Introduction

The posttranslational modification of proteins with ubiquitin and Small Ubiquitin-like MODifier (SUMO) plays an important role in promoting and coordinating DNA repair [1,2,3]. SUMO conjugation to proteins can modulate their DNA binding, enzymatic turnover, interaction with other proteins, subcellular localization and stability [4,5,6,7,9]. SUMO is covalently attached to conserved lysine residues of target proteins by an enzymatic cascade, which involves an activating enzyme (E1), a conjugating enzyme (E2) and a protein ligase (E3) [10]. In the fission yeast *Schizosaccharomyces pombe* two SUMO E3 protein ligases, Pli1 and Nse2, have been identified [11,12]. Pli1 is the major SUMO ligase being responsible for most of the SUMO conjugates detected in cell extracts [13]. It is important for telomere maintenance, but not for the repair of genotoxin-induced DNA damage [12,13]. Nse2 is part of the Snc5-Snc6 complex and promotes DNA repair [14].

The discovery of the SUMO-targeted ubiquitin ligase (STUbL) Skx8 revealed that there is interplay between the SUMO and ubiquitylation pathways. Skx8 was shown to ubiquitylate SUMOylated proteins to mark them for proteasomal degradation [13,15,16,17,18,19,20,21]. This function appears to play an important role in ensuring genome stability during DNA replication, since Skx8 colocalizes with PCNA in replication foci and limits recombination at the programmed replication fork barrier of the rDNA locus [22]. Recent work in the fission yeast *Schizosaccharomyces pombe* demonstrated that Nse2/Skx8 mediated SUMOylation/Ubiquitylation functions to suppress spontaneous Topoisomerase I (Top1) mediated genome instability [23].

Top1 plays an important role in the relaxation of supercoiled DNA that forms ahead of both the replication and transcription machinery [24]. It does this by cleaving one DNA strand to generate a covalent protein-DNA intermediate, the so-called Top1cc, which can then rotate around the intact complementary strand. Rounds of strand rotation are usually followed by the re-ligation of the single strand nick, however, in the presence of DNA lesions, such as single-strand breaks and abasic sites, or the Top1 poison camptothecin (CPT), re-ligation is inhibited resulting in the persistence of the Top1cc, which in turn can inhibit transcription and lead to replication fork stalling and chromosome breakage [25,26,27]. Thus mechanisms for the removal of trapped Top1cc are essential for ensuring genome stability. In *S. pombe* processing of Top1cc appears to rely on either the tyrosyl-DNA phosphodiesterase Tdp1 or a pathway involving Nse2, Skx8 and the SUMO mimetic Rad60, which are thought to somehow promote the activity of the nucleotide excision repair endonuclease Rad16-Swi10 in removing Top1cc [23].

Top1 is SUMOylated by Pli1, however Pli1 is seemingly not required for processing Top1cc and the function of this SUMOylation in fission yeast remains unclear [23]. Intriguingly the presence of Top1 without Pli1 (or in budding yeast Siz1 and...
Siz2) engenders a dependency on homologous recombination (HR) factors, including Rad51, for cell viability [28,29]. This suggests that SUMOylation of Top1 and/or other proteins is needed to govern a Top1-dependent process, which otherwise necessitates the need for HR. Interestingly Pli1-dependent SUMOylation also necessitates a requirement for Siz8 to prevent the accumulation of toxic SUMO chains [13,20].

Here we confirm that the presence of Top1 results in a need for Pli1-dependent SUMOylation to limit spontaneous recombination. Intriguingly, Pli1 SUMOylation is dispensable at a programmed replication fork barrier RTS1, which is a potential recombination hotspot [30,31,32,33,34]. This suggests that in the absence of Siz8, SUMOylated proteins, including Top1, accumulate in a Pli1-dependent manner. Although the failure to process SUMOylated Top1 by Siz8 is not the only cause of the heightened spontaneous genome instability and reduced cell viability, it significantly contributes to elevated recombination levels when forks stall at the programmed replication barrier RTS1.

Materials and Methods

S. pombe strains

S. pombe strains are listed in Table 1. The slx8Δ deletion strain was made by PCR-based gene targeting [35].

Media and genetic methods

Media and genetic methods followed standard protocols [36]. The complete and minimal media were yeast extract with supplements (YES) and Edinburgh minimal medium plus 3.7 mg/ml sodium glutamate (EMMG) plus appropriate amino acids (0.25 mg/ml), respectively. Low adenine media (YELA) was supplemented with 0.01 mg/ml adenine. Ade+ recombinants were selected on YES lacking adenine and supplemented with 0.2 mg/ml guanine to prevent uptake of residual adenine.

Spot assays

Exponentially growing cells from liquid cultures were harvested, washed and resuspended in water at a density of $1 \times 10^7 \text{ cells/ml}$. Aliquots (10 μl) of the cell suspensions were spotted onto agar plates containing genotoxins as indicated. For UV, plates were irradiated using a Stratalinker (Stratagene). Plates were photographed after 5–7 days growth at 25°C, 30°C or 37°C as indicated.

Microscopy

Cells from asynchronously growing cultures were fixed with 70% ethanol and stored at 4°C for later analysis. Fixed cells were rehydrated, stained with DAPI and then analysed using an Olympus BX50 epifluorescence microscope equipped with the appropriate filter set to detect blue fluorescence (Chroma Technology Corp., VT). Black and white images were acquired with a CoolSNAP HQ CCD camera (Photometrics, AZ) controlled by MetaMorph software (v7.7.3.0, Molecular Devices Inc., CA).

Recombination assays

The direct repeat recombination assay was performed as described [30,37,38]. Two sample t tests were used to determine the statistical significance of differences in recombination values between strains.

Western blots

Whole-cell protein extracts were made from asynchronously growing yeast cultures as described [39]. Western blots were probed with rabbit anti-Pmt3 (a gift from F. Watts), mouse anti-tubulin (Sigma), and mouse anti-C-myc (Sigma) antibodies as indicated.

2D gels

The protocol for analysis of replication intermediates by 2D gel electrophoresis has been described previously [40].

Results

Pli1-dependent SUMOylation in the absence of Siz8 results in reduced cell viability, hypersensitivity to genotoxins and defects in chromosome segregation

The biological importance of Siz8 in S. pombe has mainly been investigated using a hypomorphic temperature-sensitive mutant, and therefore experiments have involved a temperature shift to study a partially impaired Siz8 protein at the restrictive temperature (36°C) [13]. Heat shock can induce SUMOylation and may lead to the formation of specific SUMO conjugates as a stress response [41,42]. Whether Siz8 preferentially targets such stress-induced SUMO conjugates or SUMO conjugates in general is not entirely clear. To be able to characterize the effects of Siz8 deficiency on SUMOylation at 30°C we constructed a strain in which the siz8Δ gene was fully deleted. The siz8Δ mutant is viable but exhibits slow growth, elongated cells, temperature sensitivity (at 37°C) and hypersensitivity to ultraviolet light (UV), hydroxyurea (HU), CPT and the alkylating agent methyl methanesulfonate (MMS) (Fig. 1A and B). Strikingly, deletion of pli1 in the siz8Δ background fully or partially suppressed all of these phenotypes (Fig. 1A and B). Analysis of cells stained with the DNA-specific dye DAPI revealed a high percentage of binucleate and septated siz8Δ cells with abnormalities, including cut phenotypes, missegregated chromosomes and multinucleated cells (Fig. 1C and D). Again these phenotypes are largely suppressed by deleting pli1 (Fig. 1D). Western blot analysis of whole-cell extracts from asynchronously growing yeast cultures showed an accumulation of SUMOylated protein conjugates in siz8Δ cells compared to wild-type (Fig. 2A). In pli1Δ cells SUMOylation was barely detectable, which is consistent with an earlier report [43]. Likewise SUMOylation was virtually absent in the pli1Δ siz8Δ double mutant (Fig. 2A). These results suggest that Siz8 is needed to remove Pli1-dependent SUMO conjugates under normal growth conditions, and in its absence these SUMO conjugates accumulate and cause toxicity.

Loss of fitness in nse2-SA siz8Δ mutant cells correlates with aberrant hyper-SUMOylation

Next we investigated whether deficiency in Nse2-mediated SUMOylation also alleviates the poor growth and genotoxic sensitivity of siz8Δ cells. As Nse2 is an essential protein we made use of the Nse2-SA mutant, which is deficient in SUMO conjugation [14]. Unlike the pli1Δ siz8Δ double mutant, the nse2-SA siz8Δ double mutant exhibits a synergistic reduction in growth and increased hypersensitivity to CPT and MMS when compared to its parental single mutant strains (Fig. 2B). Intriguingly when we analysed the level of SUMO conjugates in the double mutant we observed that they accumulated to even higher levels than in a siz8Δ single mutant (Fig. 2A). This suggests that in the absence of Nse2, Pli1-dependent SUMOylation is further stimulated and may even start to act on Nse2 target proteins. This aberrant hyper-SUMOylation is probably the cause of the severe growth defect and genotoxic sensitivity of the nse2-SA siz8Δ double mutant.
Pli1 and Slx8 Promote Genome Stability

Previous studies have shown that deletion of either slx8 or $slx8^2$, which encode SUMO E3 ligases, results in increased levels of spontaneous mitotic recombination in budding yeast [22,29]. Similarly loss of Pli1 results in hyper-recombination in fission yeast [44]. However, the $slx8^2$ temperature sensitive mutant ($slx8^2-1$) has only been tested at its permissive temperature where it exhibits no significant change in direct repeat recombination compared to wild-type [23]. To clarify the relative importance of Pli1-dependent SUMOylation and Slx8-dependent SUMO-targeted ubiquitylation for limiting recombination in fission yeast we used strains harbouring a direct repeat of ade6$^+$ heteroalleles with an intervening $his3^+$ gene and RTS1 element (Fig. 3A) [30,37]. In these strains the frequency of recombination between the two copies of ade6 can be monitored by the appearance of Ade$^+$ prototrophs, which arise either from a gene conversion event or from a deletion event, with the former being distinguished from the latter by the retention of the $his3^+$ gene. RTS1 is a strong polar replication fork barrier (RFB), which when positioned in orientation 2 blocks replication forks traversing the ade6 locus causing a large increase in direct repeat recombination (Fig. 3A) [30,37]. However, due to the placement of replication origins, the ade6 locus is replicated unidirectionally and therefore when positioned in orientation 1 RTS1 does not impede the passage of the replication fork and consequently has no effect on the local frequency of recombination [30,37,45]. In wild-type cells with the ade6-L469 and nse2-SA::ura4 double mutants, both the conversion-type and deletion-type prototroph frequencies are increased (conversion-type to deletion-type ratio 1.5:8.5) (Fig. 3B and Table S1). The conversion-type to deletion-type ratio is 2 in 10,000 viable cells with a $P<0.01$ increase in Ade$^+$ prototroph frequency, whereas Pli1 and Slx8 deficient cells showed an approximately 12-fold ($P<0.01$) increase in Ade$^+$ prototroph frequency as compared to wild-type. The increase in Ade$^+$ prototroph frequency is independent of Rad52 (formerly known as Rad22) [46].

Table 1. $S. pombe$ strains used in this study.

| Strain          | Relevant genotype                           |
|-----------------|---------------------------------------------|
| MCW1221         | $h^+ uro4-D18 leu1-32 his3-D1 arg3-D4       |
| FO986           | $h^+ slx8A::kanMx6 ura4-D18 leu1-32 his3-D1 arg3-D4 |
| MCW4568         | $h^+ pli1::ura4$ ura4-D18 leu1-32 his3-D1 arg3-D4 |
| MCW4688         | $h^+ pli1::ura4$ slx8A::kanMx6 ura4-D18 leu1-32 his3-D1 arg3-D4 |
| MCW5057         | $h^+ nse2-SA::ura4$ ura4-D18 leu1-32 his3-D1 arg3-D4 |
| MCW5122         | $h^+ nse2-SA::ura4$ slx8A::kanMx6 ura4-D18 leu1-32 his3-D1 arg3-D4 |
| MCW5663         | $h^+ pli1::kanMx6 nse2-SA::ura4$ ura4-D18 leu1-32 his3-D1 arg3-D4 |
| MCW6514         | $h^+ top1::natMx4 slx8A::kanMx6 ura4-D18 leu1-32 his3-D1 arg3-D4 |
| MCW6516         | $h^+ top1::natMx4 ura4-D18 leu1-32 his3-D1 arg3-D4 |
| MCW4712         | $h^+ uro4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3$ RTS1 site A orientation 1 ade6-L469 |
| MCW4713         | $h^+ uro4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3$ RTS1 site A orientation 2 ade6-L469 |
| MCW4774         | $h^+ pli1::ura4$ ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3$ RTS1 site A orientation 1 ade6-L469 |
| MCW4776         | $h^+ pli1::ura4$ ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3$ RTS1 site A orientation 2 ade6-L469 |
| MCW5131         | $h^+ nse2-SA::ura4$ ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3$ RTS1 site A orientation 1 ade6-L469 |
| MCW5133         | $h^+ nse2-SA::ura4$ ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3$ RTS1 site A orientation 2 ade6-L469 |
| MCW5463         | $h^+ pli1::kanMx6 nse2-SA::ura4$ ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3$ RTS1 site A orientation 1 ade6-L469 |
| MCW5466         | $h^+ pli1::kanMx6 nse2-SA::ura4$ ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3$ RTS1 site A orientation 2 ade6-L469 |
| MCW4852         | $h^+ slx8A::kanMx6 ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3$ RTS1 site A orientation 1 ade6-L469 |
| MCW4828         | $h^+ slx8A::kanMx5 ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3$ RTS1 site A orientation 2 ade6-L469 |
| MCW4830         | $h^+ pli1::ura4+ slx8A::kanMx6 ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3$ RTS1 site A orientation 1 ade6-L469 |
| MCW4832         | $h^+ pli1::ura4+ slx8A::kanMx6 ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3$ RTS1 site A orientation 2 ade6-L469 |
| MCW5650         | $h^+ top1::natMx4 ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3$ RTS1 site A orientation 1 ade6-L469 |
| MCW6093         | $h^+ top1::natMx4 ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3$ RTS1 site A orientation 2 ade6-L469 |
| MCW5631         | $h^+ top1::LEU2$ pli1::ura4$ ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3$ RTS1 site A orientation 1 ade6-L469 |
| MCW5633         | $h^+ top1::LEU2$ pli1::ura4$ ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3$ RTS1 site A orientation 2 ade6-L469 |
| MCW6549         | $h^+ top1::natMx4 slx8A::kanMx6 ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3$ RTS1 site A orientation 1 ade6-L469 |
| MCW6551         | $h^+ top1::natMx4 slx8A::kanMx6 ura4-D18 leu1-32 his3-D1 arg3-D4 ade6-M375 int::pUC8/his3$ RTS1 site A orientation 2 ade6-L469 |
| MCW5987         | $h^+ top1::myc::natMx4 ura4-D18 leu1-32 his3-D1 arg3-D4 |
| MCW6242         | $h^+ top1::myc::natMx4 pli1::ura4$ ura4-D18 leu1-32 his3-D1 arg3-D4 |
| MCW6345         | $h^+ top1::myc::natMx4 slx8A::kanMx6 ura4-D18 leu1-32 his3-D1 arg3-D4 |
| MCW6284         | $h^+ top1::myc::natMx4 pli1::ura4$ slx8A::kanMx6 ura4-D18 leu1-32 his3-D1 arg3-D4 |

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However, we were unable to explore the genetic dependency of the hyper-recombination in a slx8Δ mutant as both slx8Δ rad51Δ and slx8Δ rad52Δ double mutants were not viable (data not shown). The pli1Δ nse2-SA and pli1Δ slx8Δ double mutants produced a similar Ade<sup>+</sup> prototroph frequency and conversion-type to deletion-type ratio as the pli1Δ single mutant (Fig. 3B and Table S1). These results indicate that Pli1-dependent SUMOylation and Slx8-dependent processing of SUMO conjugates function in a common pathway for limiting spontaneous recombination, with the former process being more important than the latter for restricting the overall number of recombinants that are formed. In contrast Nse2-dependent SUMOylation appears to have relatively little impact on the frequency of direct repeat recombination.

Slx8 promotes genome stability at the polar replication fork barrier RTS1

Having established that both Pli1 and Slx8 play an important role in limiting spontaneous recombination we next tested whether they are similarly important for restricting recombination that is induced by replication fork blockage at RTS1. In line with previous data RTS1 in orientation 2 causes a ~65-fold increase in Ade<sup>+</sup> frequency compared to the spontaneous level of recombination, with slightly more than half of the recombinants being conversion-types [30] (Fig. 3C and Table S1). Similar to what we observed for spontaneous recombination the nse2-Δ1 mutant exhibited no significant change in recombinant frequency, whereas the slx8Delta mutant showed a ~2-fold increase (P<0.01) with a slight bias towards deletion-type recombinants (P<0.01) (Fig. 3C and Table S1). Surprisingly, and in marked contrast to its effect on spontaneous recombination, deletion of pli1 had little effect on the level of RTS1-induced recombinants (Fig. 3C and Table S1). The same is true for a pli1 nse2-SA double mutant indicating that the lack of a marked effect is not due to redundancy between the two E3 ligases (Fig. 3C and Table S1). A previous study found a dependency on SUMO for establishment/maintenance of the RTS1 RFB [47]. However, analysis of replication fork blockage at RTS1 by native two-dimensional gel electrophoresis showed that neither pli1Δ nor nse2-Δ1 (either as single or double mutants) caused a significant reduction in barrier strength (Fig. 4A–D). Importantly, the hyper-recombination in a slx8Δ mutant is reduced to wild-type levels by deletion of pli1, and again this
SUMOylated Top1 accumulates in a slx8Δ mutant

The aforementioned data imply that Pli1-dependent SUMOylation prevents Top1 from causing a need for HR. One way it could achieve this is by marking Top1 for degradation through the Slx8-dependent pathway. This idea has been largely discounted as western blot analysis showed that SUMOylated Top1 does not accumulate in a slx8Δ temperature sensitive mutant [23]. However, using a strain expressing Myc-tagged Top1 we were able to detect SUMO conjugated Top1 in a slx8Δ mutant, but not in wild-type, pli1Δ or pli1Δ sld2Δ strains (Fig. 6). This suggests that Pli1-dependent SUMOylation does target Top1 for Slx8-dependent degradation. Interestingly we also observed that tubulin, which was used as a loading control and is known to be SUMOylated in budding yeast and human cells [48,49], likewise accumulates in a high molecular weight SUMOylated form in a slx8Δ mutant, and that this is again dependent on Pli1 (Fig. 6).

Removal of Top1-SUMO conjugates by Slx8 helps to limit local hyperrecombination at RTS1

If the removal of Pli1-dependent Top1-SUMO conjugates by Slx8 promotes genome stability, then, similar to pli1Δ, the hyper-recombination of a slx8Δ mutant should be suppressed by deleting top1. Indeed, the deletion of top1 does partially suppress (P<0.05) the increased RTS1-induced recombination in a slx8Δ mutant (Fig. 5B and Table S1), and this effect is not due to any reduction in RTS1 barrier strength (Fig. 4C–D). In contrast, the spontaneous Ade6 recombinant frequency and ratio of deletion- to conversion-types is essentially the same in slx8Δ and slx8Δ top1Δ strains (Fig. 5A and Table S1). Moreover, apart from CPT hypersensitivity, deletion of top1 does not suppress the poor growth and genotoxin hypersensitivity of a slx8Δ mutant (Fig. 7). These data indicate that removal of Top1-SUMO conjugates contributes to limiting local hyper-recombination at a programmed replication fork barrier but that accumulating Top1-SUMO conjugates are not the major cause of the spontaneous recombination and poor growth observed in Slx8 deficient cells.

Discussion

We have shown that Pli1’s role in limiting spontaneous direct repeat recombination is needed only in the presence of Top1, and is also negated in the vicinity of a blocked replication fork. Like Pli1, Slx8 is required for constraining direct repeat recombination, however this role is largely independent of Top1, and instead relates to a need for preventing the accumulation of Pli1-dependent SUMO conjugates.

How does Pli1 limit spontaneous recombination?

How Pli1-dependent SUMOylation limits recombination in the presence of Top1 is unclear. One possibility is that SUMOylation of Top1 and/or other factors is needed for the removal of Top1cc, which could otherwise lead to replication fork stalling and breakage necessitating repair by HR involving both the Mus81-Eme1 structure-specific endonuclease and Rad51 [50,51,52]. Consistent with this model Pli1 deficient cells are dependent on Mus81 and Rad51 for viability in the presence of Top1 [11,13,23]. However, at present there is no direct evidence that Pli1 is involved in the removal of Top1cc, which in fission yeast appears to depend on either Tdp1 or a pathway involving Nse2, Rad60 and Slx8 [23]. In humans SUMOylation of Top1 is enhanced when it is trapped in the cleavage complex, and this might, along with other potential functions, play a role in helping to target its ubiquitination and subsequent degradation by the proteasome thereby enabling access for other DNA repair factors to repair the
Figure 3. Spontaneous and RTS1-induced direct repeat recombination in cells deficient for SUMOylation and/or STUbL activity. (A) Schematic of the ade6^− direct repeat on chromosome 3 and two classes of Ade^+ recombinant. (B and C) Ade^+ recombinant frequencies (left panels) and the percentage of recombinants that are conversion types (right panels). The strains are MCW4712, MCW4774, MCW5131, MCW5463, MCW4826, MCW4830, MCW4713, MCW4776, MCW5133, MCW5466, MCW4828 and MCW4832. Error bars are the standard deviations about the mean. doi:10.1371/journal.pone.0071960.g003
Localized suppression of Pli1’s antirecombinogenic role by RTS1

One of the intriguing results of our study is the localized suppression of pli1Δ hyper-recombination by replication fork blockage at RTS1. How this is achieved is unclear, but presumably involves either a localized reduction in the formation of Top1ccs or activation of an alternative pathway for limiting their recombinogenic impact. One way in which Top1cc formation could be reduced is if replication fork blockage at RTS1 allows the dissipation of positive supercoils that build up ahead of the advancing fork. For example, cleavage of the stalled fork by Mus81-Eme1 would provide an alternative for Top1-mediated relaxation of supercoiled DNA [52]. However, in wild-type cells only a very small percentage of forks blocked at RTS1 are subject to breakage [30,33]; most are either resolved by passive replication from the opposing fork or restarted by a DSB-independent recombination process [31,38,40,45]. Moreover, if there was a significant increase in fork breakage in a pli1Δ mutant we would expect to see an increase in recombinant formation and a greater proportion of deletion-types [22, unpublished data].

SIX8’s role in suppressing spontaneous recombination

Unlike Pli1, SIX8’s main role in promoting genome stability is not to prevent unscheduled HR brought about by Top1 activity. This finding appears incongruous with the idea that Pli1’s key role in preventing HR might be to target Top1 for SIX8-dependent degradation. However, it is conceivable that an overall increase in Pli1-dependent SUMO-conjugates in a pli1Δ mutant could result in the dissipation of positive supercoils that build up ahead of the advancing fork. For example, cleavage of the stalled fork by Mus81-Eme1 would provide an alternative for Top1-mediated relaxation of supercoiled DNA [52]. However, in wild-type cells only a very small percentage of forks blocked at RTS1 are subject to breakage [30,33]; most are either resolved by passive replication from the opposing fork or restarted by a DSB-independent recombination process [31,38,40,45]. Moreover, if there was a significant increase in fork breakage in a pli1Δ mutant we would expect to see an increase in recombinant formation and a greater proportion of deletion-types [22, unpublished data].

Figure 4. Analysis of replication fork blockage at RTS1 on a plasmid in cells deficient for SUMOylation, SUMOylation, STUbL activity and Top1. (A) Schematic of plasmid pREP3 containing RTS1, which is orientated so that it blocks the replication fork that approaches it from the replication origin (ars1) on its right as drawn [66]. The bottom panel shows a replication fork originating from ars1 moving toward RTS1 and the position of the probe used for the analysis in C. (B) Schematic showing the main features of the 2D gel analysis of replication intermediates in C. These are: the arc of Y-shaped replication forks (Y); replication forks blocked/paused at RTS1 (P); replication termination where two opposing forks merge (T); and a spike of X-shaped DNA molecules that represent fully replicated conjoined DNAs. (C) 2D gel analysis of replication intermediates in the PstI-SacI fragment shown in A from wild-type or mutant cells as indicated. The strains are MCW1221, MCW4568, MCW5057, FO986, MCW6516, MCW6563, MCW4688 and MCW6514. (D) Amount of replication fork blockage/pausing as a percentage of the total Y-arc relative to wild-type. Values are the means of three independent experiments. Error bars represent standard deviations.

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may be needed for the removal of SUMOylated Top1 to avoid unnecessary HR.

A note about SUMO chains

The function of SUMO chains remains largely enigmatic, and it is even unknown whether the majority of high molecular weight SUMO conjugates detected in yeast and higher eukaryotes represent chains that are attached or unattached to substrate proteins [60]. Our observation that two known SUMO targets (Top1 and tubulin) are part of the high molecular weight mass of SUMO conjugates that accumulate in a slx8Δ mutant suggests that SUMO chains may generally be attached to substrate proteins. However, whether this reflects a deliberate mechanism to regulate protein turnover in all cases, or simply a need to counter the effects of an overactive SUMOylation system is unclear [60].

Is SUMOylation needed for proficient replication restart at RTS1?

There are a number of documented examples of how SUMOylation plays a role in both promoting and controlling HR. For example, in budding yeast Ubc9- and Mms21-dependent SUMOylation influences the ability of Sgs1 and Top3 to prevent the accumulation of recombinogenic structures on replicating chromosomes [61], and in human cells PIAS1- and PIAS4-dependent SUMOylation appears to target RNF4 to promote the stepwise progression of DSB repair by mediating the turnover of RPA bound to single-stranded DNA so that BRCA2 and RAD51 can take its place [62]. However, SUMOylation does not appear to be critical for HR-mediated replication restart in fission yeast. This assertion is based on our observation that neither pli1Δ nor nse2-S4 mutant exhibit a significant reduction in the frequency of RTS1-induced recombination, which provides a readout for attempted replication restart of persistently stalled forks. It is also surprising that the deletion of both known E3 SUMO ligases does not manifest a more dramatic hyper-recombination phenotype, given that there are several proteins, which are likely to be influenced either directly or indirectly by SUMOylation, that strongly suppress RTS1-induced recombinant formation [45]. One example is the DNA helicase Srs2, which in budding yeast is recruited to stalled replication forks by SUMOylated PCNA and there acts to limit recombination by displacement of Rad51 and/or Polδ and Polη [63,64,65]. It should be noted that the majority of recombination induced by RTS1 occurs accurately between sister chromatids and is therefore “genetically silent”, whereas only a minority occurs between the two ade6 heteroalleles and can therefore give rise to a genetically detectable recombinant. It is
therefore possible that SUMOylation or a STUbL-dependent process is required for promoting both replication restart proficiency and fidelity, and the net effect of losing both these activities could be a recombinant frequency that is similar to wild-type. Clarification of this awaits further studies.

Supporting Information

Table S1  Direct repeat recombinant frequencies.  (DOC)

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Author Contributions

Conceived and designed the experiments: RS FO MCW. Performed the experiments: RS FO AL CB. Analyzed the data: RS FO AL CB MCW. Contributed reagents/materials/analysis tools: RS FO AL MCW. Wrote the paper: RS MCW.

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