B) Schematic to highlight the mutual suppression between *srs2Δ* and *rfa1* mutants and the underlying effects on the RPA-ssDNA levels in cells.
(C) *srs2-ΔPIM* and *-3KR* suppress *rfa1-zm2* sensitivity to CPT. A 10-fold serial dilution of cells of the indicated genotypes were spotted and growth was assessed after incubation at 30°C for 40 hours unless otherwise noted.
(D) Several *srs2* mutants did not suppress *rfa1-zm2* sensitivity to CPT. Experiments were performed as in panel 2C.
(E) *srs2-ΔPIM* and *-3KR* suppress *rfa1-t33* sensitivity to CPT. Top: schematic of the Rfa1 protein domains and *rfa1* mutant alleles. Bottom: *srs2-ΔPIM* and *-3KR* improved *CPT* sensitivity of *rfa1-t33* cells. Experiments were performed as in panel 2C.

also improved by *srs2-ΔPIM* or *srs2-3KR* (**Fig. 2E**). As seen in the case of *rfa1-zm2*, *srs2-ΔPIM* conferred better rescue than *srs2-3KR* toward *rfa1-t33* (**Fig. 2E**). When comparing the two *rfa1* alleles, *srs2* rescue was stronger toward *rfa1-zm2*, likely because *rfa1-t33* has other defects besides reduced ssDNA binding (**Fig. 2C**, 2E). In either case, *srs2* mutants only conferred partial suppression, consistent with the notion that Srs2 has roles beyond RPA regulation (Bronstein *et al*., 2018; Niu and Klein, 2017). Nevertheless, the observed rescue of both *rfa1-zm2* and *rfa1-t33* provided strong genetic evidence that Srs2 binding to PCNA and sumoylation are involved the regulation of RPA during the DNA damage response.

Srs2 regulation of RPA during DDC is not only observed under CPT treatment, but is also seen in treatment with MMS (Dhingra *et al*., 2021), which induces both DDS and the DNA replication checkpoint and thus delaying both S phase progression and G2/M phase exit (Paciotti *et al*, 2000; Tercero *et al*, 2003). Consistent with observation made in CPT conditions, *srs2-ΔPIM* moderately improved the MMS sensitivity of *rfa1-zm2* and *rfa1-t33*, while *srs2-3KR* had a small effect on the MMS sensitivity of *rfa1-zm2* (Fig. S1B, S1C). The degrees of suppression seen in MMS treatment were less than those observed in CPT conditions, and we address likely reasons for this difference in Discussion. Collectively, the multiple genetic results are consistent among themselves and provide evidence that the roles of Srs2 binding to PCNA and sumoylation in RPA antagonism can be applicable to more than one genomic stress situation.

srs2-ΔPIM and *-3KR* rescue checkpoint abnormality of *rfa1-zm2* cells

We next investigated whether *srs2-ΔPIM* and *-3KR* suppression of the DNA damage sensitivity of *rfa1-zm2* cells pertained to DDC regulation. We employed two readouts for DDC that are widely used. First, we used the F9 antibody, which specifically recognizes phosphorylated Rad53, to measure the levels of activated Rad53 (Fiorani *et al*, 2008). Second, we measure cellular ability to timely exit the G2/M phase after CPT treatment using FACS analyses. We note that CPT-induced DDC leads to G2/M delay rather than an arrest as shown in previous studies (Dhingra *et al*., 2021; Menin *et al*, 2018; Redon *et al*, 2003). We have recently reported that *srs2Δ* cells exhibit increased levels of phosphorylated Rad53 and diminished ability to timely exit G2/M phase in CPT, and that both are suppressed by *rfa1-zm2* (Dhingra *et al*., 2021; Menin *et al*., 2018; Redon *et al*., 2003). Interestingly, *rfa1-zm2* leads to similar defects and these are rescued by *srs2Δ* (Dhingra *et al*., 2021) (**Fig. 3A**). The mutual suppression seen in these assays is consistent with that seen for CPT sensitivity (Dhingra *et al*., 2021). We note that several other RPA ssDNA binding mutants, such as *rfa1-t33*, also exhibit phenotypes indicative of increased DDC levels (Pellicioli *et al*., 2001; Seeber *et al*, 2016; Smith and Rothstein, 1995; Zou and Elledge, 2003). This is likely because compromised ssDNA protection can lead to increased levels of DNA lesions thus enhancing the activation of Mec1 and/or its homolog Tel1.

We reasoned that if *srs2-ΔPIM* and *srs2-3KR* compromise Srs2's ability to remove RPA from DNA in cells, they should alleviate *rfa1-zm2* cells' defects in ssDNA protection and the associated DDC abnormalities (**Fig. 3A**). Indeed, while *rfa1-zm2* exhibited about 5-fold increase in Rad53 phosphorylation levels in CPT compared with wild-type cells as seen before (Dhingra *et al*., 2021), *srs2-ΔPIM* and *-3KR* reduced this level by about 50% and 80%, respectively (**Fig. 3B**). Similar to earlier data, we observed that wild-type cells arrested in G_2/M after 1 hour of CPT treatment and about 25% of these cells entered G_1 after another hour (**Fig. 3C**) (Dhingra *et al*., 2021). The ability to timely exit from G_2/M phase was reduced in *rfa1-zm2* cells so that only 13% cells moved to G1 phase after 2 h treatment with CPT, as seen before (**Fig. 3C**) (Dhingra *et al*., 2021). Significantly, *srs2-ΔPIM* and *-3KR* increased the percentage of *rfa1-zm2* cells transitioning into the G1 phase (**Fig. 3C**). The suppressive effects seen in both assays were stronger for *srs2-ΔPIM* than *srs2-3KR* as seen in genotoxicity assays (Fig. S1B; 3B, 3C).

Collectively, the genetic and DDC assay data are consistent with each other and suggest that Srs2 binding to PCNA and its sumoylation contribute to RPA antagonism in DDC regulation.

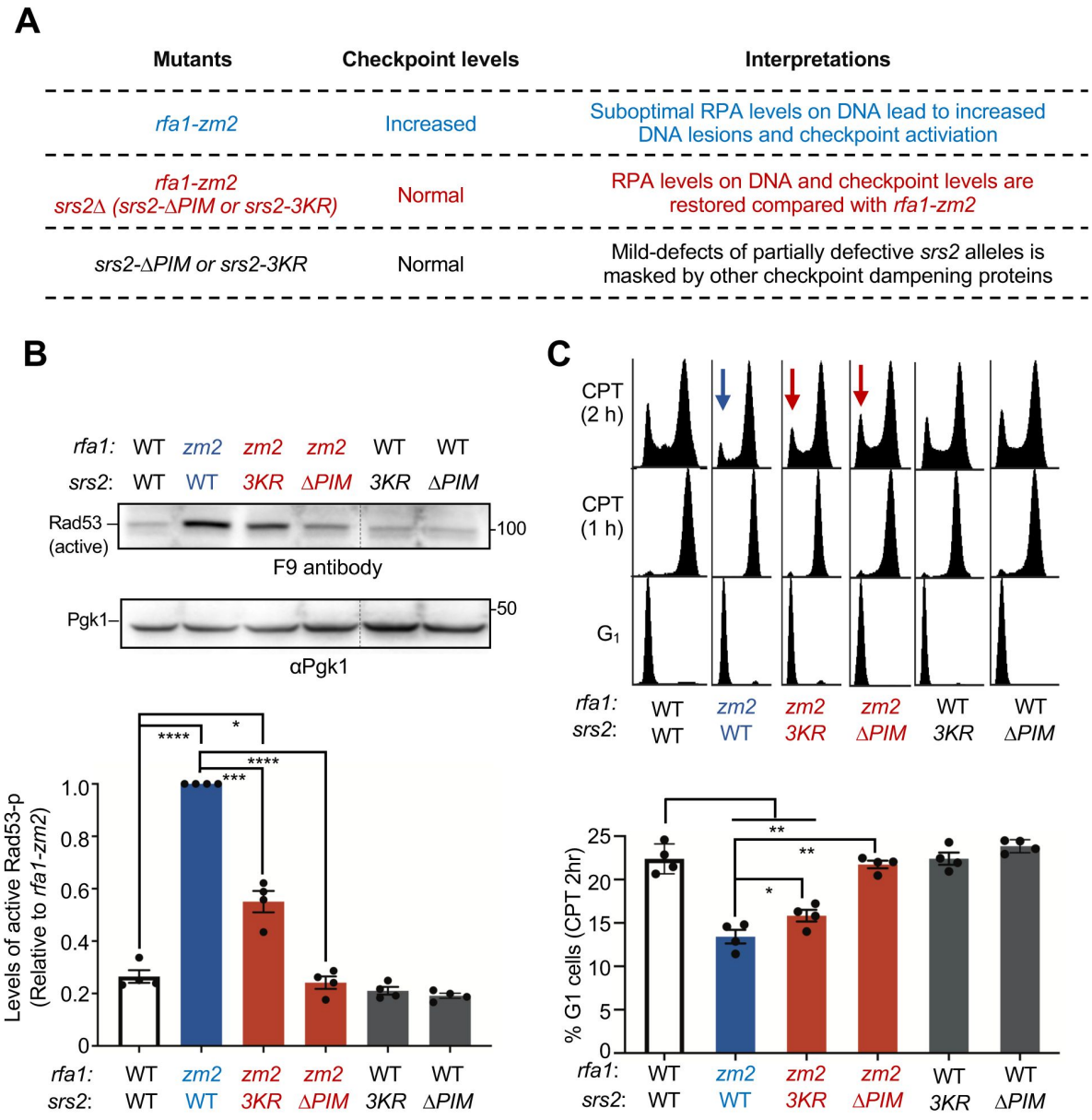

Figure 3.

srs2-ΔPIM and *-3KR* correct checkpoint abnormalities in *rfa1-zm2* cells.

(A) A table summarizing DDC levels observed in *rfa1-zm2*, *srs2* null or partially defective mutants, and their combined mutants in this work and in a previous study (Dhingra *et al.*, 2021) and interpretations.

(B) *srs2-ΔPIM* and *-3KR* reduce the levels of phosphorylated Rad53 in *rfa1-zm2* cells. Protein extracts were examined after G1 cells were released into cycling in the presence of 16 ug/ml CPT for 2 hours. Phosphorylated Rad53 (active form) was detected by the F9 antibody by immunoblotting. Phosphorylated Rad53 signals in each indicated strain were compared to the Pgk1 loading control and normalized to those of *rfa1-zm2*. Two biological isolates per genotype were examined in two technical repeats and results are graphed with error bars representing SD. Statistical analysis was performed using pairwise Student's t-test. *, p <0.05; ***, p <0.001; ****, p <0.0001.

(C) *srs2-ΔPIM* and *-3KR* allow better G1 entry of *rfa1-zm2* cells. Experiments were performed as in panel A and samples were collected at indicated time points. FACS profiles of the samples are shown at the top and percentages of G1 cells after 2 hours of CPT treatment (CPT 2 h) are plotted at the bottom. Two biological isolates per genotype were examined in two technique repeats and results are graphed with error bars representing SD. Statistical analysis was performed using pairwise Student's t-test. *, p <0.05; **, p <0.01.

srs2-ΔPIM is additive with a *slx4* mutant defective in DDC recovery

We found that *srs2-ΔPIM* and *srs2-3KR* mutants on their own behaved normally in the two DDC assays described above, which differs from *srs2Δ* cells that exhibit increased levels of Rad53 phosphorylation and reduced G1 entry (**Fig. 3B**, 3C) (Dhingra *et al*., 2021). One plausible interpretation of this difference is that unlike *srs2*Δ, these partially defective *srs2* mutants only mildly impair Srs2-mediated RPA regulation during DDC downregulation, such that their defects can be compensated for by other DDC dampening systems. To test this idea, we examined the consequences of removing the Slx4-mediated anti-checkpoint function in *srs2-ΔPIM* cells. Slx4 is known to bind the scaffold protein Rtt107, and their complex can compete with the DDC protein Rad9 for binding to damaged chromatin, consequently disfavoring Rad9's role in promoting Rad53 phosphorylation (Ohouo *et al*, 2010; Ohouo *et al*, 2013). Slx4 uses an Rtt107 interaction motif (RIM) to bind Rtt107 and a previously established RIM mutant, $slx4^{RIM}$ (T423A, T424A, and S567A), abolishes Rtt107 binding without affecting other Slx4 protein attributes (Wan *et al*, 2019). $slx4^{RIM}$ was tagged with a TAP tag at its C-terminal end and examined together with a control wherein the wild-type Slx4 was tagged in the same manner. Compared with the control, $slx4^{RIM}$ exhibited mild sensitivity toward CPT (**Fig. 4A**). Significantly, when *srs2-ΔPIM* was combined with $slx4^{RIM}$, the double mutant exhibited stronger CPT sensitivity compared with corresponding single mutants (**Fig. 4A**).

We further examined DDC levels when cells were exposed to CPT using both Rad53 phosphorylation and ability to timely exit G2/M as readouts. Compared with *Slx4-TAP* cells, $slx4^{RIM}$*-TAP* cells exhibited 2-fold increase in Rad53 phosphorylation level, consistent with a role of Slx4 in downregulating DDC (**Fig. 4B**). In *Slx4-TAP* background, *srs2-ΔPIM* led to 3-fold increase in Rad53 phosphorylation level (**Fig. 4B**). As cells containing untagged Slx4 and *srs2-ΔPIM* did not show Rad53 phosphorylation changes (**Fig. 3B**), tagging Slx4 likely sensitizes *srs2-ΔPIM*. Significantly, the double mutant of *srs2-ΔPIM* and $slx4^{RIM}$*-TAP* exhibited further increase in Rad53 phosphorylation (~5.5 fold) compared with *srs2-ΔPIM* and $slx4^{RIM}$*-TAP* single mutant (**Fig. 4B**). Congruous with this finding, the *srs2-ΔPIM* $slx4^{RIM}$*-TAP* double mutant also reduced the percentage of cells entering G_1 compared with their single mutants after 2-hour treatment with CPT (**Fig. 4C**). Together, these three lines of evidence support the notion that defects in the Srs2-mediated DDC downregulation can be compensated for by Slx4-mediated mechanisms.

Srs2 binding to PCNA facilitates Srs2 sumoylation

We next addressed whether the two Srs2 features involved in RPA antagonism are functionally related. To this end, we tested if Srs2 binding to PCNA affected Srs2 sumoylation in cells. We confirmed that endogenous sumoylation of Srs2 could be detected in cell extracts after enriching SUMOylated proteins using 8His-tagged SUMO (8His-Smt3) (**Fig. 5A**). Briefly, proteins were extracted from cells under denaturing conditions to disrupt protein-protein association and prevent desumoylation, and sumoylated proteins were enriched on Ni-NTA (nickel-nitrilotriacetic acid) resin (Ulrich and Davies, 2009). Consistent with previous findings, sumoylated forms of Srs2 in the Ni-NTA eluate were detected on immunoblots using an anti- Srs2 antibody only when cells contained 8His-Smt3, where unmodified Srs2 was seen in the presence or absence of 8His-Smt3 due to nonspecific binding to the resin (Fig. S2A) (Saponaro *et al*., 2010). These bands were only seen in wild-type cells but not *srs2Δ* cells (Fig. S2A). We found that Srs2 sumoylation level was reduced in *srs2-3KR* strains in CPT conditions, as expected from mutating the three major sumoylation sites (**Fig. 5A**) (Saponaro *et al*., 2010).

Interestingly, *srs2-ΔPIM* cells exhibited a similar reduction in Srs2 sumoylation as seen in *srs2-3KR* cells (**Fig. 5A**). This result suggests that Srs2 sumoylation largely depends on Srs2 binding to PCNA, supporting the notion that the two events occur sequentially.

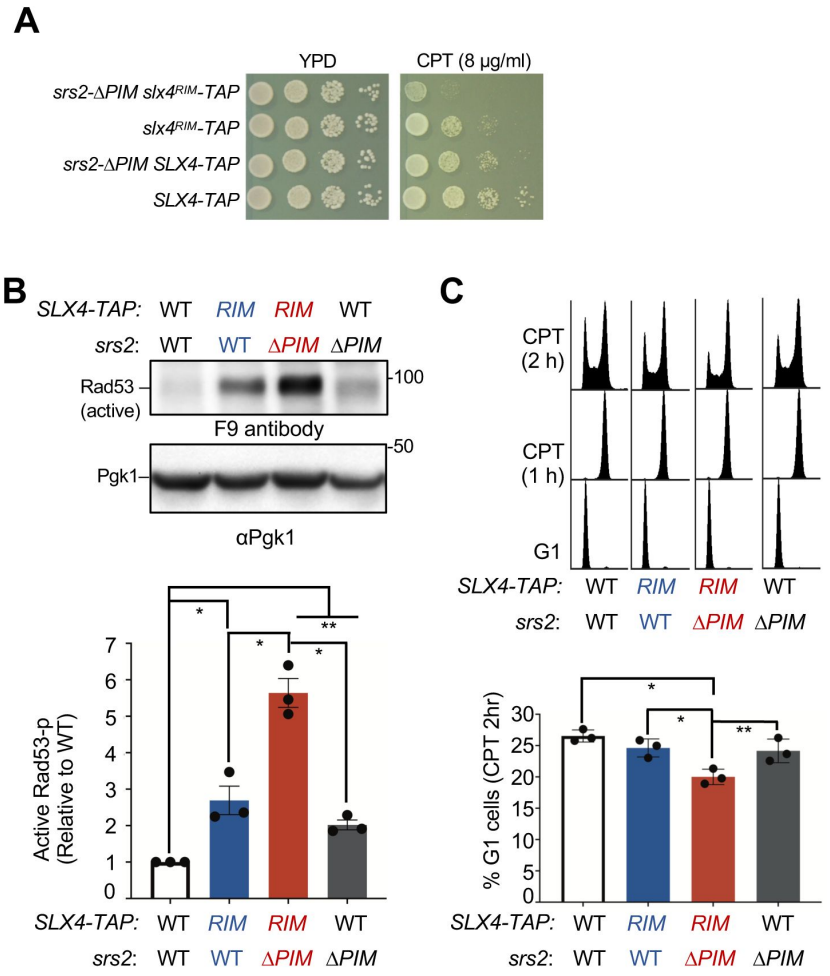

Figure 4.

srs2-ΔPIM and *slx4^{RIM}* are additively defective in DDC down-regulation.

(A) The *srs2-ΔPIMΔ slx4^{RIM}* double mutant shows stronger CPT sensitivity than either single mutant. Experiments are performed as in **Figure 2C**.
(B) *srs2-ΔPIM* and *slx4^{RIM}* are additive for increasing the level of phosphorylated Rad53 when cells are treated with CPT. Experiments were conducted and signals are quantified as those described in **Figure 3B**. Results from three biological isolates per genotype are graphed with error bars representing SD. Statistical analysis was performed using pairwise Student's t- test. *, p <0.05; **, p <0.01.
(C) *srs2-ΔPIM* and *slx4^{RIM}* are additive for reducing cells exiting from G2/M arrest at 2 hours post CPT treatment. Experiments were conducted and signals are quantified as those described in **Figure 3C**. Results from three biological isolates per genotype are graphed with error bars representing SD. Statistical analysis was performed using pairwise Student's t- test. *, p <0.05; **, p <0.01.

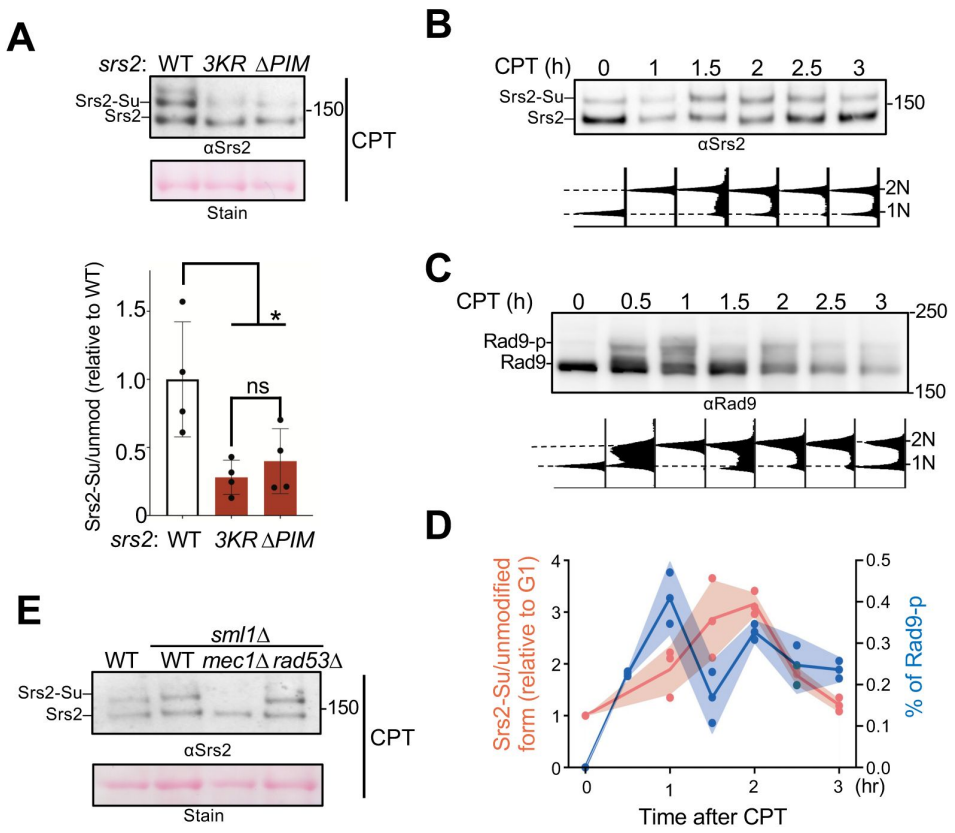

Figure 5.

Srs2 sumoylation depends on Srs2 PIM motif and the Mec1 kinase.

(A) Srs2 sumoylation level is reduced in *srs2-ΔPIM* cells. Samples were collected after CPT treatment as in **Figure 3A** and sumoylated proteins were enriched as described in the text and methods. Sumoylated form of Srs2 (Srs2-Su) migrated slower than unmodified Srs2 on gel and both were detected using anti-Srs2 antibody in immunoblotting. Equal loading is indicated by Ponceau-S stain (Stain). The levels of sumoylated Srs2 relative to those of unmodified Srs2 were normalized to wild-type cells and plotted. Two biological isolates per genotype were examined in two technique repeats and results are graphed with error bars representing SD. Statistical analysis was performed using pairwise Student's t-test. ns, not significant; *, p <0.05.
(B) Srs2 sumoylation level during CPT treatment. Samples were collected as described in **Figure 3B**. Experiments were conducted, and immunoblotting data is presented as those in panel A. Time 0 samples are G1 cells before CPT treatment. FACS analysis at corresponding time points are included below the plot.
(C) Rad9 phosphorylation level during CPT treatment. Samples used in panel B were examined for Rad9 phosphorylation using an anti-Rad9 antibody. Unmodified and phosphorylated (Rad9-p) Rad9 forms are indicated. FACS analysis at corresponding time points are indicated below blots.
(D) Quantification of Srs2 sumoylation and Rad9 phosphorylation level changes during CPT treatment. Srs2 sumoylation signals were normalized based on unmodified form. Values at each timepoint were relative to that of G1 sample (Time 0) before CPT treatment, and fold changes were indicated on left y-axis. Rad9 signals were normalized to non-modified form and percentage of phosphorylated form were indicated on right y-axis. Results from three biological isolates per genotype are graphed.
(E) Mec1 is required for Srs2 sumoylation. Experiments were performed and data are presented as in panel A.

Srs2 sumoylation peaks after maximal Mec1 activation and depends on Mec1

While RPA can engage with ssDNA generated in different processes, such as transcription, DNA repair, and DNA replication, only a subset of ssDNA regions is adjacent to 3'-junctions loaded with PCNA. This is because PCNA loading requires an ssDNA and dsDNA junction site with a 3' DNA end and occurs mainly during S phase (Moldovan *et al*., 2007). We thus reasoned that Srs2 binding to PCNA may prevent Srs2 from removing RPA bound to ssDNA that forms transiently or is not flanked by PCNA, such as within R-loop regions and negatively supercoiling DNA regions, both of which are generated abundantly in CPT conditions due to topological stress (Pommier *et al*, 2022). This could provide a means for spatial control of Srs2's anti-RPA role. We explored whether this role could also be regulated temporally. As the genetic data described above suggest that Srs2 sumoylation acts downstream of Srs2 binding to PCNA in antagonizing RPA, we examined the timing of Srs2 sumoylation after CPT treatment.

We released G1 synchronized cells into media containing CPT and examined Srs2 sumoylation at several time points (**Fig. 5B**). A similar experimental scheme was used to monitor the level of Mec1 activation using Rad9 phosphorylation as a readout (**Fig. 5C**).

Quantification of Srs2 sumoylated forms relative to its unmodified forms showed that its sumoylation level peaked between 1.5 and 2 h after cells were released into CPT (**Fig. 5D**). Quantification of phosphorylated forms of Rad9 relative to its unmodified form showed that checkpoint activation peaked 1 hour after cell release (**Fig. 5D**). That checkpoint level peaked before Srs2 sumoylation level peaked suggests a possible dependence of the latter on the checkpoint. In testing this idea, we found that *mec1Δ* cells, which contain *sml1Δ* to support viability (Zhao *et al*, 1998), abolished Srs2 sumoylation (**Fig. 5E**). Neither *sml1Δ* alone nor removal of Rad53 affected Srs2 sumoylation (**Fig. 5E**), suggesting that Mec1's effect on this modification does not require the downstream Rad53 effector kinase. As Mec1 is not generally required for protein sumoylation during the DNA damage response (Cremona *et al*, 2012), its involvement in Srs2 sumoylation presents a rather unique effect.

Examination of Mec1-S1964 phosphorylation in Srs2-mediated DDC regulation

The combined results that Srs2 sumoylation levels peak after Rad9 phosphorylation reaches maximal level and that Srs2 sumoylation depends on Mec1 raised the possibility that Mec1 hyper-activation during the late part of the DNA damage response may favor Srs2's role in DDC dampening. Recently, Mec1 protein bound to Ddc2 was shown to be auto-phosphorylated at Ser1964 in a late part of the DNA damage response after a single DNA break is induced by the HO endonuclease (Memisoglu *et al*, 2019). Further, the phosphorylation-defective mutant *mec1-S1964A* impaired checkpoint downregulation in this condition, leading to the model that Mec1-S1964 phosphorylation contributes to DDC dampening at least after HO-induced break (Memisoglu *et al*., 2019). We thus tested whether Mec1-S1964 phosphorylation is also involved in Srs2-mediated DDC regulation under CPT conditions.

First, we asked whether Mec1-S1964 phosphorylation occurs under CPT conditions using a time course scheme similar to the one used in testing Srs2 sumoylation as described above. Ddc2-bound Mec1 was recovered after immunoprecipitating Ddc2 at each timepoint and the proteins were examined by immunoblotting. To detect Mec1-S1964 phosphorylation, we used an antibody raised against the peptide containing this modification provided kindly by the Haber lab (Memisoglu *et al*., 2019). The antibody detected the Mec1 S1964 phosphorylation band in immunoprecipitated fractions only when Ddc2 was tagged with the Myc tag but not when Ddc2 was untagged (Fig. S2B). Further, the Mec1 S1964 phosphorylation band was largely abolished in the *mec1-S1964A* cells.

(Fig. S2B; 6A). Given that the antibody also detects a closely spaced background band (Fig. S2B; 6A), we calculated the relative level of Mec1 S1964 phosphorylation in each sample by normalizing to this band. Quantification results showed that Mec1 S1964 phosphorylation level peaked around the same time as seen for Rad9 phosphorylation, which was 1 hour after CPT treatment (**Fig. 6A**).

After confirming that Mec1 S1964 phosphorylation also took place under CPT conditions, we tested whether the *mec1-S1964A* or the phosphorylation-mimetic mutant, *mec1- S1964E*, influenced *rfa1-zm2* or *srs2* growth on media containing CPT. If Mec1 S1964 phosphorylation aids Srs2-mediated RPA antagonism in CPT, we would expect *mec1-S1964A* to behave similarly to *srs2* mutants in rescuing *rfa1-zm2* CPT sensitivity, while *mec1-S1964E* should have the opposite effects. We found that *mec1-S1964A,* which showed normal CPT resistance on its own, did not affect the growth of *rfa1-zm2* cells on CPT-containing media (**Fig. 6B**). *mec1-S1964A* also did not influence the growth of *srs2-ΔPIM* and *srs2-3KR* cells regardless of *rfa1-zm2* (**Fig. 6B**; S3A).

Though *mec1-S1964E* on its own did not cause CPT sensitivity, it led to slight but reproducible slow growth in cells containing *srs2-3KR* and *srs2-ΔPIM* with or without *rfa1-zm2* (Fig. S3B). We found that neither *mec1-S1964A* nor *mec1-S1964E* affected Srs2 sumoylation levels (Fig. S3C). Thus, Mec1, but not its S1964 phosphorylation, is required for Srs2 sumoylation. These data provide evidence that even though an HO-induced DNA break and CPT treatment both induce Mec1 S1964 phosphorylation, this modification appears to not affect Srs2-mediated DDC dampening in the latter situation.

Genetic data suggest that known RPA phosphorylation sites do not affect DDC recovery

While we mainly focused on the identification of Srs2 features that contribute to RPA antagonism during DDC regulation, we also considered the possibility that certain regulatory RPA features may contribute to this control as well. In particular, RPA is known to be phosphorylated by Mec1 (Chen *et al*, 2010; Faca *et al*., 2020; Lanz *et al*, 2021). We thus tested whether Mec1-mediated phosphorylation may render RPA more susceptible to be removed from ssDNA by Srs2. To this end, we mutated previously identified Mec1 phosphorylation sites on the Rfa1 and Rfa2 subunits. These include Rfa1 Ser178 and three sites on Rfa2 (S122, S187, S189) (Albuquerque *et al*, 2015; Chen *et al*., 2010). We generated corresponding non- phosphorylatable mutants, *rfa1-S178A* and *rfa2-3SA (*S122A, S187A, and S189A), and phosphomimetic mutants, *rfa1-S178D* and *rfa2-3SD*.

Using genetic suppression as a readout for possible involvement of RPA mutants in Srs2-based DDC regulation, we examined the interactions between the mutants affecting RPA phosphorylation sites as described above and *srs2*Δ. If RPA phosphorylation promote its removal by Srs2, the *SD* mutants may show suppressive interactions with *srs2*Δ, while *SA* mutants would behave like *srs2* mutants. We found that neither *rfa1-S178D* nor *S178A*, both of which showed normal CPT resistance on their own, affected *srs2*Δ cell growth on CPT- containing media (**Fig. 6C**). In contrast, *rfa2-3SD* or *rfa2-3SA*, which also behaved like wild-type regarding CPT sensitivity, sensitized *srs2Δ* growth on CPT-containing media (**Fig. 6D**). This negative genetic interaction contrasts with the positive interaction seen between *srs2*Δ and *rfa1- zm2* or *rfa1-t33* (Fig. S1A) (Dhingra *et al*., 2021). The differential Srs2 genetic interactions among distinct RPA alleles likely reflect the multifunctional natures of Srs2 and RPA. Collectively, these analyses suggest that the known phosphorylation sites on RPA are unlikely to be involved in Srs2-based DDC regulation.

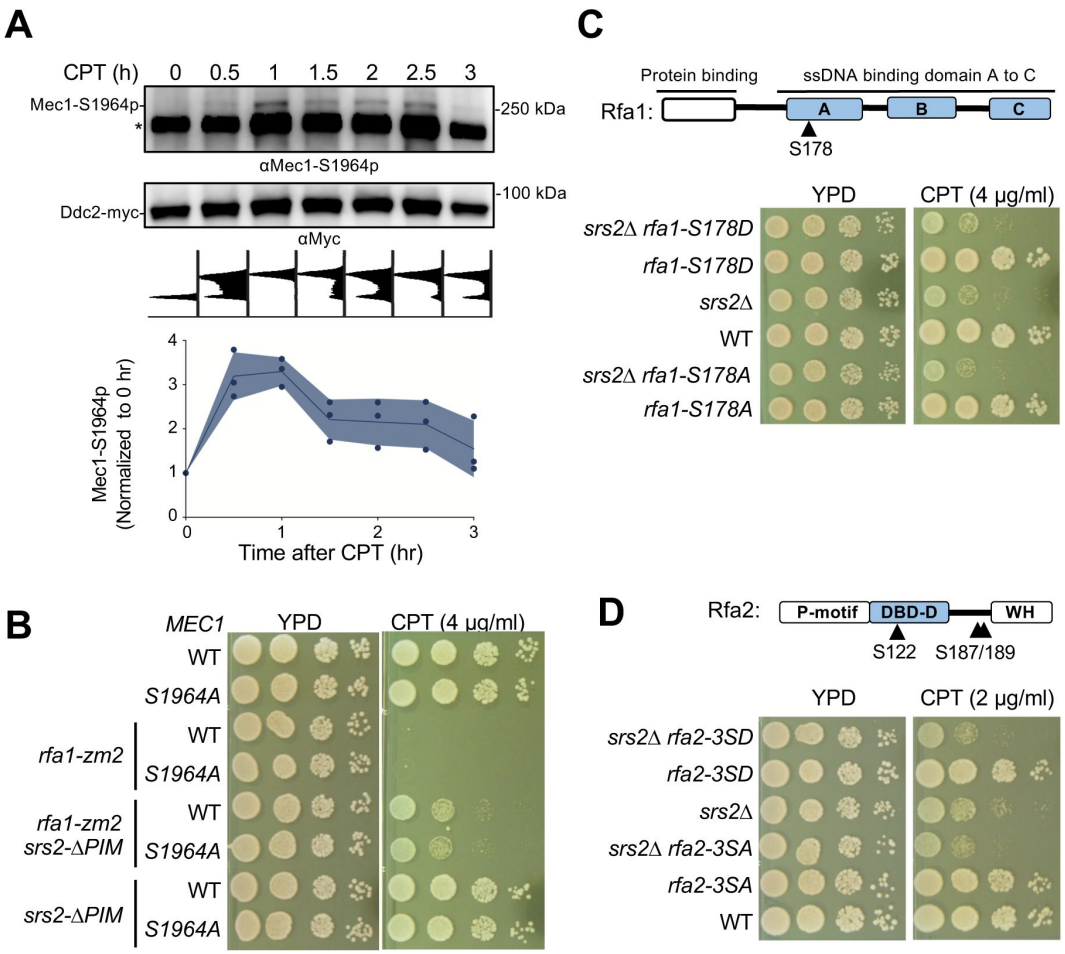

Figure 6.

Mec1 and RPA phosphorylation did not link to Srs2 anti-checkpoint function.

(A) Mec1 autophosphorylation at S1964 during CPT treatment. Samples were collected as described in **Figure 3B**. Ddc2-myc was immunoprecipitated and the associated Mec1 was co-purified. The anti-Mec1-S1964-p antibody detected the phosphorylated form of Mec1 and a non-specific band (*). Quantification below the blot was based on results from two biological isolates.
(B) *mec1-S1964A* did not rescue *rfa1-zm2* sensitivity toward CPT. Experiments were performed as in **Figure 2C**.
(C) Examination of the *rfa1-S178A* and *-S178D* mutants. Top: schematic of the Rfa1 protein domains and position of Ser178. ssDNA binding domain (DBD) A-C are shown. Bottom, *rfa1-S178A* and *-S178D* had no effect on *srs2Δ*'s sensitivity to CPT. Experiments were performed as in **Figure 2C**.
(D) Examination of the *rfa2-3SA* and *-3SD* mutants. Top, schematic of the Rfa2 protein domains and position of Ser122, Ser187 and Ser189 mutated in *rfa2-3SA* and *-3SD*. ssDNA binding domain (DBD)-D is indicated. Bottom, *rfa2-3SA* has no effect on *srs2Δ*'s sensitivity toward CPT while *rfa2-3SD* showed additive effect. Experiments were performed as in **Figure 2C**.

Discussion

Timely termination of DDC is crucial for cell survival, yet the underlying mechanisms are not well understood. Using the budding yeast model system, we investigated how the Srs2-mediated DDC dampening through antagonizing RPA can be regulated. Our previous work reports a requirement of Srs2's ATPase activity in this role; here we examined additional Srs2 features involved in either binding to other proteins or post-translational modifications. Our results suggest that among the examined features, Srs2 binding to PCNA and its sumoylation are involved in RPA antagonism during DDC downregulation. We showed that mutants impairing these features, namely *srs2Δ-PIM* and *srs2-3KR*, suppressed both DDC abnormalities and CPT sensitivity of *rfa1-zm2* cells. Interestingly, these features are functionally linked, since optimal Srs2 sumoylation requires its binding to PCNA. Further, the Srs2 sumoylation level peaks after Mec1 activity reaches its maximum and that Mec1 is required for this modification. We posit a model that can interpret previous and current data. In this model, Srs2 removal of RPA is favored at a subset of ssDNA regions that have proximal PCNA (**Fig. 7**, top). PCNA recruits Srs2 toward the adjacent RPA-ssDNA filament, and promotes Srs2 sumoylation together with hyperactive Mec1, and both effects foster downregulation of the Mec1 checkpoint (**Fig. 7**, bottom).

RPA-ssDNA filaments can be separated into two groups based on PCNA loading status

The first group contains 3' DNA end at the ss-dsDNA junction to permit PCNA loading (**Fig. 7**, top, green). These regions can include ssDNA gaps produced from perturbed replication (Sogo *et al*, 2002)or from excision and repair of trapped Top1 (Sun *et al*, 2020). Due to PCNA proximity, this group of ssDNA regions can favor Srs2 recruitment and RPA removal. Since they often also contain 5' DNA ends required for loading the 9-1-1 complex (Majka *et al*, 2006), these sites can be ideal for Srs2's action in checkpoint dampening. The second group of ssDNA regions are devoid of 3' DNA end thus PCNA loading. These regions can be within R-loops or negatively supercoiled regions, which are induced by CPT treatment due to increased topological stress associated with the depletion of the functional pool of Top1 (Pommier *et al*., 2022). These regions are unlikely to contain 5' DNA ends at ss-dsDNA junctions; thus RPA there mainly supports DNA protection and repair and not DDC. We reason that these ssDNA sites can be shielded from Srs2 removal of RPA to allow better repair and protection. The notion that ssDNA regions close to PCNA are preferred for Srs2 anti-checkpoint action provides a rationale for how Srs2 could remove RPA at some sites and minimize unnecessary RPA loss from other sites. Quantitative assessment of RPA residence on DNA and genome-wide mapping of Srs2 action sites in the future can further test the proposed models and provide more clarity. Regardless, results presented in this work support the conclusion that Srs2 can distinguish between two types of ssDNA regions using PCNA proximity as a guide for RPA removal.

Genetic analysis showed that *srs2Δ-PIM* also suppressed *rfa1-zm2* sensitivity toward MMS, though to a less degree as seen in CPT conditions. While more than one factor could contribute to this difference, we note that CPT only activates the DNA damage checkpoint, while MMS additionally induces DNA replication checkpoint (Menin *et al*., 2018; Redon *et al*., 2003; Tercero *et al*., 2003). It is thus possible that the Srs2-RPA antagonism is more important for the DNA damage checkpoint compared with the DNA replication checkpoint. Further investigation of this possibility among others will shed light on differential suppressive effects seen here.

An involvement of PCNA binding in Srs2-based RPA regulation could compensate for a lack of Srs2 interaction with RPA. This scenario differs from Srs2-based Rad51 stripping that requires their interaction (Antony *et al*., 2009; Colavito *et al*., 2009; Krejci *et al*, 2003). It is thus likely that

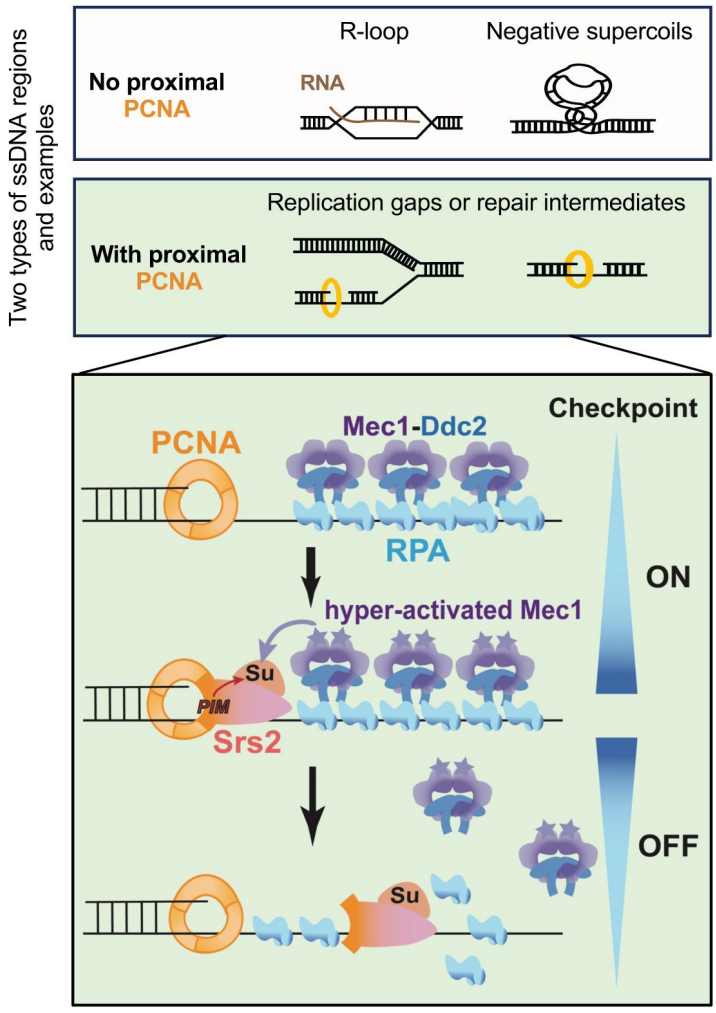

Figure 7.

A working model for the regulation of Srs2-mediated checkpoint dampening.

RPA-coated ssDNA regions generated from multiple DNA metabolic processes include those without proximal PCNA, such as ssDNA within R-loops and negatively supercoiled regions, and those with proximal PCNA, such as ssDNA gaps associated with perturbed DNA replication or repair. In latter situations, the resultant RPA-ssDNA can recruit the Mec1/Ddc2 kinase complex. Mec1 is subsequently activated by other DDC proteins, such as the 9-1-1 complex that demarcates the 5′ end junctions adjacent to ssDNA regions (not drawn). After DDC is activated for a prolonged period, Srs2 recruited to PCNA that demarcates the 3′ end junction flanking the ssDNA regions can be sumoylated. Srs2 sumoylation level increases as Mec1 activation heightens and can result in more efficient RPA removal, leading to Mec1 downregulation.

though both the anti-recombinase and anti-checkpoint roles of Srs2 benefit from selectivity, they are achieved via different means. Our previous work has provided several lines of evidence to support that Rad51 removal by Srs2 is separable from the Srs2-RPA antagonism (Dhingra *et al*., 2021). Consistent with this conclusion, deleting the Rad51 binding domain in Srs2 had no effect on *rfa1-zm2* phenotype in CPT (**Fig. 2D**). This is in sharp contrast to mutating the PCNA binding and the sumoylation sites of Srs2, which suppressed *rfa1-zm2* defects (**Fig. 2C**). This data provides additional evidence that Srs2 regulation of Rad51 is separable from the Srs2-RPA antagonism. For this reason, the Srs2-based Rad51 function was not further examined in this study. However, future studies to address how Srs2 regulates RPA and Rad51 in different manners and whether there is a crosstalk between them in specific contexts will help to generate a more comprehensive understanding of the Srs2's anti-checkpoint vs. anti- recombination functions.

Previous studies have suggested that both PIM and SIM aid Srs2 recruitment to sumoylated form of PCNA (Papouli *et al*., 2005; Pfander *et al*., 2005). Our data reveal that at least in CPT conditions, Srs2-PIM, but not Srs2-SIM, is involved in RPA regulation. This observation is congruent with phenotypic differences between Srs2's PIM and SIM seen in other studies and here, such as much stronger genotoxic sensitivity of the latter (Fig. S1A) (Fan *et al*, 2023; Kolesar *et al*., 2016; Kolesar *et al*., 2012). Considering that only a small percentage of PCNA is sumoylated and mainly during S phase (Papouli *et al*., 2005; Pfander *et al*., 2005), it is probably advantageous for Srs2 not being restricted to sumoylated PCNA in order to achieve efficient RPA regulation.

How could Srs2 binding to PCNA helps Srs2 sumoylation? While a full answer awaits future studies, we speculate that this interaction may render Srs2 more permissive for sumoylation and/or position Srs2 in proximity with its sumoylation E3 ligase Siz2 that binds RPA (Chung and Zhao, 2015; Kolesar *et al*., 2012). Considering that Mec1, Ddc2, and RPA contain SUMO interaction motifs (Psakhye and Jentsch, 2012), it is possible that Srs2 sumoylation could help enrich the helicase at ssDNA-RPA sites via interacting with these motifs, though other effects are also possible. Compared with *srs2-3KR*, *srs2-ΔPIM* showed better suppression of *rfa1-zm2* defects. This indicates that during RPA regulation, PCNA plays additional roles besides promoting Srs2 sumoylation.

We found that Srs2 sumoylation in cells also requires Mec1, which does not promote sumoylation in general (Cremona *et al*., 2012). On the contrary, Mec1 loss causes increased sumoylation levels (Cremona *et al*., 2012). It is currently unclear how Mec1 can specifically promote Srs2 sumoylation, but this may involve Mec1-mediated phosphorylation of SUMO substrates and/or enzymes. A requirement of Mec1 for Srs2 sumoylation and the dynamic nature of sumoylation suggest that Srs2 sumoylation may be a potential timing regulator. It is possible that hyperactivation of Mec1 favors Srs2 sumoylation that may aid its own downregulation. While fully testing this idea requires time-course phosphor-proteomic studies under genotoxin treatment, we examined a Mec1 auto-phosphorylation site implicated in its downregulation after induction of a single DNA break (Memisoglu *et al*., 2019). We did not find evidence that it is involved in Mec1 dampening in CPT condition, raising the possibility that distinct mechanisms may be used in different genomic stress conditions. We also found that two phosphorylation sites on Srs2 that showed Mec1 dependency in proteomic studies had no effect in the CPT situation (Albuquerque *et al*., 2015; Faca *et al*., 2020); however we note that these sites do not fit with the typical S/TQ Mec1 consensus sites, thus may not be the bona fide Mec1 sites. Other phosphorylation sites examined here include five Mec1 sites on Rfa1 and Rfa2 (Albuquerque *et al*., 2015; Chen *et al*., 2010). Genetic analyses did not support their roles in the Srs2 and RPA antagonism. Thus, future studies to comprehensively map DDC kinase phosphorylation sites on Srs2 and RPA, as well as their regulators, such as the RPA chaperone Rtt105 (Li *et al*, 2018), will be needed to test how the kinases can communicate with Srs2- based RPA regulation during the DNA damage response.

While we provide multiple lines of evidence in support of the involvement of Srs2 binding to PCNA and its sumoylation in RPA regulation during DDC downregulation, *srs2-ΔPIM* or *-3KR* alone behaved normally in checkpoint assays. A lack of defect here can be due to buffering effects, both from not-yet characterized Srs2 features and from other checkpoint dampening pathways. In addressing the former possibility, we tested the separation pin of Srs2 (Y775), which was shown to enables its *in vitro* helicase activity during the revision of our work (Meir *et al*, 2023). We found that the *srs2-Y775A* mutation that severely compromised the Srs2 helicase activity rescued *rfa2-zm2* sensitivity to CPT (Fig. S4). This result suggests that Y775 may directly contribute to *in vivo* RPA removal; future examination of this feature, alone and in conjunction with PCNA binding and sumoylation, can extend our understanding of Srs2- mediated DDC regulation. We also provided evidence for the latter possibility, since *srs2-ΔPIM* or *-3KR* showed additive phenotype when combined with a *slx4* mutant defective in DDC dampening function. The presence of multiple checkpoint dampening factors that target different checkpoint modules highlights the importance of the process. Recent demonstration of other DNA helicases, such as the human HELB, HELQ, and BLM proteins, in stripping of RPA from ssDNA in vitro suggests that Srs2-like checkpoint downregulation may present in human cells as well (Hormeno *et al*., 2022; Jenkins *et al*., 2021; Shorrocks *et al*., 2021). Since RPA-ssDNA binding not only plays a key role in checkpoint, but also in many other processes, the Srs2 features uncovered here may help to better understand how RPA dynamics is controlled by helicases and other factors during additional processes beyond DDC.

Methods

Yeast strains and genetic techniques

Standard procedures were used for cell growth and media preparation. The strains used in this work are listed in Table S1and are isogenic to W1588-4C, a *RAD5* derivative of W303 (Zhao and Blobel, 2005). Mutant alleles of *srs2*, *rfa1*, *rfa2* and *mec1* used in this work were generated by CRISPR-Cas9-based gene replacement, except for *srs2-3KR* that was introduced using a PCR-based method (DiCarlo *et al*, 2013). All mutations were verified by sequencing. Standard procedures were used for tetrad analyses and genotoxin testing. For each assay, at least two biological duplicates were used per each genotype. Cells were grown at 30 °C unless otherwise stated.

Cell synchronization and cell cycle analyses

Cells from log-phase cultures were treated with 5 μg/mL α factor (GenScript RP01002) for 1 h, followed by an additional 2.5 μg/mL α factor for 30 min. When at least 95% cells were arrested in G1 phase based on the percentage of unbudded cells, they were released into yeast extract–peptone–dextrose (YPD) media containing 100 μg/mL Protease (Millipore 53702) and 16 μg/mL CPT (Sigma C9911) for 2 h. Cell cycle progression was monitored by standard flow cytometry analyses as described previously (Zhao and Rothstein, 2002). To derive percentage of G1 cells in **Figure 3B**, the G1 region of the "G1 sample" was used to demarcate the G1 region of the "CPT 2h" sample of the same strain in the same experiment.

Protein extraction using trichloroacetic acid (TCA)

To examine the protein levels of Srs2, the phosphorylation form of Rad53, and Rad9, cell extracts were prepared as reported (Regan- Mochrie *et al*, 2022) . Briefly, 2×10^8 cells were collected at indicated time points. Cell pellets were resuspended in 20% TCA and lysed by glass bead beating. The lysate was then centrifuged to remove supernatant. Precipitated proteins were suspended in Laemmli buffer (65 mM Tris-HCl at pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.025% bromophenol blue) with 2 M Tris added to neutralize the solution. Prior to loading, samples were boiled for 5 min and spun down at 14,000 rpm for 10 min to remove insoluble materials. Samples

were separated on 4-20% Tris-glycine gels (Bio-Rad 456-1096) to examine Srs2 level and phosphorated Rad53 form or 7% Tris-acetate gels (Thermo Fisher EA03555) to detect Rad9 phosphorylation.

Detection of Srs2 sumoylation

Sumoylated proteins were pulled down from cells containing 8His-tagged SUMO expressed from its endogenous locus using the standard Ni-NTA pull-down method as previously described (Ulrich and Davies, 2009). Briefly, protein extracts prepared in 55% TCA were incubated in buffer A (6 M guanidine HCl, 100 mM sodium phosphate at pH 8.0, 10 mM Tris-HCl at pH 8.0) with rotation for 1 h at room temperature. The cleared supernatant was obtained after centrifuging for 20 min and was then incubated overnight at room temperature with Ni-NTA resin (Qiagen 30210) in the presence of 0.05% Tween-20 and 4.4 mM imidazole with rotation. Beads were washed twice with buffer A supplemented with 0.05% Tween 20 and then four times with buffer C (8 M urea, 100 nM sodium phosphate at pH 6.3, 10 mM Tris-HCl at pH 6.3) supplemented with 0.05% Tween 20. Proteins were eluted from the beads using HU buffer (8 M urea, 200 mM Tris-HCl at pH 6.8, 1 mM EDTA, 5% SDS, 0.1% bromophenol blue, 1.5% DTT, 200 mM imidazole). Samples were loaded onto NuPAGE^{TM} 3– 8% Tris-acetate gels (Thermo Fisher EA03752) for immunoblotting to detect both sumoylated and unmodified Srs2. Equal loading was verified using Ponceau S staining.

Detection of Mec1 S1964 phosphorylation during CPT treatment

G1-arrested cells were filtered and resuspended in prewarmed YPD media containing 16 µg/mL CPT to allow entry into S phase. Samples were collected at indicated time points. Cells were disrupted by glass bead beating in lysis buffer, followed by centrifugation at 20,000 g for 15 min to obtain whole-cell extract. The lysis buffer consisted of 25 mM K-HEPES (pH 7.6), 100 mM NaCl, 100 mM K- glutamate, 5 mM $Mg(OAc)_2$, 0.02% NP40, and 0.5% Triton X-100 supplemented by protease inhibitor cocktail (Sigma P8215) and Complete Ultra EDTA-free protease inhibitor (Roche 11836145001). The activated form of Mec1 was enriched by immunoprecipitating myc-tagged Ddc2 using Protein A beads and an anti-Myc antibody (Thermo Fisher 9E10) for 2–4 h at 4°C. Beads were washed five to six times with lysis buffer, and proteins were eluted using Laemmli buffer (65 mM Tris-HCl at pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.025% bromophenol blue). After boiling for 5 min, eluted proteins were loaded onto NuPAGE^{TM} 3–8% Tris-acetate gels for SDS-PAGE and subsequent immunoblotting analyses.

Immunoblotting analysis and antibodies

After SDS-PAGE, proteins were transferred to a 0.2-µm nitrocellulose membrane (GE, #G5678144) for immunoblotting. Pgk1 was used as a loading control. Antibodies used were anti-myc (Thermo, Fisher 9E10). anti-Srs2 (Santa Cruz, yC-18), anti-Pgk1 (Invitrogen, 22C5D8), F9 (a kind gift from Marco Foiani and Daniele Piccini, The FIRC Institute of Molecular Oncology, Milan, Italy) (Pellicioli *et al*., 2001), anti-Rad9 (a kind gift from John Petrini, MSKCC, NY, USA) (Usui *et al*, 2009), anti-Mec1-S1964-p (a kind gift from James E. Haber, Brandeis University, MA, USA) (Memisoglu *et al*., 2019). Validation of antibodies is provided either by the manufacturers or in the cited references. Membranes were scanned with a Fujifilm LAS-3000 luminescent image analyzer, which has a linear dynamic range of 10^4. The signal intensities of non-saturated bands were measured using ImageJ software. For graphs, data are shown as mean and SD. Statistical differences were determined using two-sided Student's t tests.

Acknowledgements

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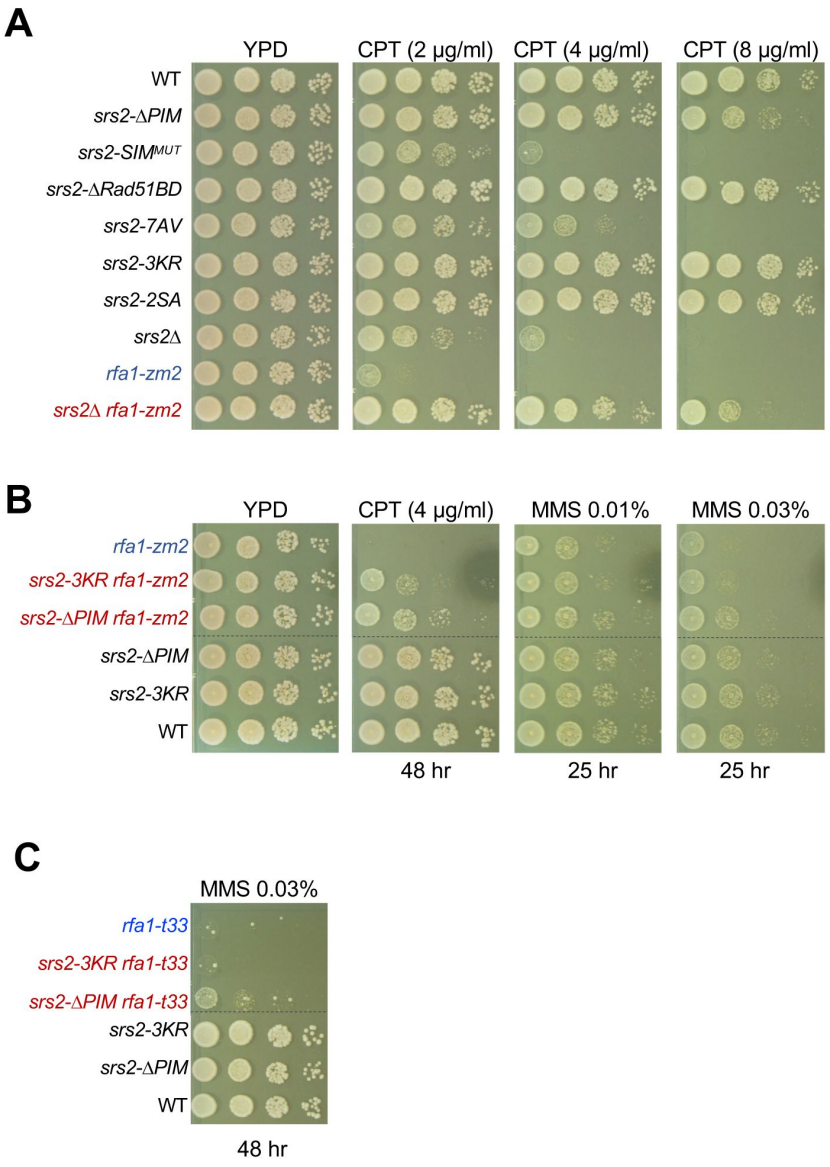


Figure S1.

srs2 mutants examined for CPT sensitivity and interactions with _rfa1_ mutant.

(A) *srs2* mutants exhibit different levels of CPT sensitivity. *srs2-ΔPIM* showed slow growth on 8 µg/ml CPT. Sensitivity *srs2-SIM*^{MUT} toward CPT was seen at three CPT concentrations. *srs2- 7AV* showed slow growth on media containing 4 and 8 µg/ml CPT. *srs2Δ* and *rfa1-zm2* are mutually suppressive for CPT sensitivity. Experiments are performed as in **Figure 2C**.
(B) *srs2-ΔPIM* confers better suppression of *rfa1-zm2's* CPT and MMS sensitivity than *srs2-3KR*. We note that mild suppression of *rfa1-zm2* by *srs2-3KR* was only seen at 0.03% MMS plates. Experiments are performed as in **Figure 2C**, and incubation times are noted. Dashed lines indicate the removal of superfluous rows.
(C) *srs2-ΔPIM* suppresses *rfa1-t33* sensitivity toward MMS. Dashed lines indicate the removal of superfluous rows.

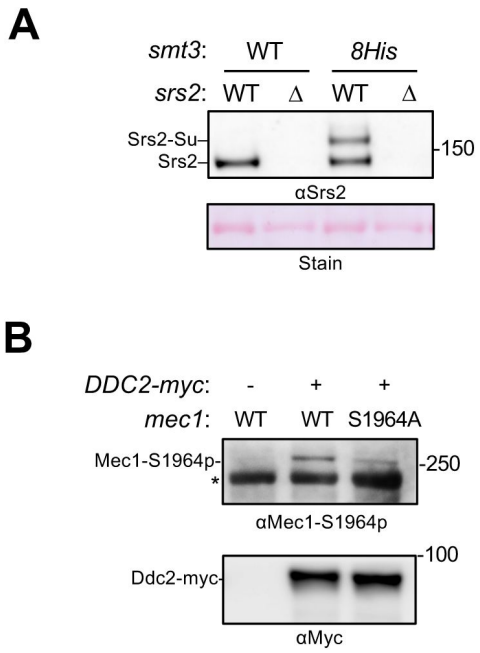


Figure S2.

Confirmation of Srs2 sumoylation and Mec1-S1964 phosphorylation.

(A) Detection of Srs2 sumoylation. Protein extracts from asynchronous cells were bound to Ni- NTA beads and the elutes were examined by immunoblotting using an anti-Srs2 antibody. Sumoylated Srs2 form was detected in wild-type cells expressing 8His-tagged SUMO (Smt3) but not in cells lacking this construct or in *srs2Δ* cells. Unmodified Srs2 showed unspecific Ni-NTA bead binding, thus being detectable in cells containing Srs2 regardless of the SUMO status.
(B) Detection of Mec1-S1964 phosphorylation. Cells were treated with CPT for 2 hours before immunoprecipitating Ddc2-myc was conducted. Ddc2 and the co-immunoprecipitated Mec1 were examined by immunoblotting. Phosphorylation of Ser1964 of the Mec1 protein was detected using the anti-Mec1-S1964-p antibody. This antibody also detected a non-specific band (*). Cells containing untagged Ddc2 or the *mec1-S1964A* mutation were used as controls.

Figure S3.

Examination of Mec1-S964 phosphorylation.

(A) mec1-S1964A does not affect the CPT sensitivity of srs2-3KR or srs2-3KR rfa1-zm2 cells. Experiments were performed as in Figure 2C.
(B) The effects of *mec1-S1964E* on the CPT sensitivity of *srs2-3KR* and *srs2-ΔPIM* cells with or without *rfa1-zm2*. Experiments were performed as in **Figure 2C**.
(C) *mec1-S1964A* or *-S1964E* does not affect Srs2 sumoylation. Experiments were performed as in **Figure 4A**. Mean values of three biological isolates per genotype are graphed, with error bars representing SD. Statistical analysis was performed using pairwise Student's t- test; no significance was found between values of wild-type and mutant strains.

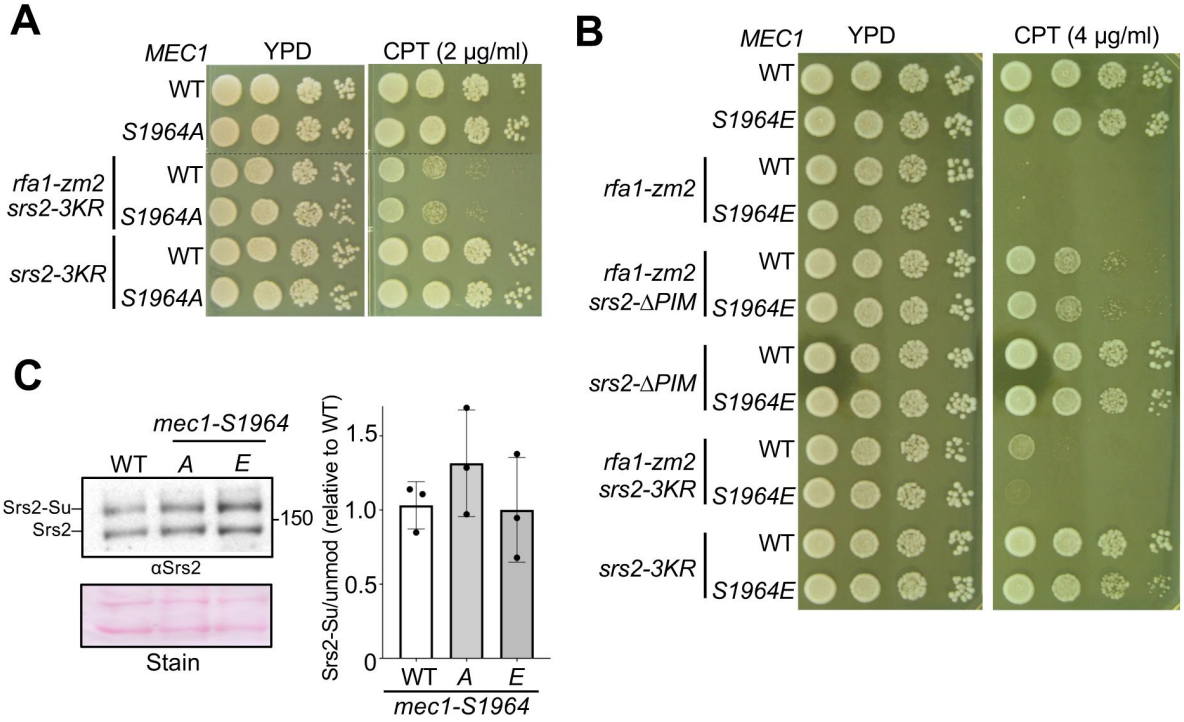
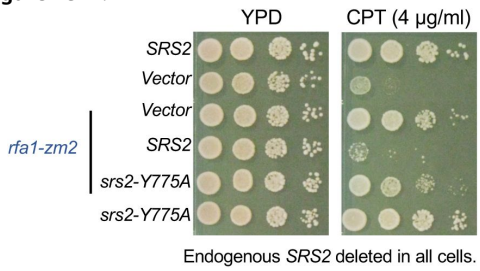


Figure S4.

srs2-Y775A improves *rfa1-zm2* mutant growth on CPT containing media.

The endogenous *SRS2* gene was deleted in cells, while the wild-type *SRS2* or the *srs2-Y775A* allele was integrated at the *HIS3* locus (Meir *et al*., 2023). The control strain contains vector sequence integrated at the *HIS3* locus (Meir *et al*., 2023). Experiments were performed as in **Figure 2C**.



Strain	Genotype	Source	Used In Figures
G16	Wild-type W303 *RAD5* strain	Lab collection	1B, 2C-E, 3B-C, 5C, 6B-D, S1-3
X8047-14B	*srs2Δ*	(Dhingra *et al.*, 2021)	6C-D, S1A, S2A
X8665-12-22D	*MATa srs2-ΔPIM*	This study	1B, 2C, 3B-C, 6B, S1A-C
X8198-15B	*srs2-SIM^{MUT}*	This study	1B, 2D, S1A
G1114	*MATa srs2-7AV::HIS3*	(Saponaro *et al.*, 2010)	1B, 2D, S1A
X8650-2-1D	*MATa srs2-3KR::NAT*	This study	1B, 2C, 3B-C, S1A-C
X8659-14A	*MATa srs2-2SA*	This study	1B, 2D, S1A
G854	*MATα srs2-Δ(875-902)::HPH*	(Colavito *et al.*, 2009)	1B, 2D, S1A
X8047-1B	*MATα rfa1-zm2*	(Dhingra *et al.*, 2021)	2C, 2C-D, 3B-C, 6B, S1A-B, S3B
X8650-2C	*MATa srs2-3KR::NAT rfa1-zm2*	This study	2C, 3B-C, S1B, S3A-B
X8666-35-6A	*MATa srs2-ΔPIM rfa1-zm2*	This study	2C, 3B-C, 6B, S1B, S3B
X8210-2B	*MATa rfa1-t33*	(Dhingra *et al.*, 2021)	2E, S1C
X8745-2A	*MATa srs2-3KR::NAT rfa1-t33*	This study	2E, S1C
X8743-13A	*MATα srs2-ΔPIM rfa1-t33*	This study	2E, S1C
X8198-15A	*srs2-SIM^{MUT} rfa1-zm2*	This study	2D
X8637-6B	*MATa srs2-7AV::HIS3 rfa1-zm2*	This study	2D
X8137-10B	*MATa srs2-Δ875-902::HPH rfa1-zm2*	This study	2D
X8660-4B	*MATα srs2-2SA rfa1-zm2*	This study	2D
T1358-2	*MATa 8His-SMT3::TRP1*	Lab Collection	5A-B, 5E
X8898-19D	*MATa srs2-Δ::HIS3 8His-SMT3::TRP1*	This study	S2A
X8808-2D	*MATa srs2-ΔPIM 8His-SMT3::TRP1*	This study	5A
X8795-9D	*MATa srs2-3KR::NAT 8His-SMT3::TRP1*	This study	5A
X8807-6A	*MATa sml1Δ::URA3 8His-SMT3::TRP1*	This study	5E
X8746-4D	*MATa sml1Δ::URA3 mec1Δ::TRP1 8His-SMT3::TRP1*	This study	5E
X8807-7A	*MATa sml1Δ::URA3 rad53Δ::HIS3 8His-SMT3::TRP1*	This study	5E
X9055-1D	*MATa DDC2-Myc18::HIS3*	This study	6A, S2B
X9190-8A	*MATa DDC2-Myc18::HIS3 mec1-S1964A*	This study	S2B
X9225-3A	*MATa mec1-S1964A*	This study	6B
X9225-3C	*MATa mec1-S1964A rfa1-zm2*	This study	6B
X9088-38C	*MATa mec1-S1964A srs2-ΔPIM*	This study	6B
X9225-1B	*MATα mec1-S1964A srs2-ΔPIM rfa1-zm2*	This study	6B
X8495-5D	*MATa rfa1-S178D*	This study	6C
X8495-2D	*MATa srs2Δ::HIS3 rfa1-S178D*	This study	6C
X8494-3B	*MATa rfa1-S178A*	This study	6C
X8494-3A	*MATa srs2Δ::HIS3 rfa1-S178A*	This study	6C

Table S1

Strains Sources.

All strains are derived from W1588-4C, a *RAD5* derivative of W303 (*MATα ade2-1 can1-100 ura3-1 his3-11,15, leu2-3, 112 trp1-1 rad5-535*). Only one strain is listed per each genotype, but at least two independent isolates of each genotype were used in the experiments.

X8658-14-22D	*MATa rfa2-3SA*	This study	6D
X8658-14-13C	*MATa srs2Δ::HIS3 rfa2-3SA*	This study	6D
X8779-13D	*MATa rfa2-3SD*	This study	6D
X8779-13B	*MATa srs2Δ rfa2-3SD*	This study	6D
X9265-4D	*MATa SLX4-TAP::HIS3*	(Wan *et al.*, 2019)	4A-C
X9265-4A	*MATa srs2-ΔPIM SLX4-TAP::HIS3*	This study	4A-C
X8956-7C	*MATa slx4^{RIM}-TAP::HIS3*	(Wan *et al.*, 2019)	4A-C
X8956-5D	*MATa srs2-ΔPIM slx4^{RIM}-TAP::HIS3*	This study	4A-C
X9228-2D	*MATa mec1-S1964A srs2-3KR::NAT*	This study	S3A
X9228-5A	*MATα mec1-S1964A srs2-3KR::NAT rfa1-zm2*	This study	S3A
X9090-9C	*MATa mec1-S1964E*	This study	S3B
X9091-14A	*MATa mec1-S1964E rfa1-zm2*	This study	S3B
X9090-19A	*MATa mec1-S1964E srs2-ΔPIM*	This study	S3B
X9132-15B	*MATa mec1-S1964E srs2-ΔPIM rfa1-zm2*	This study	S3B
X9091-13A	*MATa mec1-S1964E srs2-3KR::NAT*	This study	S3B
X9132-10B	*MATα mec1-S1964E srs2-3KR::NAT rfa1-zm2*	This study	S3B
X9137-3B	*MATa mec1-S1964A 8His-SMT3::TRP1*	This study	S3C
X9136-2B	*MATa mec1-S1964E 8His-SMT3::TRP1*	This study	S3C
X9486-5B	*MATa his3::pRS303-p500-SRS2::HIS3 srs2Δ::KanMX*	This study	S4
X9485-8D	*MATa his3::pRS303-p500::HIS3 srs2Δ::KanMX*	This study	S4
X9485-8B	*MATa rfa1-zm2 his3::pRS303-p500::HIS3 srs2Δ::KanMX*	This study	S4
X9486-1D	*MATa rfa1-zm2 his3::pRS303-p500-SRS2::HIS3 srs2Δ::KanMX*	This study	S4
X9484-9C	*MATa rfa1-zm2 his3::pRS303-p500-Srs2-Y775A::HIS3 srs2Δ::KanMX*	This study	S4
X9484-18D	*MATa his3::pRS303-p500-Srs2-Y775A::HIS3 srs2Δ::KanMX*	This study	S4

Table S1 (continued)

Additional information

Author contributions

All authors were involved in research design and data analyses, J. F., N. D., T. Y., and V. Y. performed research. J. F. and X.Z. wrote the paper with all authors' input.

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Reviewer #1 (Public review):

Overall, the data presented in this manuscript is of good quality. Understanding how cells control RPA loading on ssDNA is crucial to understanding DNA damage responses and genome maintenance mechanisms. The authors used genetic approaches to show that

disrupting PCNA binding and SUMOylation of Srs2 can rescue the CPT sensitivity of rfa1 mutants with reduced affinity for ssDNA. In addition, the authors find that SUMOylation of Srs2 depends on binding to PCNA and the presence of Mec1.

Comments on revisions:

I am satisfied with the revisions made by the authors, which helped clarify some points that were confusing in the initial submission.

https://doi.org/10.7554/eLife.98843.2.sa3

Reviewer #2 (Public review):

This revised manuscript mostly addresses previous concerns by doubling down on the model without providing additional direct evidence of interactions between Srs2 and PCNA, and that "precise sites of Srs2 actions in the genome remain to be determined." One additional Srs2 allele has been examined, showing some effect in combination with rfa1-zm2.

Many of the conclusions are based on reasonable assumptions about the consequences of various mutations, but direct evidence of changes in Srs2 association with PNCA or other interactors is still missing. There is an assumption that a deletion of a Rad51-interacting domain or a PCNA-interacting domain have no pleiotropic effects, which may not be the case. How SLX4 might interact with Srs2 is unclear to me, again assuming that the SLX4 defect is "surgical" - removing only one of its many interactions.

One point of concern is the use of t-tests without some sort of correction for multiple comparisons - in several figures. I'm quite sceptical about some of the $p < 0.05$ calls surviving a Bonferroni correction. Also in 4B, which comparison is **? Also, admittedly by eye, the changes in "active" Rad53 seem much greater than 5x. (also in Fig. 3, normalizing to a non-WT sample seems odd).

What is the WT doubling time for this strain? From the FACS it seems as if in 2 h the cells have completed more than 1 complete cell cycle. Also in 5D. Seems fast...

I have one over-arching confusion. Srs2 was shown initially to remove Rad51 from ssDNA and the suppression of some of srs2's defects by deleting rad51 made a nice, compact story, though exactly how srs2's "suppression of rad6" fit in isn't so clear (since Rad6 ties into Rad18 and into PCNA ubiquitylation and into PCNA SUMOylation). Now Srs2 is invoked to remove RPA. It seems to me that any model needs to explain how Srs2 can be doing both. I assume that if RPA and Rad51 are both removed from the same ssDNA, the ssDNA will be "trashed" as suggested by Symington's RPA depletion experiments. So building a model that accounts for selective Srs2 action at only some ssDNA regions might be enhanced by also explaining how Rad51 fits into this scheme.

As a previous reviewer has pointed out, CPT creates multiple forms of damage. Foiani showed that 4NQO would activate the Mec1/Rad53 checkpoint in G1- arrested cells, presumably because there would be single-strand gaps but no DSBs. Whether this would be a way to look specifically at one type of damage is worth considering; but UV might be a simpler way to look.

As also noted, the effects on the checkpoint and on viability are quite modest. Because it isn't clear (at least to me) why rfa1 mutants are so sensitive to CPT, it's hard for me to understand how srs2-zm2 has a modest suppressive effect: is it by changing the checkpoint response or facilitating repair or both? Or how srs2-3KR or srs2-dPIM differ from Rfa1-zm2 in this respect. The authors seem to lump all these small suppressions under the rubric of "proper

levels of RPA-ssDNA" but there are no assays that directly get at this. This is the biggest limitation.

Srs2 has also been implicated as a helicase in dissolving "toxic joint molecules" (Elango et al. 2017). Whether this activity is changed by any of the mutants (or by mutations in Rfa1) is unclear. In their paper, Elango writes: "Rare survivors in the absence of Srs2 rely on structure-specific endonucleases, Mus81 and Yen1, that resolve toxic joint-molecules" Given the involvement of SLX4, perhaps the authors should examine the roles of structure-specific nucleases in CPT survival?

Experiments that might clarify some of these ambiguities are proposed to be done in the future. For now, we have a number of very interesting interactions that may be understood in terms of a model that supposes discriminating among gaps and ssDNA extensions by the presence of PCNA, perhaps modified by SUMO. As noted above, it would be useful to think about the relation to Rad6.

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Reviewer #3 (Public review):

The superfamily I 3'-5' DNA helicase Srs2 is well known for its role as an anti-recombinase, stripping Rad51 from ssDNA, as well as an anti-crossover factor, dissociating extended D-loops and favoring non-crossover outcome during recombination. In addition, Srs2 plays a key role in in ribonucleotide excision repair. Besides DNA repair defects, srs2 mutants also show a reduced recovery after DNA damage that is related to its role in downregulating the DNA damage signaling or checkpoint response. Recent work from the Zhao laboratory (PMID: 33602817) identified a role of Srs2 in downregulating the DNA damage signaling response by removing RPA from ssDNA. This manuscript reports further mechanistic insights into the signaling downregulation function of Srs2.

Using the genetic interaction with mutations in RPA1, mainly rfa1-zm2, the authors test a panel of mutations in Srs2 that affect CDK sites (srs2-7AV), potential Mec1 sites (srs2-2SA), known sumoylation sites (srs2-3KR), Rad51 binding (delta 875-902), PCNA interaction (delta 1159-1163), and SUMO interaction (srs2-SIMmut). All mutants were generated by genomic replacement and the expression level of the mutant proteins was found to be unchanged. This alleviates some concern about the use of deletion mutants compared to point mutations. Double mutant analysis identified that PCNA interaction and SUMO sites were required for the Srs2 checkpoint dampening function, at least in the context of the rfa1-zm2 mutant. There was no effect of this mutants in a RFA1 wild type background. This latter result is likely explained by the activity of the parallel pathway of checkpoint dampening mediated by Slx4, and genetic data with an Slx4 point mutation affecting Rtt107 interaction and checkpoint downregulation support this notion. Further analysis of Srs2 sumoylation showed that Srs2 sumoylation depended on PCNA interaction, suggesting sequential events of Srs2 recruitment by PCNA and subsequent sumoylation. Kinetic analysis showed that sumoylation peaks after maximal Mec1 induction by DNA damage (using the Top1 poison camptothecin (CPT)) and depended on Mec1. This data are consistent with a model that Mec1 hyperactivation is ultimately leading to signaling downregulation by Srs2 through Srs2 sumoylation. Mec1-S1964 phosphorylation, a marker for Mec1 hyperactivation and a site found to be needed for checkpoint downregulation after DSB induction, did not appear to be involved in checkpoint downregulation after CPT damage. The data are in support of the model that Mec1 hyperactivation when targeted to RPA-covered ssDNA by its Ddc2 (human ATRIP) targeting factor, favors Srs2 sumoylation after Srs2 recruitment to PCNA to disrupt the RPA-Ddc2-Mec1 signaling complex. Presumably, this allows gap filling and disappearance of long-lived ssDNA as the initiator of checkpoint signaling, although the study does not extend to this step.

Strengths
(1) The manuscript focuses on the novel function of Srs2 to downregulate the DNA damage signaling response and provide new mechanistic insights.
(2) The conclusions that PCNA interaction and ensuing Srs2-sumoylation are involved in checkpoint downregulation are well supported by the data.

Weaknesses
(1) Additional mutants of interest could have been tested, such as the recently reported Pin mutant, srs2-Y775A (PMID: 38065943), and the Rad51 interaction point mutant, srs2-F891A (PMID: 31142613).
(2) The use of deletion mutants for PCNA and RAD51 interaction is inferior to using specific point mutants, as done for the SUMO interaction and the sites for post-translational modifications.
(3) Figure 4D and Figure 5A report data with standard deviations, which is unusual for n=2. Maybe the individual data points could be plotted with a color for each independent experiment to allow the reader to evaluate the reproducibility of the results.

Comments on revisions:

In this revision, the authors adequately addressed my concerns. The only issue I see remaining is the site of Srs2 action. The authors argue in favor of gaps and against R-loops and ssDNA resulting from excessive supercoiling. The authors do not discuss ssDNA resulting from processing of one-sided DSBs, which are expected to result from replication run-off after CPT damage but are not expected to provide the 3'-junction for preferred PCNA loading. Can the authors exclude PCNA at the 5'-junction at a resected DSB?

https://doi.org/10.7554/eLife.98843.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***eLife Assessment***
>
> *This manuscript reports valuable findings on the role of the Srs2 protein in turning off the DNA damage signaling response initiated by Mec1 (human ATR) kinase. The data provide solid evidence that Srs2 interaction with PCNA and ensuing SUMO modification is required for checkpoint downregulation. However, experimental evidence with regard to the model that Srs2 acts at gaps after camptothecin-induced DNA damage is currently lacking. The work will be of interest to cell biologists studying genome integrity but would be strengthened by considering the possible role of Rad51 and its removal.*

We thank editors and reviewers for their constructive comments and address their main criticisms below.

(1) *Srs2 action sites.* Our data provide support to the model that Srs2 removal of RPA is favored at ssDNA regions with proximal PCNA, but not at ssDNA regions lacking proximal PCNA. A prominent example of the former type of ssDNA regions is an ssDNA gap with a 3' DNA end permissive for PCNA loading. Examples of the latter type of ssDNA sites include those within R-loops and negatively supercoiled regions, both lacking 3' DNA end required for PCNA loading. The former type of ssDNA regions can recruit other DNA damage checkpoint proteins, such as 9-1-1, which requires a 5' DNA end for loading; thus, these ssDNA regions are ideal for Srs2's action in checkpoint dampening. In contrast, ssDNA within supercoiled and Rloop regions, both of which can be induced by CPT treatment (Pommier *et*

al, 2022), lacks the DNA ends required for checkpoint activation. RPA loaded at these sites plays important roles, such as recruiting Rloop removal factors (Feng and Manley, 2021; Li *et al*, 2024; Nguyen *et al*, 2017), and they are not ideal sites for Srs2's checkpoint dampening functions. Based on the above rationale and our data, we suggest that Srs2 removal of RPA is favored only at a subset of ssDNA regions prone to checkpoint activation and can be avoided at other ssDNA regions where RPA mainly helps DNA protection and repair. We have modified the text and model drawing to better articulate the implications of our work, that is, Srs2 can distinguish between two types of ssDNA regions by using PCNA proximity as a guide for RPA removal_._ We noted that the precise sites of Srs2 actions in the genome remain to be determined.

(2) *Rad51 in the Srs2-RPA antagonism*. In our previous report (Dhingra *et al*, 2021), we provided several lines of evidence to support the conclusion that Rad51 is not relevant to the Srs2-RPA antagonism, despite it being the best-studied protein that is regulated by Srs2. For example, while *rad51Δ* rescues the hyperrecombination phenotype of *srs2Δ* cells as shown by others, we found that *rad51Δ* did not affect the hypercheckpoint phenotype of *srs2Δ*. In contrast, *rfa1-zm1/zm2* have the opposite effects. The differential effects of *rad51Δ* and *rfa1-zm1/zm2* were also seen for the *srs2-ATPase* dead allele (*srs2-K41A*). For example, *rfa1-zm2* rescued the hyper-checkpoint defect and the CPT sensitivity of *srs2-K41A*, while *rad51Δ* had neither effect. These and other data described by Dhingra et al (2021) suggest that Srs2's effects on checkpoint vs. recombination can be separated and that Rad51 removal by Srs2 is distinct from the Srs2RPA antagonism in checkpoint regulation. Given the functional separation summarized above, in our current work investigating which Srs2 features affect the Srs2-RPA antagonism, we did not focus on the role of Rad51. However, we did examine all known features of Srs2, including its Rad51 binding domain. Consistent with our conclusion summarized above, deleting the Rad51 binding domain in Srs2 (*srs2ΔRad51BD*) has no effect on *rfa1-zm2* phenotype in CPT (Figure 2D). This data provides yet another evidence that Srs2 regulation of Rad51 is separable from the Srs2-RPA antagonism. Our work provides a foundation for future examination of how Srs2 regulates RPA and Rad51 in different manners and if there is a crosstalk between them in specific contexts. We have added this point to the revised text.

Public Reviews:

Reviewer #1.

Overall, the data presented in this manuscript is of good quality. Understanding how cells control RPA loading on ssDNA is crucial to understanding DNA damage responses and genome maintenance mechanisms. The authors used genetic approaches to show that disrupting PCNA binding and SUMOylation of Srs2 can rescue the CPT sensitivity of rfa1 mutants with reduced affinity for ssDNA. In addition, the authors find that SUMOylation of Srs2 depends on binding to PCNA and the presence of Mec1. Noted weaknesses include the lack of evidence supporting that Srs2 binding to PCNA and its SUMOylation occur at ssDNA gaps, as proposed by the authors. Also, the mutants of Srs2 with impaired binding to PCNA or impaired SUMOylation showed no clear defects in checkpoint dampening, and in some contexts, even resulted in decreased Rad53 activation. Therefore, key parts of the paper would benefit from further experimentation and/or clarification.

We thank the reviewer for the positive comments, and we address her/his remark regarding ssDNA gaps below. In addition, we provide evidence that redundant pathways can mask checkpoint dampening phenotype of the *srs2-ΔPIM* and *-3KR* alleles.

> *Major Comments*
>
> *(1) The central model proposed by the authors relies on the loading of PCNA at the 3' junction of an ssDNA gap, which then mediates Srs2 recruitment and RPA removal. While*

> *several aspects of the model are consistent with the data, the evidence that it is occurring at ssDNA gaps is not strong. The experiments mainly used CPT, which generates mostly DSBs. The few experiments using MMS, which mostly generates ssDNA gaps, show that Srs2 mutants lead to weaker rescue in this context (Figure S1). How do the authors explain this discrepancy? In the context of DSBs, are the authors proposing that Srs2 is engaging at later steps of HRdriven DSB repair where PCNA gets loaded to promote fill-in synthesis? If so, is RPA removal at that step important for checkpoint dampening? These issues need to be addressed and the final model adjusted.*

Our data provide supports to the model that Srs2 removal of RPA is favored at ssDNA regions with proximal PCNA, but not at ssDNA regions lacking proximal PCNA (Figure 7). A prominent example of the former type is ssDNA gap with 3' DNA end permissive for PCNA loading. Examples of the latter type of ssDNA sites are present within R-loops and negatively supercoiled regions, and these ssDNA sites lack 3' DNA ends required for PCNA loading. In principle, the former can recruit other DNA damage checkpoint proteins, such as 9-1-1, which requires 5' DNA end for loading, thus it is ideal for Srs2's action in checkpoint dampening. In contrast, ssDNA within supercoiled and R-loop regions, which can be induced by CPT treatment (Pommier *et al.*, 2022), lacks DNA ends required for checkpoint activation. RPA loaded at these sites plays important roles such as recruiting R-loop removal factors (Feng and Manley, 2021; Li *et al.*, 2024; Nguyen *et al.*, 2017), and these are not ideal sites for Srs2 removal of RPA to achieve checkpoint dampening. Our work suggests that Srs2 removal of RPA is favored only at a subset of ssDNA regions prone to checkpoint activation and can be avoided at other ssDNA regions where RPA mainly helps DNA protection and repair. We have modified the text and the model to clarify our conclusions and emphasized that Srs2 can distinguish between two types of ssDNA regions using PCNA proximity as a guide for RPA removal.

We note that in addition to DSBs, CPT also induces both types of ssDNA mentioned above. For example, CPT can lead to ssDNA gap formation upon excision repair or DNA-protein crosslink repair of trapped Top1 (Sun *et al*, 2020). The resultant ssDNA regions contain 3' DNA end for PCNA loading, thus favoring Srs2 removal of RPA. CPT treatment also depletes the functional pool of Top1, thus causing topological stress and increased levels of DNA supercoiling and R-loops (Petermann *et al*, 2022; Pommier *et al.*, 2022). As mentioned above, R-loops and supercoiled regions do not favor Srs2 removal of RPA due to a lack of PCNA loading. We have now adjusted the text to clarify that CPT can lead to the generation of two types of ssDNA regions as stated above. We have also adjusted the model drawing to indicate that while ssDNA gaps can be logical Srs2 action sites, other types of ssDNA regions with proximal PCNA (e.g., resected ssDNA tails) could also be targeted by Srs2. Our work paves the way to determine the precise ssDNA regions for Srs2's action.

Multiple possibilities should be considered in explaining the less potent suppression of *rfa1* mutants by *srs2* alleles in MMS compared to CPT conditions. For example, MMS and CPT affect checkpoints differently. While CPT only activates the DNA damage checkpoint, MMS additionally induces DNA replication checkpoint (Menin *et al*, 2018; Redon *et al*, 2003; Tercero *et al*, 2003). It is possible that the Srs2-RPA antagonism is more relevant to the DNA damage checkpoint compared with the DNA replication checkpoint. Further investigation of this possibility among other scenarios will shed light on differential suppression seen here. We have included this discussion in the revised text.

> *(2) The data in Figure 3 showing that Srs2 mutants reduce Rad53 activation in the rfa1-zm2 mutant are confusing, especially given the claim of an anti-checkpoint function for Srs2 (in which case Srs2 mutants should result in increased Rad53 activation). The authors propose that Rad53 is hyperactivated in rfa1-zm2 mutant because of compromised ssDNA protection and consequential DNA lesions, however, the effects sharply contrast with the central model. Are the authors proposing that in the rfa1-zm2*

> *mutant, the compromised protection of ssDNA supersedes the checkpoint-dampening effect? Perhaps a schematic should be included in Figure 3 to depict these complexities and help the reader. The schematic could also include the compensatory dampening mechanisms like Slx4 (on that note, why not move Figure S2 to a main figure?... and even expand experiments to better characterize the compensatory mechanisms, which seem important to help understand the lack of checkpoint dampening effect in the Srs2 mutants)*

Partially defective alleles often do not manifest null phenotype. In this case, while *srs2Δ* increases Rad53 activation (Dhingra *et al.*, 2021), *srs2-ΔPIM* and *-3KR* did not (Figure 3A-3B). However, *srs2-ΔPIM* did increase Rad53 activation when combined with another checkpoint dampening mutant $slx4^{RIM}$ (now Figure 4B-4C). This result suggests that defects of partially defective *srs2* alleles can be masked by Slx4. Further, *srs2-ΔPIM* and *3KR* rescued *rfa1-zm2*'s checkpoint abnormality (now Figure 3B-3C), suggesting that Srs2 binding to PCNA and its sumoylation contribute to the Srs2-RPA antagonism in the DNA damage checkpoint response.

Partially defective alleles that impair specific features of a protein without producing null phenotype have been used widely to reveal biological mechanisms. For example, a partially defective allele of the checkpoint protein Rad9 perturbing binding to gamma-H2A (*rad9-K1088M*) does not cause DNA damage sensitivity on its own, due to the compensation from other checkpoint factors (Hammet *et al*, 2007). However_, rad9-K1088M_ rescues the DNA damage sensitivity and persistent G2/M checkpoint of *slx4* mutants, providing strong evidence for the notion that Slx4 dampens checkpoint via regulating Rad9 (Ohouo *et al*, 2013).

We have now indicated that our model highlights the checkpoint recovery process and does not depict another consequence of the Srs2-RPA antagonism, that is, *rfa1* DNA binding mutants can lead to increased levels of DNA lesions and consequently stronger checkpoint activation, which are rescued by lessening Srs2's ability to strip RPA from DNA (Dhingra *et al.*, 2021). We have stated these points more clearly in the text and added a schematic (Figure 3A) to outline the genetic relationship and interpretations. We also moved Figure S2 to the main figures (Figure 4), as suggested by the reviewer. Better characterizing the compensatory mechanisms among the multiple checkpoint dampening pathways requires substantial amounts of work that will be pursued in the future.

> *(3) The authors should demarcate the region used for quantifying the G1 population in Figure 3B and explain the following discrepancy: By inspection of the cell cycle graph, all mutants have lower G1 peak height compared to WT (CPT 2h). However, in the quantification bar graph at the bottom, ΔPIM has higher G1 population than the WT.*

We now describe how the G1 region of the FACS histogram was selected to derive the percentage of G1 cells in Figure 3B (now Figure 3C). Briefly, the G1 region from the "G1 sample" was used to demarcate the G1 region of the "CPT 2h" sample. We noticed that a mutant panel was mistakenly put in the place of wild-type, and this error is now corrected. The conclusion remains that *srs2-ΔPIM* and *srs2-3KR* improved *rfa1-zm2* cells' ability to exit G2/M, while they themselves do not show difference from the wild-type control for the percentage of G1 cells after 2hr CPT treatment. We have added statistics in Figure 3C that support this conclusion.

> ***Reviewer #2:***
>
> *This is an interesting paper that delves into the post-translational modifications of the yeast Srs2 helicase and proteins with which it interacts in coping with DNA damage. The authors use mutants in some interaction domains with RPA and Srs2 to argue for a model in which there is a balance between RPA binding to ssDNA and Srs2's removal of RPA. The idea that a checkpoint is being regulated is based on observing Rad53 and*

> *Rad9 phosphorylation (so there are the attributes of a checkpoint), but evidence of cell cycle arrest is lacking. The only apparent delay in the cell cycle is the re-entry into the second S phase (but it could be an exit from G2/M); but in any case, the wild-type cells enter the next cell cycle most rapidly. No direct measurement of RPA residence is presented.*

We thank the reviewer for the helpful comments. Previous studies have shown that CPT does not induce the DNA replication checkpoint, and thus does not slow down or arrest S phase progression; however, CPT does induce the DNA damage checkpoint, which causes a delay (not arrest) in G2/M phase and re-entering into the second G1 (Menin *et al.*, 2018; Redon *et al.*, 2003). Our result is consistent with these findings, showing that CPT induces G2/M delay but not arrest. We have now made this point clearer in the text.

We have previously reported chromatin-bound RPA levels in *rfa1-zm2*, *srs2*, and their double mutants, as well as *in vitro* ssDNA binding by wild-type and mutant RPA complexes (Dhingra *et al.*, 2021). These data showed that Srs2 loss or its ATPase dead mutant led to 4-6-fold increase of RPA levels on chromatin, which was rescued by *rfa1-zm2* (Dhingra *et al.*, 2021). On its own, *rfa1-zm2* did not cause defective chromatin association, despite modestly reducing ssDNA binding *in vitro* (Dhingra et al., 2021). This discrepancy could be due to a lack of sensitivity of the chromatin fractionation assay in revealing moderate changes of RPA residence on DNA *in vivo*. Our functional assays (Figure 2-3) were more effective in identifying the Srs2 features pertaining to RPA regulation.

> *Strengths:*
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> *Data concern viability assays in the presence of camptothecin and in the post-translational modifications of Srs2 and other proteins.*
>
> *Weaknesses:*
>
> *There are a couple of overriding questions about the results, which appear technically excellent. Clearly, there is an Srs2-dependent repair process here, in the presence of camptothecin, but is it a consequence of replication fork stalling or chromosome breakage? Is repair Rad51-dependent, and if so, is Srs2 displacing RPA or removing Rad51 or both? If RPA is removed quickly what takes its place, and will the removal of RPA result in lower DDC1-MEC1 signaling?*

Srs2 can affect both the checkpoint response and DNA repair processes in CPT conditions. However, *rfa1zm2* mainly affects the former role of Srs2; this allows us to gain a deeper understanding of this role, which is critical for cell survival in CPT (Dhingra *et al.*, 2021). Building on this understanding, our current study identified two Srs2 features that could afford spatial and temporal regulation of RPA removal from DNA, providing a rationale for how cells can properly utilize an activity that can be beneficial yet also dangerous if it were to lack regulation. Study of Srs2-mediated DNA repair in CPT conditions, either in Rad51-dependent or -independent manner, to deal with replication fork stalling or DNA breaks will require studies in the future.

> *Moreover, it is worth noting that in single-strand annealing, which is ostensibly Rad51 independent, a defect in completing repair and assuring viability is Srs2-dependent, but this defect is suppressed by deleting Rad51. Does deleting Rad51 have an effect here?*

We have previously shown that *rad51Δ* did not rescue the hyper-checkpoint phenotype of *srs2Δ* cells in CPT conditions, while *rfa1-zm1* and *-zm2* did (Dhingra *et al.*, 2021). This differential effect was also seen for the *srs2* ATPase-dead allele (Dhingra *et al.*, 2021). These and other data described by Dhingra et al (2021) suggest that Srs2's effects on checkpoint vs.

recombination are separable at least in CPT condition, and that the Srs2-RPA antagonism in checkpoint regulation is not affected by Rad51 removal (unlike in SSA).

> *Neither this paper nor the preceding one makes clear what really is the consequence of having a weakerbinding Rfa1 mutant. Is DSB repair altered? Neither CPT nor MMS are necessarily good substitutes for some true DSB assay.*

We have previously showed that *rfa1-zm1/zm2* did not affect the frequencies of rDNA recombination, gene conversation, or direct repeat repair (Dhingra *et al.*, 2021). Further, *rfa1-zm1/zm2* did not suppress the hyperrecombination phenotype of *srs2Δ*, while *rad51Δ* did (Dhingra *et al.*, 2021). In a DSB system, wherein the DNA repeats flanking the break were placed 30 kb away from each other, *srs2Δ* led to hyper-checkpoint and lethality, both of which were rescued by *rfa1-zm* mutants (Dhingra *et al.*, 2021). In this assay, *rfa1-zm1/zm2* did not show sensitivity, suggesting largely proficient DNA repair. Collectively, these data suggest that moderately weakening DNA binding of Rfa1 does not lead to detectable effect on the recombinational repair examined thus far, rather it affects Srs2-mediated checkpoint downregulation. In-depth studies of *rfa1-zm* mutations in the context of various DSB repair steps will be interesting to pursue in the future.

> *With camptothecin, in the absence of site-specific damage, it is difficult to test these questions directly. (Perhaps there is a way to assess the total amount of RPA bound, but ongoing replication may obscure such a measurement). It should be possible to assess how CPT treatment in various genetic backgrounds affects the duration of Mec1/Rad53-dependent checkpoint arrest, but more than a FACS profile would be required.*

Quantitative measurement of RPA residence time on DNA in cellular context and the duration of the

Mec1/Rad53-mediated cell cycle delay/arrest will be informative but requires further technology development. Our current work provides a foundation for such quantitative assessment.

> *It is also notable that MMS treatment does not seem to yield similar results (Fig. S1).*

Figure S1 showed that *srs2-ΔPIM* and *srs2-3KR* had weaker suppression of *rfa1-zm2* growth on MMS plates than on CPT plates. Multiple possibilities should be considered in explaining the less potent suppression of *rfa1* mutants by *srs2* in MMS compared with CPT conditions. For example, MMS and CPT affect checkpoints differently. While CPT only activates the DNA damage checkpoint, MMS additionally induces DNA replication checkpoint (Menin *et al.*, 2018; Redon *et al.*, 2003; Tercero *et al.*, 2003). It is therefore possible that the Srs2RPA antagonism is more relevant for the DNA damage checkpoint control compared with the DNA replication checkpoint. Further investigation of this possibility will shed light on differential suppression seen here. We have included this discussion in the revised text.

> ***Reviewer #3:***
>
> *The superfamily I 3'-5' DNA helicase Srs2 is well known for its role as an anti-recombinase, stripping Rad51 from ssDNA, as well as an anti-crossover factor, dissociating extended D-loops and favoring non-crossover outcome during recombination. In addition, Srs2 plays a key role in ribonucleotide excision repair. Besides DNA repair defects, srs2 mutants also show a reduced recovery after DNA damage that is related to its role in downregulating the DNA damage signaling or checkpoint response. Recent work from the Zhao laboratory (PMID: 33602817) identified a role of Srs2 in downregulating the DNA damage signaling response by removing RPA*

> *from ssDNA. This manuscript reports further mechanistic insights into the signaling downregulation function of Srs2.*
>
> *Using the genetic interaction with mutations in RPA1, mainly rfa1-zm2, the authors test a panel of mutations in Srs2 that affect CDK sites (srs2-7AV), potential Mec1 sites (srs2-2SA), known sumoylation sites (srs2-3KR), Rad51 binding (delta 875-902), PCNA interaction (delta 1159-1163), and SUMO interaction (srs2SIMmut). All mutants were generated by genomic replacement and the expression level of the mutant proteins was found to be unchanged. This alleviates some concern about the use of deletion mutants compared to point mutations. The double mutant analysis identified that PCNA interaction and SUMO sites were required for the Srs2 checkpoint dampening function, at least in the context of the rfa1-zm2 mutant. There was no effect of these mutants in a RFA1 wild-type background. This latter result is likely explained by the activity of the parallel pathway of checkpoint dampening mediated by Slx4, and genetic data with an Slx4 point mutation affecting Rtt107 interaction and checkpoint downregulation support this notion. Further analysis of Srs2 sumoylation showed that Srs2 sumoylation depended on PCNA interaction, suggesting sequential events of Srs2 recruitment by PCNA and subsequent sumoylation. Kinetic analysis showed that sumoylation peaks after maximal Mec1 induction by DNA damage (using the Top1 poison camptothecin (CPT)) and depended on Mec1. These data are consistent with a model that Mec1 hyperactivation is ultimately leading to signaling downregulation by Srs2 through Srs2 sumoylation. Mec1-S1964 phosphorylation, a marker for Mec1 hyperactivation and a site found to be needed for checkpoint downregulation after DSB induction did not appear to be involved in checkpoint downregulation after CPT damage. The data are in support of the model that Mec1 hyperactivation when targeted to RPA-covered ssDNA by its Ddc2 (human ATRIP) targeting factor, favors Srs2 sumoylation after Srs2 recruitment to PCNA to disrupt the RPA-Ddc2-Mec1 signaling complex. Presumably, this allows gap filling and disappearance of long-lived ssDNA as the initiator of checkpoint signaling, although the study does not extend to this step.*
>
> *Strengths*
>
> *(1) The manuscript focuses on the novel function of Srs2 to downregulate the DNA damage signaling response and provide new mechanistic insights.*
>
> *(2) The conclusions that PCNA interaction and ensuing Srs2-sumoylation are involved in checkpoint downregulation are well supported by the data.*

We thank the reviewer for carefully reading our work and for his/her positive comments.

> *Weaknesses*
>
> *(1) Additional mutants of interest could have been tested, such as the recently reported Pin mutant, srs2Y775A (PMID: 38065943), and the Rad51 interaction point mutant, srs2-F891A (PMID: 31142613).*

Residue Y775 of Srs2 was shown to serve as a separation pin in unwinding D-loops and dsDNA with 3' overhang in vitro; however, *srs2-Y775A* lacks cellular phenotype in assays for gene conversion, crossover, and genetic interactions. As such, the biological role of this residue has not been clear. In addressing reviewer's comment, we obtained *srs2-Y775A*, and the control strains as described in the recent publication (Meir *et al*, 2023). While *srs2-Y775A* on its own did not affect CPT sensitivity, it improved _rfa1-zm_2 mutant growth on media containing CPT. This result suggests that Y775 can influence RPA regulation during in checkpoint dampening. Given that truncated Srs2 (ΔCter 276 a.a.) containing Y775A showed normal RPA stripping activity *in vitro*, it is possible that cellular assay using *rfa1-zm2* is more sensitive for revealing defect of this activity or full-length protein is required for manifest

Y775A effect. Future experiments distinguishing these possibilities can provide more clarity. Nevertheless, our result reveals the first phenotype of Srs2 separation pin mutant. We have added this new result (Figure S4) and our interpretation.

We have already included data showing that a *srs2* mutant lacking the Rad51 binding domain (*srs2ΔRad51BD*, Δ875-902) did not affect *rfa1-zm2* growth in CPT nor caused defects in CPT on its own (Figure 2D). This data suggest that Rad51 binding is not relevant to the Srs2-RPA antagonism in CPT, a conclusion fully supported by data in our previous study (Dhingra *et al.*, 2021). Collectively, these findings do not provide a strong rationale to test a point mutation within the Rad51BD region.

> *(2) The use of deletion mutants for PCNA and RAD51 interaction is inferior to using specific point mutants, as done for the SUMO interaction and the sites for post-translational modifications.*

We generally agree with this view. However, it is less of a concern in the context of the Rad51 binding site mutant (*srs2-ΔRad51BD*) since it behaved as the wild-type allele in our assays. The *srs2-ΔPIM* mutant (lacking 4 amino acids) has been examined for PCNA binding *in vitro* and *in vivo* (Kolesar *et al*, 2016; Kolesar *et al*, 2012); to our knowledge no detectable defect was reported. Thus, we believe that this allele is suitable for testing whether Srs2's ability to bind PCNA is relevant to RPA regulation.

> *(3) Figure 4D and Figure 5A report data with standard deviations, which is unusual for n=2. Maybe the individual data points could be plotted with a color for each independent experiment to allow the reader to evaluate the reproducibility of the results.*

We have included individual data points as suggested and corrected figure legend to indicate that three independent biological samples per genotype were examined in both panels.

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> ***Reviewer #1 (Recommendations For The Authors):***
>
> *(1) "the srs2-ΔPIM (Δ1159-1163 amino acids)". "11" should not be italic.*

Corrected.

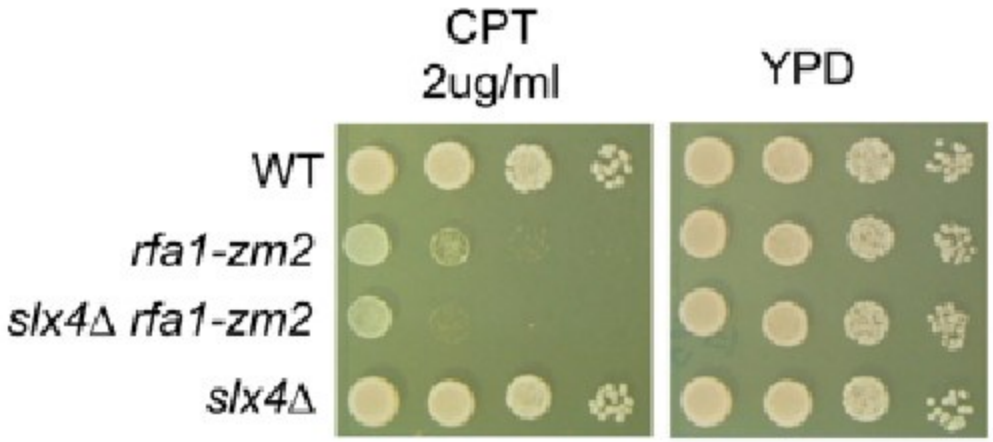
> *(2) "the srs2-SIMmut (1170 IIVID 1173 to 1170 AAAAD 1173)". "1173" should be 1174.*

Corrected.

> *(3) Can Slx4-RIM mutant rescue rfa1-zm2 CPT sensitivity?*

We found that unlike *srs2Δ*, *slx4Δ* failed to rescue *rfa1-zm2* CPT sensitivity (picture on the right). On the other hand, *slx4Δ* counteracts Rad9-dependent Rad53 activation as shown by Ohouo et al (2013).

Author response image 1.



> *(4) One genotype (rfa1-zm2 srs2-3KR) is missing in Figure 5B.*

Corrected.

> *(5) In Fig. S2C, FACS plots do not match the bar graph (see major concern 3).*

Corrected and is described in more detail in Major Concern #3.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *Figure 1. The colors in A are not well-conserved in B.*

Colors for *srs2-7AV* and *-2SA* in panel B are now matched with those in panel A.

> *Figure 2. Is srs2-SIMmut the same as srs2-sim?*

This mutant allele is now referred to as *srs2-SIM*^{mut} throughout the text and figures.

> *The suppression of rfa1-zm2 and (less strongly) rfa-t33 by the Srs2 mutants is interesting. Based on previous data, the suppression is apparently mutual, though it isn't shown here, unless we misunderstand.*

We have previously shown that *rfa1-zm2* and *srs2Δ* showed mutual suppression (Dhingra et al 2021 PNAS) and have included an example in Figure S1A. Unlike *srs2Δ*, *srs2-ΔPIM* and *-3KR* showed little damage sensitivity and DDC defects, likely due to the compensation by the Slx4-mediated checkpoint dampening (detailed in the Public Review section). Suppression is not applicable toward mutants lacking a phenotype, though the mutants could confer suppression when there is a functional relationship with another mutant, as we see here toward *rfa1-zm2*.

> *Is Srs2 interaction with PCNA dependent on its ubiquitylation or SUMO? Does PCNA mutant K164R mimic this mutation? (this may well be known; our ignorance).*

It was known that Srs2 can bind unmodified PCNA, though SUMO enhances this interaction; however, a very small percentage of PCNA is sumoylated in cells and PCNA sumoylation

affects both Srs2-dependent and independent processes (e.g., (Papouli *et al*, 2005). As such, the genetic interaction of K164R with *rfa1-zm2* can be difficult to interpret.

> *Why srs2-7AV or srs2-sim make* rfa1-zm2 *even more sensitive is also not obvious. The authors take refuge in the statement that Srs2 "has multiple roles in cellular survival of genotoxic stress" but don't attempt to be more precise.*

Our understanding of *srs2-7AV* and *-sim* is limited; thus, more specific speculation cannot be made at this time.

> *Figure 3. It is striking (Figure 3A) that all the cells have reached G2 an hour after releasing from alpha-factor arrest, even though presumably CPT treatment must impair replication. It is even more striking that there is apparently no G2/M arrest in the presumably damaged cells as the WT (Figure 3B) has the most rapid progression through the cell cycle. How does this compare with cells in the absence of CPT? The idea that CPT is triggering Rad53-mediated response is hard to understand if there is in fact no delay in the cell cycle. Instead, the several mutants appear to delay re-entry into S... Or maybe it is actually an exit from G2/M?*
>
> *This phenomenon needs a better explanation.*

CPT does not induce the DNA replication checkpoint nor S phase delay, explaining apparent G2 content by the one hour time point; however, CPT does induce the DNA damage checkpoint, and a delay (not arrest) in G2/M (Menin *et al.*, 2018; Redon *et al.*, 2003; Tercero *et al.*, 2003). We confirmed these findings. In our hand, wildtype G1 cells released into the cell cycle in the absence of CPT complete the first cell cycle within 80 minutes, such that most cells are in the second G1 phase by 90 min. In contrast, when wild-type cells were treated with CPT, G2/M exit was only partial at 120min (e.g., Figure 3B). These features differentiate CPT treatment from MMS treatment, which induces both types of checkpoints and lengthening the time that cells reach G2. We have highlighted this unique feature of CPT in checkpoint induction.

> *What is "active Rad53"? If the authors mean they are using a phospho-specific Ab versus Rad53, they should explain this. It's impossible to know if total Rad53 is altered from Figure 3A. A blot with an antibody that detects both phosphorylated and nonphosphorylated Rad53 would help.*

The F9 antibody used here detects phosphorylated Rad53 forms induced by Mec1 activation and does not detect unphosphorylated Rad53 (Fiorani *et al*, 2008). We changed “active Rad53” to “phosphorylated Rad53”. We used Pgk1 as a loading control to ensure equal loading, which help to quantify the relative amount of “active Rad53” in cells. This method has been used widely in the field.

> *Also is there a doublet of Rad53 in the right two lanes and in WT? Rad53 often shows more than one slowmigrating species, so this isn't necessarily a surprise. Were both forms used in quantitation?*

Both forms are used for quantification.

> *Figure 4A. Is there a di-SUMO form above the band marked Srs2-Su? Is this known? Is it counted?*

Mono-sumoylated form of Srs2 is the most abundant form of sumoylated Srs2, though we detected a sumoylated Srs2 band that can represent its di-sumo form. We did quantify both

forms in the plot.

> *B. The dip at 1.5 h in Rad9-P is curious. It would be useful to know what % of Rad9 is phosphorylated in a repair-defective (rad52?) background with CPT treatment. And would such rad52 cells show a long arrest?*

This dip is reproducible and may reflect that a population of cells escape G2/M delay at this timepoint.

> *Figure 5. It seems clear that the autophosphorylation site of Mec1, which was implicated in turning off a longdelayed G2/M arrest has no effect here, but presumably, a kinase-dead Mec1 (or deletion) does? The idea that a checkpoint is being regulated seems to come more from an assumption than from any direct data; as noted above, the only apparent delay in the cell cycle is the re-entry into S. There clearly is Rad53 and Rad9 phosphorylation so there are the attributes of a checkpoint. If PI3KK phosphorylation is important, can this be accomplished by Tel1 as well as Mec1?*

A *mec1* helicase dead or null would not activate the checkpoint at the first place, therefore will not be useful to address whether Mec1 autophosphorylation is implicated in turning off checkpoint. A recent study from the Haber lab provided evidence that Mec1 autophosphorylation at S1964 helps to turn off the checkpoint in a DSB situation (Memisoglu *et al*, 2019). The role of Tel1 in checkpoint dampening will be interesting to examine in the future.

> *Figure 6. Two Rfa1 phospho-sites don't appear to be important, but do the known multiple phosphorylations of Rfa2 play a role?*

Figure 6D examined three Rfa2 phosphorylation sites and found no genetic interaction with *srs2Δ*.

> *Summary: There are a lot of interesting data here, but they don't strongly support the author's model in the absence of a more direct way to monitor RPA binding and removal. This could be done using some sitespecific damage, but hard to do with CPT or MMS (which themselves don't appear to have the same effect). The abstract suggests Srs2 is "temporally and spatially regulated to both allow timely checkpoint termination and to prevent superfluous RPA removal." But where is the checkpoint termination if there's no evident checkpoint? And "superfluous" is probably not the right word (= unnecessary); probably the authors intend "excessive"? As noted above, it also isn't clear if the displacement is of RPA or of Rad51, which normally replaces RPA and which is well-known to be itself displaced by Srs2. Again, if CPT is causing enough damage to kill orders of magnitudes of cells (are the plate and liquid concentrations comparable, we suddenly wonder) then why isn't there some stronger evidence for a cell cycle response to the DDC?*

As described in the Public Review section, we have previously shown that a lack of Srs2-mediated checkpoint downregulation leads to a 4-6 fold increase of RPA on chromatin, which was rescued by *rfa1-zm2* (Dhingra *et al.*, 2021). On its own, *rfa1-zm2* did not cause defective chromatin association in our assays, despite modestly reducing ssDNA binding in *vitro* (Dhingra *et al.*, 2021). This discrepancy could be due to a lack of sensitivity of chromatin fractionation assay in revealing moderate changes of RPA residence on DNA. Considering this, we decided to employ functional assays (Figure 2-3) that are more effective in identifying the specific Srs2 features pertaining to RPA regulation.

We respectfully disagree with the reviewer's point that there is "no evident checkpoint" in CPT. Previous studies have shown that CPT induces the DNA damage checkpoint as evidenced by Mec1 activation and phosphorylation of Rad53 and Rad9, and delaying exit from G2/M (Dhingra *et al.*, 2021; Menin *et al.*, 2018; Redon *et al.*, 2003). Our data are fully consistent with these reports. It is important to note that DNA damage checkpoint can manifest at a range of strengths depending on the genotoxic conditions and treatment, but the fundamental principles are the same. For example, we found that the Srs2-RPA antagonism not only affects the checkpoint downregulation in CPT, but also does so in MMS treatment and in a DSB system. We focused on CPT condition in this work, since CPT only induces the DNA damage checkpoint but not DNA replication checkpoint while MMS induces both. Further investigating the Srs2-RPA antagonism in a DSB system can be interesting to pursue in the future.

We believe that "superfluous removal" is appropriately used when discussing RPA regulation at genomic sites wherein it supports ssDNA protection and DNA repair, rather than DDC. Examples of these sites include R-loops and negatively supercoiled regions. These sites lack 3' and 5' DNA ends at the ss-dsDNA junctions for loading PCNA and the 9-1-1 checkpoint factors, and thus are not designated for checkpoint regulation.

We addressed the reviewer's point regarding Rad51 in the Public Review section. We disagree with reviewer's view that "Rad51 normally replaces RPA". RPA is involved in many more processes than Rad51 wherein it is not replaced by Rad51.

Regarding toxicity of CPT, our view is that it stems from a combination of checkpoint regulation and other processes that also involve the Srs2-RPA antagonism. While this work focused on the checkpoint aspect of this antagonism, future studies will be conducted to address the latter.

> *One reference is entered as Lee Zhou and Stephen J. Elledge as opposed to "Zhou and Elledge."*

Corrected.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *(1) It would be nice to see the additional point mutants (srs2-Y775A, srs2-F891A) be tested, as they showed little to no phenotypes in the previously reported analyses, which did not specifically test the function surveyed here.*

This point is addressed in the Public Reviews section.

> *(2) Maybe the caveat of using deletion versus point mutations could be discussed.*

This point is addressed in the Public Reviews section.

> *(3) Please plot individual data points of the two independent experiments in Figures 4D and 5A so that the reader can evaluate reproducibility. N=2 does not really allow deriving SD.*

This point is addressed in the Public Reviews section and three individual data points are now included in both panels.

> *(4) It will help the reader to have the exact strains used in each experiment listed in each figure legend. Minor point.*

The strain table is now updated to address this point.

> *(5) Page 7 middle paragraph: The reference to Figure 4A in line 11 should probably be Figure S3A.*

Corrected.

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