The SOSS1 single-stranded DNA binding complex promotes DNA end resection in concert with Exo1

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The human SSB homologue 1 (hSSB1) has been shown to facilitate homologous recombination and double-strand break signalling in human cells. Here, we compare the DNA-binding properties of the SOSS1 complex, containing SSB1, with Replication Protein A (RPA), the primary single-strand DNA (ssDNA) binding complex in eukaryotes. Ensemble and single-molecule approaches show that SOSS1 binds ssDNA with lower affinity compared to RPA, and exhibits less stable interactions with DNA substrates. Nevertheless, the SOSS1 complex is uniquely capable of promoting interaction of human Exo1 with double-strand DNA ends and stimulates its activity independently of the MRN complex *in vitro*. Both MRN and SOSS1 also act to mitigate the inhibitory action of the Ku70/80 heterodimer on Exo1 activity *in vitro*. These results may explain why SOSS complexes do not localize with RPA to replication sites in human cells, yet have a strong effect on double-strand break resection and homologous recombination.

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Introduction

DNA double-strand breaks (DSBs) are induced by exogenous factors such as ionizing radiation (IR), and genotoxic agents as well as endogenous factors including replication fork collapse and oxidative stress. Eukaryotic cells possess two primary mechanisms to repair DNA DSBs: non-homologous end joining (NHEJ) and homologous recombination (HR) (Symington and Gautier, 2011). In vertebrates, NHEJ is the major pathway for DSB repair that occurs in all phases of the

cell cycle and repairs DSBs by direct joining of the broken DNA ends. In contrast, HR occurs primarily in the S and G₂ phases of the cell cycle and is initiated by processing of the 5' strand at DSB ends to produce a 3' single-stranded DNA (ssDNA) that is essential for Rad51 filament formation and strand invasion.

The molecular mechanisms of 5' strand processing are well understood in bacteria where the RecBCD pathway and the RecQ/RecJ pathway exist to recognize DSBs and process the 5' strand of DSB ends to generate 3' ssDNA for subsequent HR repair (Dillingham and Kowalczykowski, 2008). RecBCD does not exist in eukaryotes, but several partially redundant pathways function to perform resection of 5' strands (Mimitou and Symington, 2009). In budding yeast, the Mre11/Rad50/Nbs1(Xrs2) (MRN(X)) complex cooperates with the Sae2 endonuclease to initiate short-range processing of 5' strands, then the Exo1 and Dna2 exonuclease function redundantly to continue the resection of DNA ends from a few hundred to several thousand nucleotides from the original break site (Gravel *et al*, 2008; Mimitou and Symington, 2008; Zhu *et al*, 2008). *In vitro* studies with the archaeal MR complex and the eukaryotic MRN/X complex have demonstrated that Mre11/Rad50 proteins perform at least two important roles in resection: short-range endonucleolytic resection of 5' strands at DSB ends, and also stimulation of extensive resection through recruitment of 5' to 3' exonucleases and helicases (Hopkins and Paull, 2008; Nimmonkar *et al*, 2008; Nicolette *et al*, 2010; Niu *et al*, 2010).

Single-stranded DNA binding proteins (SSBs) are critical for DNA replication, recombination, DNA damage signalling, and repair in all organisms. In bacteria, SSBs are essential because of their roles in replication, but also interact with and stimulate many other factors involved in recombination and repair (Shereda *et al*, 2008). For instance, SSB was shown to stimulate the 5' to 3' exonucleolytic activity of RecJ on both ssDNA and double-stranded DNA (dsDNA) substrates *in vitro*, using proteins from *E. coli* and *H. influenzae* (Butland *et al*, 2005; Han *et al*, 2006; Handa *et al*, 2009; Sharma and Rao, 2009).

In eukaryotes, the heterotrimeric complex Replication Protein A (RPA) is considered to be the primary ssDNA-binding activity in eukaryotic cells (Wold, 1997; Richard *et al*, 2009). However, two novel SSB proteins hSSB1 and hSSB2 were recently identified in the human genome (Richard *et al*, 2008). hSSB1 exists as a member of a heterotrimeric complex called Sensor of Single-Stranded DNA complex 1 (SOSS1), together with SOSSA(INTS3) and SOSSC(C9orf80) (Huang *et al*, 2009; Skaar *et al*, 2009). Unlike RPA, SOSS1 does not localize to replication foci, suggesting that the novel SOSS1 complex functions in DNA damage repair through HR but not in DNA replication (Richard *et al*, 2008). Both RPA and SOSS1 are recruited to DNA DSB sites but, paradoxically, they exhibit low co-localization (Richard *et al*, 2008). Depletion of SOSS1 results in loss of

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checkpoint activation, increased sensitivity to IR, defects in DSB resection, and reduced HR (Richard *et al*, 2008; Huang *et al*, 2009), suggesting a role for SOSS1 in HR-mediated repair. Recent *in vitro* studies have suggested an important role for RPA in promoting DSB resection through stimulation of the nuclease-helicase complex Dna2/Sgs1 in yeast and Dna2/BLM in human cells (Nimonkar *et al*, 2008; Niu *et al*, 2010; Cejka *et al*, 2010a). However, the role of the SOSS1 complex in resection and HR-mediated repair has not been characterized and the functional relationship between SOSS complexes and RPA in regulating DSB repair is unclear. Recent reports have shown that the hSBB1 protein localizes at DSB sites very early after DNA damage and binds directly to the MRN complex, suggesting a role in the initiating stages of DSB processing (Richard *et al*, 2010, 2011).

In this study, we examine the DNA-binding properties of the SOSS1 complex in comparison to RPA, using both ensemble and single-molecule techniques. While RPA exhibits significantly higher affinity for ssDNA and a smaller minimum binding site, SOSS1 has a unique ability to stimulate DSB resection by human Exonuclease 1 (hExo1), a member of the Rad2 family of nucleases which is implicated in numerous DNA repair pathways including mismatch repair (MMR), post-replication repair, meiotic and mitotic recombination, and HR repair (Tran *et al*, 2004). SOSS1 stimulates the enzymatic activity of hExo1 on DNA by increasing hExo1 recruitment to DNA ends *in vitro*, whereas RPA specifically activates Dna2, as reported previously (Nimonkar *et al*, 2008; Niu *et al*, 2010; Cejka *et al*, 2010a). In addition, we demonstrate that MRN in concert with either SOSS1 or RPA can overcome Ku inhibition of hExo1 and Dna2. Overall, we find that SOSS1 binds DNA and affects the activity of DNA processing enzymes in a distinct manner compared to RPA, and propose that the divergent functions of these complexes can be understood in light of these differences.

Results

RPA binds ssDNA with higher affinity compared to SOSS1 T117E

Both RPA and SOSS1 are recruited to DNA DSBs although they exhibit low co-localization (Richard *et al*, 2008). Unlike RPA, SOSS1 does not localize to replication sites (Huang *et al*, 2009), indicating that RPA and SOSS1 may have different affinity and specificity for DNA substrates. To address this question, we expressed and purified human RPA and SOSS1 complexes from *E. coli* and insect cell expression systems, respectively. High level expression of the SOSS1 complex required a phosphomimic mutation at threonine 117 of SSB1 (T117E), consistent with previous observations that ATM phosphorylation at this residue stabilizes the SSB1 protein (Richard *et al*, 2008). All the experiments shown here utilize the T117E form of the SOSS1 complex. The requirement for complex formation between the SOSSA(IntS3) component and the SOSSB (hSSB1) component of SOSS1 has been disputed (Skaar *et al*, 2009), but here we found that both SOSSB and SOSSC (C9orf80) co-purify with GST-tagged SOSSA with ~1:1:1 stoichiometry, consistent with complex formation between these factors as previously reported (Huang *et al*, 2009).

With the purified recombinant complexes (Supplementary Figure S1), gel mobility shift assays were performed on

several [³²P]-labelled DNA substrates containing different lengths and configurations of ssDNA (Figure 1). Both RPA and SOSS1 complexes bound specifically to ssDNA and showed little binding to double-stranded duplex DNA (Figure 1A and B). However, RPA showed significantly higher affinity for the ssDNA overhangs compared to SOSS1, with a K_d of ~15 nM on the substrate containing a 44-nt ssDNA overhang, compared with ~45 nM for SOSS1 (Figure 1A). DNA substrates containing either 3' or 5' ssDNA overhangs were also tested, showing that neither complex exhibits a polarity preference (Figure 1B). At least two distinct RPA-ssDNA complexes were apparent in these gels, whereas the complexes of SOSS1 were not resolved under these conditions. The minimum length of ssDNA required for SOSS1 binding was ~35 nt, consistent with previous results with SSB1 protein alone (Richard *et al*, 2008), which is much larger than the 10 nt minimal binding site reported for RPA (Fanning *et al*, 2006). SOSS1 binding was also observed with shorter regions of ssDNA (28 nt) when present adjacent to duplex DNA, and RPA binding also increased in efficiency with overhangs present (Figure 1C).

Since RPA binding in cells is specific for replication foci (Huang *et al*, 2009), we also analysed a DNA substrate with an internal ssDNA gap flanked by double-stranded junctions, which recapitulates the form of DNA present at replication sites (Figure 1D and E). Both RPA and SOSS1 exhibited robust binding to this substrate, and again showed that the affinity of RPA is higher than that of SOSS1 (~7 nM for RPA compared to ~35 nM for SOSS1). Taken together, these results suggest that RPA binds to ssDNA with higher affinity than SOSS1, and also can bind more efficiently to a smaller length of ssDNA. With all the ssDNA substrates tested, it was apparent that SOSS1 exhibits marked cooperativity in binding (Hill coefficients calculated from binding data ranged from 3.0 to 6.8 depending on the length of the ssDNA and the presence of adjacent duplex), whereas RPA exhibited less cooperativity in binding ($H = 1.6$ – 3.1) (Supplementary Table S1).

Less stable, more dynamic binding of SOSS1 to ssDNA compared to RPA

To measure the binding of RPA and SOSS1 to ssDNA with greater accuracy, we employed single-molecule Fluorescence Resonance Energy Transfer (smFRET), a single-molecule method to sensitively monitor the distribution and changes of distance between a donor and an acceptor fluorophore in the range of 3–8 nm (Roy *et al*, 2008). We used partial duplex DNA molecules containing a 3' Poly (T) tail ((dT)_{m+n}; Figure 2A). The donor (Cy3) and the acceptor (Cy5) were attached to the ss-dsDNA junction and the middle of the ssDNA tail, respectively, separated by (dT)_m. A small distance between the two fluorophores would result in high FRET efficiencies and vice versa.

We first tested RPA and SOSS1 binding to a partial duplex containing a 3' 40 nt tail ((dT)₁₆₊₂₄; Cy3 and Cy5 were separated by 16 nt). A relatively high FRET peak centred at 0.53 was initially observed in the single-molecule histogram of FRET efficiency for (dT)₁₆₊₂₄ only (i.e., without any protein; Figure 2B), due to the fact that ssDNA is very flexible (Murphy *et al*, 2004). Protein binding is expected to decrease FRET via ssDNA stretching unless ssDNA wraps around the protein. After adding 200 pM to 2 nM RPA, two new peaks at low FRET efficiencies (~0.42 and ~0.29) appeared in the

Figure 1 Gel shift assays with recombinant SOSS1 T117E and RPA. (A) Gel shift assays were performed with SOSS1 complex and RPA on a 32 P-labelled substrate containing a 44-nt ssDNA 3' overhang and a 35-bp duplex region as shown. Each reaction contained 1.125, 2.25, 4.5, 9, 18, 36, or 72 nM SOSS1 complex or RPA. The reactions were separated in a native 8% acrylamide gel which was analysed by phosphorimager. (B) DNA binding assays were performed as in (A) with substrates containing a 44-nt 5' overhang, a 44-nt 3' overhang, or a 50-bp duplex DNA, and quantification of the results from a representative experiment is shown. Reactions contained 4.5, 9, 18, 36, or 72 nM SOSS1 or RPA. (C) Binding assays were performed with SOSS1 as in (A) with various lengths of radiolabelled ssDNA (28, 31, or 34 nt) or substrates containing 28, 31, or 34 nt 5' overhangs adjacent to 31 nt duplex DNA and the quantified results from a representative set of experiments are shown. (D) Binding assays were performed with SOSS1 and RPA as in (A) with a substrate containing an internal ssDNA gap as shown. Each reaction contained 2.25, 4.5, 9, 18, 36, or 72 nM of SOSS1 or RPA. Quantification of these results is shown in (E).

FRET histogram as we increased the RPA concentration, suggesting that one and two RPA bind to each $(\text{dT})_{16+24}$ DNA molecule, respectively. We recorded the time evolution of the FRET histogram after the removal of the excess unbound RPA from our sample chamber (Figure 2C). The 0.29 FRET population shifted to the 0.42 FRET population over time, indicating one of the two bound RPA dissociated from $(\text{dT})_{16+24}$. The occluded site size for RPA is 30 nt with all of the four major ssDNA-binding domains involved, but RPA can bind to 10 nt ssDNA using only two ssDNA-binding domains with a lower affinity (Fanning *et al*,

2006). $(\text{dT})_{16+24}$ that contains a 40-nt tail is hence sufficient for two RPA binding: one RPA tightly binds in the 30-nt binding mode and the other RPA weakly binds in the 10-nt binding mode. Indeed, 95 min after flushing out unbound RPA, the 0.29 FRET peak almost disappeared. Dissociation events of the second, weakly bound RPA could also be found in some single-molecule FRET-time traces (Figure 2D). In contrast, the remaining RPA likely in the 30-nt binding mode, yielding the 0.42 FRET peak, was very stable and we could not detect its dissociation even 1–2 h after flushing out unbound RPA (Figure 2C; Supplementary Figure S2B and G).

Previously, we showed that *E. coli* SSB diffuses along ssDNA based on smFRET signal fluctuations and that the diffusion time scale can be estimated by performing a cross-correlation analysis of donor and acceptor intensity-time traces (Roy *et al*, 2009; Zhou *et al*, 2011). Here, we calculated the average cross-correlation functions between the Cy3 and Cy5 intensity-time traces separately for 0.53, 0.42, and 0.29 FRET states from many (dT)₁₆₊₂₄ molecules (Figure 2H). Other than a very short time scale anti-correlation for the 0.42 FRET state, yielding a characteristic time (τ) of 5 ± 2 ms, faster than our time resolution of 30 ms, we could not observe any evidence of anti-correlated fluctuations of the donor and acceptor intensities, indicating that movement of RPA on ssDNA is either minimal or too fast to be detected within the 30-ms time resolution.

Next, we repeated the smFRET experiment for SOSS1 binding to (dT)₁₆₊₂₄. Besides the DNA only peak (~ 0.53 FRET), only one additional peak (~ 0.37 FRET) was observed at all SOSS1 concentrations (2–60 nM), suggesting that the 40-nt tail is sufficient for only one binding unit of SOSS1 (Figure 2E). After flushing out the excess unbound SOSS1, we found that SOSS1 dissociates slowly (Figure 2F; Supplementary Figure S2). Clear and large amplitude FRET fluctuations were observed in FRET-time trace for the SOSS1 bound state (i.e., 0.37 FRET state; Figure 2G). The average cross-correlation between the Cy3 and Cy5 intensity-time traces shows a single exponential function with $\tau = 163 \pm 12$ ms, suggesting that SOSS1 on ssDNA is very dynamic in the milliseconds time scale (Figure 2H).

We further tested two other partial duplex DNA molecules, (dT)₁₆₊₄₂ and (dT)₃₂₊₃₉. The cross-correlation analysis suggests that SOSS1 is also more dynamic when bound to these two DNA molecules ($\tau = 170 \pm 15$ ms for (dT)₁₆₊₄₂ and 388 ± 22 ms for (dT)₃₂₊₃₉) compared to RPA. τ , which is the measure of the time scale of the dynamics, increases as the tail length of the partial duplex increases. We could rule out transient destabilization of the duplex region by SOSS1 as a source of FRET fluctuations (Supplementary Figure S2). Therefore, it is possible that SOSS1 diffusion on the ssDNA, as was demonstrated for *E. coli* SSB (Roy *et al*, 2009; Zhou *et al*, 2011), is responsible for the observed FRET fluctuations although a definitive demonstration requires further experiments.

Neither RPA nor SOSS1 binding to ssDNA resembles the ‘closed wrapping’ structure observed in the *E. coli* SSB-DNA complexes (Lohman and Ferrari, 1994; Supplementary Figure S2). Rather, both RPA and SOSS1 binding increase the end-to-end distance of the ssDNA region to which they bind, consistent with the extended structure of RPA-ssDNA complexes in microscopy experiments (Blackwell *et al*, 1996). Additionally, SOSS1 binds to ssDNA less tightly than RPA (30 nt binding mode) (see also Supplementary Figure S2) or *E. coli* SSB (*E. coli* SSB stays bound to ssDNA in its 65 nt binding mode for 5 h after flushing out the unbound proteins) (Roy *et al*, 2009; Zhou *et al*, 2011).

SOSS1 T117E and MRN stimulate hExo1 resection of DSBs *in vitro*

SOSS1 and RPA complexes have both been shown to influence DSB resection in human cells (Richard *et al*, 2008, 2010). *In vitro*, RPA stimulates the activity of Dna2/Sgs1 complexes (Niu *et al*, 2010; Cejka *et al*, 2010a) and also

coats ssDNA once resection has occurred, prior to loading of Rad51 (Flynn and Zou, 2010). However, the role of SOSS1 in resection and how it relates to the role of RPA is unknown.

To examine if SOSS1 and RPA function directly in 5′ strand processing, *in vitro* resection assays were performed with a 4.5-kb linear DNA and purified recombinant human Exonuclease 1 (Exo1) in the presence of SOSS1 or RPA (Figure 3A). The products of the reactions were visualized by DNA stain (SYBR Green) (top panel) and by non-denaturing southern hybridization using an RNA probe complementary to a 1-kb region of the 3′ strand at one end of the DNA substrate (middle panel). To further characterize the extent of resection, quantitative PCR (qPCR) was also used to determine the level of ssDNA generated by resection at sites located 29 and 1025 nt from the DNA end (Figure 3B) as previously described (Nicolette *et al*, 2010). With limiting concentrations of Exo1 (0.375 nM under these conditions), SOSS1 complex strongly stimulated hExo1-mediated resection of the 5′ end of DNA (Figure 3A), generating 11-fold and 55-fold higher levels of ssDNA at sites located 29 and 1025 nt from the DNA end, respectively (Figure 3B). This stimulation depends on the nuclease activity of hExo1, as SOSS1 was unable to stimulate 5′ strand resection in the presence of a nuclease-deficient mutant of hExo1 (Exo1 D78A/D173A) (Lee Bi *et al*, 2002; Orans *et al*, 2011) (Figure 3A, lane 11). In contrast, RPA was not stimulatory and in fact actually limited the resection activity of Exo1 at DNA ends (Figure 3A, lane 4) similar to previous results shown with yeast Exo1 *in vitro* (Nicolette *et al*, 2010). This was also the case when higher levels of Exo1, RPA, and substrate were used in order to compare directly to previously published data (Nimonkar *et al*, 2011; Supplementary Figure S3).

Previous studies have shown the direct involvement of Mre11/Rad50 and Mre11/Rad50/ Xrs2 complexes in DSB resection (Sartori *et al*, 2007; Hopkins and Paull, 2008; Nicolette *et al*, 2010; Langerak *et al*, 2011; Nimonkar *et al*, 2011). In addition, MRN has been reported to affect RPA foci formation at DNA damage sites *in vivo* and to interact with hSSB1 to stimulate the endonuclease activity of MRN *in vitro* (Richard *et al*, 2008, 2010). This led us to further test the effect of human MRN in DNA end processing *in vitro* with Exo1 in the presence of SOSS1 and RPA complexes. In reactions performed with MRN and Exo1, MRN stimulated Exo1 activity in 5′ strand processing 6- to 18-fold, similar to our previous results with yeast MRX and Exo1 (Nicolette *et al*, 2010). SOSS1 and MRN each stimulated Exo1-mediated 5′ strand resection independently, and did not exhibit cooperative functional interactions. In contrast to a previous report with hSSB1 and MRN (Richard *et al*, 2011), we also did not observe any stimulation of MRN nuclease activity by SOSS1 (Supplementary Figure S4). These data demonstrate that SOSS1 and RPA have very different effects on Exo1 activity. SOSS1 stimulates Exo1 activity in 5′ strand resection similar to the effect of MRN, whereas RPA limits the extent of Exo1 activity in resection.

SOSS1 overcomes RPA inhibition of hExo1-mediated resection

As RPA exhibits higher affinity for ssDNA and binds more stably compared to SOSS1, we then asked how SOSS1 would

function in resection in the presence of RPA. Consistent with the results in Figure 3, SOSS1 stimulated 5' strand processing in the presence of hExo1 (0.375–1.5 nM), while RPA limited the extent of Exo1 activity (Figure 4A and B). Notably, in reactions where SOSS1 and RPA were incubated together with Exo1, the presence of SOSS1 was sufficient to overcome the limiting effect of RPA on Exo1 to a comparable extent as MRN

(Figure 4A, compare lanes 12–13 with lanes 14–15). However, addition of SOSS1 and MRN together to reactions containing Exo1 and RPA did not have any further stimulatory effect on resection (Figure 4A, lanes 16 and 17). These results suggest that SOSS1 and MRN can independently stimulate Exo1 activity and overcome the limiting effect of RPA on Exo1 to promote 5' strand resection.

SOSS1 stimulates the *exo*- and *endonuclease* activities of *hExo1*

Human *Exo1* has been reported to exhibit both 5' to 3' exonuclease activity and 5' flap structure-specific endonuclease activity (Lee and Wilson, 1999). To characterize the resection products generated by *Exo1* in the presence of SOSS1, RPA, and MRN, resection assays were performed using a 1.7-kb DNA substrate internally labelled with [³²P]. The exonuclease activity of *Exo1* was measured by quantifying the amount of radiolabelled NMP released using thin layer chromatography. *Exo1* by itself exhibits low exonuclease activity in this assay (Figure 5A, lane 2; Supplementary Figure S5). However, addition of SOSS1 stimulated the exonuclease activity of *Exo1* by ~6-fold while RPA did not increase its activity (Figure 5A, compare lane 2 with lanes 7–8; also the quantitation of triplicate experiments in Figure 5B). Similarly to the results shown in Figures 3 and 4, MRN stimulated the exonuclease activity of *Exo1* to a comparable level as that observed with SOSS1 (Figure 5A, compare lane 2 with lanes 6–7). Both SOSS1 and MRN increased the exonuclease activity of *Exo1* in the presence of RPA but their stimulatory activity was not cooperative (Figure 5A, lanes 9–12).

To examine the effects of SOSS1 and RPA on the endonucleolytic activity of *Exo1*, nuclease assays were performed using a 5' Cy3/Cy5-labelled 717 bp DNA substrate and analysed using a denaturing sequencing gel (Figure 5B). In reactions containing both SOSS1 and *Exo1*, 5' labelled products ranging from 8 to 46 nt were formed (Figure 5B, lane 3) whereas RPA with *Exo1* did not produce any oligonucleotide products (Figure 5B, lane 4). These products were dependent on the presence of *Exo1* and can only be generated by endonucleolytic activity. Taken together, these results suggest that SOSS1 complex participates in DNA DSB resection through a functional interaction with *Exo1*, and promotes both the exonuclease and endonuclease activities of this enzyme.

SOSS1 stimulates the recruitment of *hExo1* to DNA ends

SOSS1 complexes promote a dramatic increase in the enzymatic activity of *Exo1*, but the functional mechanism underlying this process remains to be determined. Previous studies with the bacterial SSB protein have shown that SSB protein stimulates *RecJ* by increasing the affinity of *RecJ* to DNA

substrates (Han *et al*, 2006; Handa *et al*, 2009; Sharma and Rao, 2009), and we previously found that MRX and Sae2 promote the binding of yeast *Exo1* to DNA ends (Nicolette *et al*, 2010). Therefore, we examined whether SOSS1 could affect *Exo1* recruitment to DNA ends by performing gel mobility shift assays using a 717-bp double-stranded PCR product internally labelled with [³²P] and also containing three azide groups at each 5' end. The substrate was incubated with nuclease-deficient *Exo1* (D78A/D173A) in the presence or absence of SOSS1 or RPA to examine DNA binding by *Exo1* in the absence of catalysis. After incubation, the reaction was exposed to 254 nm UV light to induce crosslinking of the azide groups with amino acids located close to the azide moieties, separated in a non-denaturing agarose gel, and visualized by phosphorimager analysis. Neither *Exo1* nor SOSS1 bound to this substrate efficiently when incubated alone, but incubation of the complexes together produced a distinct gel shift product that was dependent on the concentration of *hExo1* (Figure 6A, lanes 3 and 6). In contrast, RPA did not form a gel shift product with *Exo1* D78A/D173A (Figure 6A, lanes 4 and 7), suggesting that a specific interaction between SOSS1 and *Exo1* forms on dsDNA ends.

To further confirm the formation of a cooperative complex containing *Exo1* and SOSS1, a DNA binding assay was performed using a 717-bp PCR product containing biotin on the end of one 5' strand and three azide groups on the opposite 5' strand of the DNA as shown in Figure 6B. The substrate was initially incubated with magnetic streptavidin beads for immobilization, and was then incubated with *Exo1* D78A/D173A and SOSS1 proteins and crosslinked with UV as in Figure 6A. The immobilized DNA and bound protein were released from the magnetic beads under denaturing conditions, separated by SDS-PAGE, and analysed by western blot using an antibody specific for *Exo1*. This assay showed that *Exo1* alone did not bind efficiently to DNA but SOSS1 promoted the recruitment of *hExo1* to the substrate (Figure 6B). Overall, we conclude that SOSS1 stimulates DSB processing by promoting *Exo1* recruitment to DNA ends, leading to an increased activity of *Exo1* at DSBs.

SOSS1 does not stimulate the resection activity of *Dna2* *in vitro*

RPA has been shown in previous studies to stimulate DSB resection through its interaction with *Dna2* and BLM (Niu

Figure 2 Single-molecule studies of RPA and SOSS1 binding to ssDNA. (A) A schematic representation of reaction steps for single-molecule FRET measurements. *m* and *n* are in the unit of nucleotides. (B) FRET efficiency histograms for (dT)₁₆₊₂₄ DNA only, and RPA binding to (dT)₁₆₊₂₄ at different protein concentrations. Three distinct peaks centred at FRET = 0.53, 0.42, and 0.29 were observed at varying RPA concentrations (blue arrows), representing 0, 1, and 2 RPA binding to (dT)₁₆₊₂₄, respectively. (C) Time evolution of the FRET histogram for (dT)₁₆₊₂₄ after incubating with 2 nM RPA and flushing out the excess unbound RPA, showing an RPA dissociation event (the black arrow). After the event, there was still one RPA remaining bound, giving an FRET of 0.42. (D) A single-molecule FRET-time trace for (dT)₁₆₊₂₄ obtained after incubating with 2 nM RPA and flushing out the excess unbound RPA. (E) FRET efficiency histograms for (dT)₁₆₊₂₄ DNA only, and SOSS1 binding to (dT)₁₆₊₂₄ at different protein concentration. Two distinct peaks centred at FRET = 0.53 and 0.37 were observed at varying SOSS1 concentrations (blue arrows), representing 0 and 1 SOSS binding to (dT)₁₆₊₂₄, respectively. (F) Time evolution of the FRET histogram for (dT)₁₆₊₂₄ after incubating with 60 nM SOSS1 and flushing out the excess unbound SOSS1. (G) A representative single-molecule FRET-time trace for (dT)₁₆₊₂₄ obtained after incubating with 60 nM SOSS1 and flushing out the excess unbound SOSS1. At *t* = 30 s, Cy5 was photobleached. (H) Average cross-correlations of Cy3 and Cy5 intensity-time traces for (dT)₁₆₊₂₄ DNA only, one or two RPA bound, and one SOSS1 bound. Each cross-correlation was averaged over > 100 molecules. (I) Average cross-correlations of Cy3 and Cy5 intensity-time traces for other two DNA substrates, (dT)₁₆₊₄₆ and (dT)₃₂₊₃₉. The FRET-time traces for cross-correlation analysis were obtained after flushing out unbound RPA or SOSS1, in order to assure the time-resolved anti-correlation comes only from the bound proteins. Single exponential fits are also shown (solid lines).

Figure 3 SOSS1 promotes resection by hExo1. (A) Resection reactions were performed with 0.375 nM Exo1 wild-type or nuclease-deficient (D78A/D173A), 40 nM SOSS1(T117E) complex or 40 nM RPA, and 31.25 nM wild-type MRN. Reactions were separated in a 1% non-denaturing agarose gel and further analysed by SYBR Green staining (top panel) for total DNA and by non-denaturing Southern hybridization (middle panel) using an RNA probe complementary to the 3' end of DNA adjacent to one of the break sites. The small products observed in the southern are likely generated by simultaneous digestion of the DNA from both ends of the molecule (Hopkins and Paull, 2008). (B) Resection reactions were performed with 0.375 nM Exo1 and 14, 42, or 126 nM SOSS1 and the amount of single-stranded DNA produced was quantified through quantitative PCR (qPCR) using two sets of primers to measure ssDNA levels at sites located 29 or 1025 nt from the DNA end as described previously (Nicolette *et al*, 2010). The average per cent of ssDNA from three experiments is shown; error bars indicate standard deviation.

et al, 2010; Cejka *et al*, 2010b; Nimonkar *et al*, 2011). However, we have found that RPA generally limits both yeast and human Exo1 activity *in vitro* (Figures 3 and 4;

Nicolette *et al*, 2010). To confirm that our preparation of human RPA is active in resection, we tested the protein in a resection reaction containing human Dna2 and BLM (Dna2/BLM) and found that, consistent with previous reports, RPA strongly stimulated Dna2/BLM in 5' strand processing together with MRN (Supplementary Figure S6). As we have established the stimulatory role of SOSS1 in DSB resection through Exo1, we further examined the effect of SOSS1 on Dna2/BLM in the presence or absence of MRN. Unlike RPA, SOSS1 was completely unable to stimulate DSB resection mediated by Dna2/BLM (Supplementary Figure S6), suggesting that SOSS1 and RPA promote DSB resection in a manner that is specific to each nuclease.

MRN overcomes Ku inhibition of hExo1 and Dna2-mediated resection

Processing DSB ends to generate 3' ssDNA overhangs is one of the initial steps required for repair of DSBs via HR. *In vivo* studies from yeast cells have shown that the yeast Ku70/80 heterodimer, which is required for canonical NHEJ, inhibits yeast Exo1 recruitment to DSB sites and generally blocks resection of DSBs in the G1 phase of the cell cycle (Barlow *et al*, 2008; Clerici *et al*, 2008; Zierhut and Diffley, 2008; Shim *et al*, 2010). Results *in vitro* and *in vivo* show that MRX complexes perform the unique role of displacing Ku from DNA ends and promoting resection by yExo1 (Shim *et al*, 2010; Langerak *et al*, 2011). To examine whether human Ku70/80 complexes inhibit Exo1 activity and if MRN is required to overcome the inhibitory effect of Ku on hExo1, we performed *in vitro* resection assays as described above. Even with high concentrations of Exo1 (such that Exo1 by itself shows robust resection activity), Ku exhibited strong inhibition of Exo1 (Figure 7A, lane 3). However, MRN largely recovered Exo1 resection activity, increasing resection at 29 and 1025 nt from the DNA end four-fold when compared to Exo1 + Ku in the absence of MRN (Figure 7A, compare lanes 3 and 4).

As SOSS1 and RPA promote DSB resection with Exo1 and Dna2/BLM, respectively, we also tested the abilities of each ssDNA-binding complex to overcome Ku inhibition of Exo1 and Dna2 activity. Both SOSS1 and RPA partially recovered Exo1 resection activity in the presence of Ku at 29 nt from the DNA end (1.6- and 1.8-fold, respectively) but had less effect on longer distance resection measured 1025 nt from the end (Figure 7A, compare lane 3 with lanes 7–8). As described above, MRN complex has the most dramatic stimulatory effect on Exo1 in the presence of Ku, but RPA did stimulate Exo1 activity further under these conditions (Figure 7A, lanes 4–6). As Dna2/BLM functions redundantly with Exo1 in promoting resection in eukaryotic cells (Zhu *et al*, 2008; Nimonkar *et al*, 2011), we further examined whether MRN, RPA, and SOSS1 could stimulate recombinant Dna2/BLM activity in resection in the presence of Ku. These results indicate that RPA together with MRN can overcome the inhibitory effects of Ku and stimulate the short range resection activity of Dna2/BLM while SOSS1 does not have any effect on this reaction (Figure 7B, lanes 5–9). RPA alone could not overcome the block to Dna2/BLM-mediated resection imposed by Ku, but was dramatically stimulated by the presence of MRN. Overall, these data suggest that the presence of Ku is strongly inhibitory of both Exo1 and Dna2, and

Figure 4 The SOSS1 complex can block RPA inhibition of Exo1. (A) Resection assays were performed as in Figure 3 except that each reaction contained 0.375 or 1.5 nM hExo1. (B) Resection assays as in (A) with either 0.375 nM (top) or 0.75 nM (bottom) Exo1, quantified as in Figure 3 by qPCR.

that MRN plays the most critical role in removal of this inhibition.

Discussion

Recent studies have identified SOSS1 as a novel SSB complex in human cells that exists in addition to the well-known heterotrimeric complex RPA. Both RPA and SOSS1 are critical for the regulation of DNA damage checkpoint activation as well as for DSB resection prior to HR (Richard *et al*, 2008, 2010; Huang *et al*, 2009; Oakley and Patrick, 2010). However, RPA is essential for replication and co-localizes with replication centres during S phase, while SOSS1 does not exhibit this specificity. SOSS1 co-localizes with γ -H2AX rapidly after DSB formation (Richard *et al*, 2008; Huang *et al*, 2009), while RPA is seen in foci only at later time points where it can be seen largely co-localized with Rad51 (Golub *et al*, 1998; Raderschall *et al*, 1999). Foci of SSB1, the OB-fold-containing SSB protein within the SOSS1 complex, also do not overlap with foci of RPA after IR (Richard *et al*, 2008), further suggesting a diversification of roles for these ssDNA-binding complexes. In this study, we have characterized the DNA-binding properties of SOSS1 in comparison to RPA, as well as their abilities to promote resection catalysed by Exo1 and Dna2. Overall, we find that the DNA-binding properties of RPA and SOSS1 are such that

RPA is likely to be dominant over SOSS1 at sites of ssDNA, but that SOSS1 has unique DNA binding cooperativity with Exo1 that promotes its activity on dsDNA ends.

SOSS1 and RPA display distinct modes of ssDNA binding

RPA has been shown to bind to ssDNA in several different conformations, depending on the length of the DNA, the number of OB-fold domains available for binding, and the reaction conditions (Fanning *et al*, 2006). An initial unstable interaction is formed between the A and B domain of the RPA70 subunit and 8–10 nt of DNA, followed by association of the C domain of RPA70 and the D domain of RPA32, leading to a more stable binding conformation that covers ~30 nt of ssDNA (Gomes *et al*, 1996; Bochkareva *et al*, 2002). The single-molecule FRET characterization of RPA binding to ssDNA shown here indicates two molecules of RPA binding to a 40-nt ssDNA tail, where one of the molecules is extremely stable while the other dissociates within 1–2 h. On longer ssDNA substrates (58 and 71 nt), no dissociation is observed even after several hours (Supplementary Figure S2 and data not shown). The affinity of RPA as measured in these experiments is ~1 nM, similar to previous reports (Gomes *et al*, 1996).

In contrast, the affinity of SOSS1 for ssDNA is ~10-fold lower than that of RPA (~10 nM measured in single-molecule

Figure 5 The SOSS1 complex promotes both exo- and endonucleolytic cleavage of DNA by Exo1. **(A)** Resection assays were performed using a 1.7-Kb DNA substrate internally labelled with [32 P], containing 0.16 nM wild-type Exo1 and Exo1(D78A/D173A), 15 nM MRN, 36 nM SOSS1(T117E) complex, and 36 nM RPA. Reaction products were separated by thin layer chromatography, analysed by phosphorimager, and the amount of labelled NMP released by the exonuclease activity of hExo1 was quantified by the ImageQuant software. **(B)** Resection assays as in **(A)** with 0.16 nM wild-type Exo1 and 36 nM SOSS1(T117E) complex; the average of three experiments is shown, with standard deviation. **(C)** Resection assays were performed using a Cy3/Cy5 labelled 717 bp DNA substrate, incubated with wild-type Exo1 (5 nM), SOSS1(T117E) complex (72 nM) and RPA (72 nM). Reaction products were analysed on a 20% denaturing sequencing gel and scanned for Cy5 emission.

FRET) and only one complex appears to bind to the ssDNA substrates used in this study, consistent with a larger length of ssDNA required for binding of SOSS1 (~35 nt). The presence of six OB-fold domains in RPA versus only one in SOSS1 likely accounts for the greater affinity of RPA for ssDNA, although it is remarkable that SOSS1 binds with such high affinity considering this is the case. The affinity for ssDNA measured here in gel mobility shift assays and the FRET experiments with SOSS1 is 30- to 160-fold higher than the affinity reported for SSB1 alone (Richard *et al*, 2008), thus the SOSSA(INTS3) and SOSSC(C9orf80) components of the complex contribute significantly to the DNA binding ability of the complex and may contain DNA-binding domains that are not recognizable by their sequence.

An important difference between RPA and SOSS1 is the observed fluctuation in FRET correlation values in the presence of SOSS1, indicating a more dynamic binding mode of SOSS1 on ssDNA, whereas RPA did not exhibit any movement on the DNA with either the first or second complex binding. Overall, the binding experiments suggest that RPA has a higher affinity for ssDNA sites and forms complexes that are higher in stability compared to SOSS1. In addition, quantitation of protein levels in human cells (HCT116 and a normal human fibroblast line) suggests that levels of the SSB1 protein are ~20- to 40-fold lower than RPA (data not shown). Considering this difference in concentration along with the cooperative nature of SOSS1 binding to ssDNA, RPA would be expected to predominate over SOSS1 at sites of

Figure 6 The SOSS1 complex recruits Exo1 to DNA ends. (A) hExo1 D78A/D173A binding to DNA 5' ends was tested through DNA crosslink and gel shift assays using a 717-bp double-stranded DNA substrate internally labelled with [32 P](asterisks) and containing three azide groups (N_3) at the 5' end. The substrate was incubated with 10 or 20 nM hExo1(D78A/D173A) protein in the presence of 208 nM SOSS1(T117E) complex or 208 nM RPA as indicated. After incubation, each reaction was UV crosslinked (254 nm) then loaded and separated in a 1% native agarose gel. The gel was dried and visualized by exposure to phosphorimager. Arrows indicate position of crosslinked protein-DNA species formed in the presence of both Exo1 and SOSS1. (B) DNA binding of hExo1(D78A/D173A) was measured using a 717-bp PCR product containing biotin and three azide groups as shown. The substrate was pre-incubated with magnetic streptavidin beads for immobilization. The immobilized substrate was incubated with 80 nM Exo1(D78A/D173A) and 21.6, 86.4, or 345.6 nM SOSS1(T117E) complex as indicated, the beads were washed, and bound protein was separated on a 6% SDS-PAGE and visualized by western blot using an antibody against Exo1.

ssDNA *in vivo*. Taken together, these observations likely explain why SOSS1 is not observed at replication sites with RPA and does not co-localize with RPA foci at ssDNA sites in human cells.

Functional interactions between SOSS1 and Exo1 in DSB resection

Despite the low affinity of SOSS1 for dsDNA, we observed cooperative binding of SOSS1 and human Exo1 to dsDNA substrates *in vitro*. This binding correlates with a striking

increase in Exo1 activity on linear DNA substrates in the presence of SOSS1, similarly to the effect observed with MRN. An analogous effect was previously shown with the budding yeast MRX and Sae2 complexes and yeast Exo1 *in vitro* (Nicolette *et al*, 2010). Since we did not observe any direct protein-protein interactions between yeast Exo1 and the complexes that promote its activity, our working hypothesis is that these factors contribute to the binding of Exo1 by stabilizing an opened duplex, or 'Y' structure, at the end of the DNA. In support of this idea, analysis of the degradation products of Exo1 in this study demonstrated oligonucleotide-sized initial products containing the 5' end of the 5' strand of the double-stranded substrate. These were dependent on Exo1 catalytic activity as well as the presence of SOSS1, directly demonstrating that Exo1-mediated endonucleolytic activity generates the first 5' strand product. In addition, when a Y structure is directly used as a substrate, SOSS1 exhibits relatively little stimulation of Exo1 (Supplementary Figure S7), consistent with the idea that this DNA mimics the conformation that SOSS1 stabilizes. In addition, we found that Exo1 bound preferentially to a Y-shaped, branched structure when compared with a fully paired dsDNA (Supplementary Figure S8). The structure of human Exo1 shows that the enzyme binds at a junction between single-stranded and double-stranded DNA and stabilizes a sharp bend in the helix (Orans *et al*, 2011). The frayed-end arrangement observed in the crystal structures of Exo1 on DNA could be promoted by another protein binding stably to the 3' strand, a role previously shown for yeast Sae2 (Nicolette *et al*, 2010); here, we suggest that this role is played by SOSS1.

The functional cooperativity demonstrated here for SOSS1 and Exo1 appears to be specific for Exo1 since we observed no stimulation of Dna2, another flap endonuclease that functions in lagging strand replication as well as in DSB end processing (Kang *et al*, 2010; Fortini *et al*, 2011). Budding yeast Dna2/Sgs1 complexes have been shown to remove the 5' strand of DNA at DSBs in conjunction with Rmi1/Top3 *in vivo* (Gravel *et al*, 2008; Mimitou and Symington, 2008; Zhu *et al*, 2008). Yeast RPA is critical for the strand specificity of this reaction *in vitro* and for the stimulation of Dna2/Sgs1 (Niu *et al*, 2010; Cejka *et al*, 2010a). We confirm in this study with human proteins that RPA is strongly stimulatory of Dna2 in a reaction with the BLM helicase (Nimonkar *et al*, 2008), yet RPA blocks Exo1 activity similarly to the previous findings with yeast RPA and yeast Exo1 (Nicolette *et al*, 2010). This difference also points to distinct modes of ssDNA binding by these complexes that dictates the exo-/endonuclease that each complex stimulates.

Ku inhibition of resection and release by MRN

Previous studies in budding yeast have demonstrated that the Ku heterodimer inhibits 5' strand resection *in vivo* and *in vitro* (Barlow *et al*, 2008; Clerici *et al*, 2008; Zierhut and Diffley, 2008; Shim *et al*, 2010; Langerak *et al*, 2011). Here, we show with human components that Ku70/80 complexes show a similar repression of both Exo1 and Dna2 *in vitro* and that the MRN complex facilitates the activity of both these enzymes in the presence of Ku. Although SOSS1 and RPA still stimulate Exo1 and Dna2, respectively, it is clear that MRN plays a primary role in removing or displacing Ku from DNA ends to allow resection to take place.

Figure 7 MRN promotes Exo1-mediated resection of DNA ends in the presence of Ku70/80. **(A)** Resection assays were performed as in Figure 3, except that the reactions contained higher levels of Exo1 (1.5 nM), 40 nM SOSS1(T117E) complex, 40 nM RPA, 32 nM MRN, and 100 nM Ku. **(B)** Reaction assays were performed as in **(A)** except with 16 nM Dna2, 10 nM BLM, 100 nM Ku, 80 nM RPA, 80 nM SOSS1, and 32 nM MRN.

The MRN complex interacts directly with SOSS1 through the SOSSA (INTS3) component (Huang *et al*, 2009) and the SOSSB (hSSB1) component (Richard *et al*, 2011), and is important for the formation of SOSS1 foci, particularly in S and G₂ phases of the cell cycle (Huang *et al*, 2009). From the experiments shown here it is not clear if these interactions are required for resection, since MRN and SOSS1 appear to act in similar ways on Exo1 to stimulate its activity *in vitro*. MRN interacts with several proteins that localize at DSB sites, most significantly the ATM protein kinase that phosphorylates histone H2AX and many DNA damage response factors. Phosphorylation of SSB1 by ATM on threonine 117 was shown to prevent proteasome-mediated degradation of SSB1 (Richard *et al*, 2008), thus ATM phosphorylation of SSB1 is a direct mechanism by which the kinase promotes HR. All of the experiments shown in this work were performed with SOSS1 containing the phosphomimic mutation T117E, since the wild-type protein

was not sufficiently stable during expression to allow for efficient purification of the complex. Interaction of ATM with SSB1 phosphorylated on T117 also was found to stimulate ATM kinase activity on other substrates (Richard *et al*, 2008), thus SOSS1 can promote the DNA damage response through this positive feedback loop.

In conclusion, the experiments presented here illustrate the similarities between the SOSS1 and RPA complexes in their specificity for ssDNA, but also indicate why the complexes have very different biological functions in cells. We propose that these differences are based on the affinity of the complexes for their substrates, and also on the specific conformations of dsDNA substrates when bound simultaneously by SOSS1/RPA and endonuclease enzymes. The functional cooperativity between SOSS1 and Exo1 is likely an important part of the role of SOSS1 in DNA repair, along with its stimulatory effects on ATM and Rad51 in signalling and HR, respectively (Richard *et al*, 2008).

Materials and methods

Expression constructs

Baculovirus transfer vectors used for expression of his-tagged SSB1 (SOSS1), GST-tagged SOSSA (INTS3), and SOSSC (C9orf80) have been described previously (Huang *et al*, 2009), as was the expression construct used for human Dna2 (Budd *et al*, 2000). The plasmid encoding SSB1 in pDEST10 was modified to include the T117E mutation to yield transfer vector pTP1725. Human Exo1 wild-type and D173A expression plasmids were gifts from Paul Modrich. The expression construct for human RPA was a gift from Marc Wold. Transfer vectors and viruses expressing His-Ku70 and Ku-80 were gifts from Dale Ramsden. Expression of human wild-type BLM was from transfer vector pTP1487, made by transfer of the gene from pYES2 (gift from Ian Hickson) into pFastBac1 (Invitrogen) with an N-terminal Flag tag. Point mutations were generated using QuikChange site-directed mutagenesis (Stratagene) and the mutations were confirmed by DNA sequencing. All transfer vectors were used to generate bacmids and baculovirus according to manufacturer's instructions (Invitrogen).

Protein expression and purification

See Supplementary Methods.

Oligonucleotide DNA substrates

See Supplementary Methods.

Gel mobility shift assays

Gel shift assays were performed with purified SOSS1 (T117E) complex and RPA on DNA substrates labelled with [³²P] at the 5' end of the DNA strand. The radiolabelled substrates were incubated with SOSS1 (T117E) or RPA at the concentrations indicated in the figure legends in reactions containing 2 nM DNA substrate, 25 mM MOPS pH 7.0, 1 mM DTT, 5 mM EDTA, 1 mM ATP, 0.1% Tween-20, and 60 mM NaCl for 20 min on ice. The reaction was loaded and separated in an 8% native acrylamide gel with 0.5 × TBE buffer at 100 V/cm for 2 h. The gel was dried and analysed by phosphorimager (GE).

Single-molecule FRET experiments

All single-molecule measurements were performed at 22 ± 1°C. In all, 50–100 pM of the annealed DNA substrates was immobilized on a quartz slide surface coated with polyethyleneglycol (mPEG-SC, Laysan Bio) in order to eliminate non-specific surface adsorption of proteins (Ha *et al*, 2002; Zhou *et al*, 2011). The immobilization was mediated by biotin-Neutravidin binding between biotinylated DNA, Neutravidin (Pierce), and biotinylated polymer (Bio-PEG-SC, Laysan Bio). Next, an imaging buffer containing 20 mM Tris:HCl (pH 7.2), 1 mM MgCl₂, 60 mM NaCl, 0.1 mg/ml BSA, 2% (v/v) glycerol, 0.5% (w/v) D-glucose, 165 U/ml glucose oxidase, 2170 U/ml catalase, 3 mM Trolox and with desired RPA/SOSS1 concentrations was directly added and incubated with the surface-tethered DNA substrates for 5 min before data acquisition. The DNA density immobilized on the flow chamber surface was ~500 DNA molecules per 2500 μm². A total internal reflection fluorescence (TIRF) microscope previously described (Roy *et al*, 2008) was used for data acquisition. Finally, the same imaging buffer (but with no protein) was used to flush out the excess protein from solution for another round of data acquisition. Single-molecule FRET-time traces were recorded with a time resolution of 30 ms and the FRET histograms were generated by averaging for 300 ms. The cross-correlation functions were calculated between donor and acceptor intensity-time traces (Zhou *et al*, 2011) and averaged over >100 molecules. By fitting the calculated cross-correlation functions to a single exponential function, the characteristic time, τ , can be determined.

In vitro resection assays

The resection assays with plasmid DNA were performed as described previously (Nicolette *et al*, 2010) but with the following modifications. The 4.5-kb plasmid pNO1 was linearized with *Sph*I and incubated in reactions containing 25 mM MOPS pH 7.0, 1 mM DTT, 5 mM MgCl₂, and 60 mM NaCl (30 mM in reactions containing Dna2) for 1 h at 37°C. Each reaction contained 0.135 nM linearized DNA in a total volume of 10 μl. Each reaction was terminated by adding 0.1% SDS and 10 mM EDTA. The reactions were loaded and separated on a 1% non-denaturing agarose gel and further analysed by SYBR green (Invitrogen) staining and non-denaturing southern hybridization as described previously (Nicolette *et al*, 2010).

In vitro exonuclease assays were performed using a 1.7-kb DNA substrate internally labelled with [³²P] by PCR, with conditions as described above except with 3 nM substrate. Reactions were terminated by the addition of 0.1% SDS and 10 mM EDTA and analysed by TLC as described previously (Bhaskara *et al*, 2007). The amount of labelled NMP released by the exonuclease activity of hExo1 was quantified using ImageQuant software (GE).

The *in vitro* endonuclease assay (Figure 5C) was performed with 717 bp Cy3/Cy5 labelled DNA constructed by PCR amplification of pTP466 with TP2758 (5'-CCTCT[³²P]ACAAATGTGGTATGGCTGATTATG-3') and TP2759 (5'-CTTGC[³²P]ATGCCTCAGCTATTCGGATTATTCATACCGTCCCA-3'). Each reaction was performed as described above but in a reaction buffer containing 25 mM MOPS pH 7.0, 1 mM DTT, 5 mM MgCl₂, 1 mM ATP, and 54 nM oligonucleotide duplex, in a total volume of 40 μl. Each reaction was ethanol precipitated and resuspended in formamide containing 1 mM EDTA, bromophenol blue, and xylene cyanol. The reactions were further separated in a 20% denaturing acrylamide sequencing gel and analysed by phosphorimager (GE). *In vitro* endonuclease assay with oligonucleotide substrate (Supplementary Figure S7) was performed as above but with [³²P]-labelled substrate.

Quantitative PCR

The amount of ssDNA produced by resection was quantified through qPCR as previously described (Nicolette *et al*, 2010).

Protein and DNA strand-specific crosslinking

hExo1 D78A/D173A binding to DNA 5' ends in the presence of SOSS1 or RPA was demonstrated by UV crosslinking of the protein to the DNA followed by a gel mobility shift assay. The substrate was a 717-bp PCR product internally labelled with [³²P] containing azidophenacryl bromide (APB) at the 5' end. The substrate was generated by PCR amplification of pTP466 with TP 1020 (5'-T*A*T*TCCGGATTATTCATACCGTCCC-3') and TP1030 (5'-G*A*T*CTCTAGTACTTCTCGACAAGC-3') in the presence of [³²P]dATP (NEN) (asterisks indicate positions of phosphorothioate bonds). After nucleotide removal (Qiagen), covalent incorporation of APB at phosphorothioate positions was performed as previously described (Yang and Nash, 1994). The binding reactions contained 2 nM substrate, 25 mM MOPS pH 7.0, 1 mM DTT, 5 mM EDTA, 1 mM ATP and were incubated on ice for 20 min with protein concentrations as indicated in the figure legends. After incubation, each reaction was UV crosslinked (254 nm) on ice with a handheld UV source at 3 cm for 5 min, 5% glycerol was added, and samples were separated in a 0.7% non-denaturing agarose gel in 1 × TAE buffer. The gel was dried and analysed by phosphorimager (GE).

Protein-DNA strand-specific crosslinking and pull-down assay

DNA binding assays with hExo1 D78A/D173A were performed using a 717-bp PCR product containing biotin and three azide groups on each 5' ends of the DNA strand as shown in Figure 6B. The substrate was constructed by PCR amplification of pTP466 with TP1087 (5'-Biotin-TATTCGGATTATTCATACCGTCCC-3') and TP1030. After gel purification, APB was covalently coupled to the phosphorothioate containing DNA as previously described (Yang and Nash, 1994). Approximately 250 ng of Biotin-APB-DNA was pre-incubated with Dynal streptavidin-coated magnetic beads (Invitrogen) for immobilization according to manufacturer's instructions in a total volume of 100 μl. Crosslinking experiments were performed in 25 μl reactions containing 25 mM MOPS pH 7.0, 1 mM DTT, 5 mM EDTA, 1 mM ATP, and 2 μl of Dynabead/DNA with Exo1 and SOSS1 as indicated in the figure legends. The reactions were incubated on ice for 15 min and UV crosslinked (254 nm) on ice for 5 min. The Dynabead/DNA was separated using a magnetic stand, washed three times with wash buffer (25 ml MOPS pH 7.0, 50 mM NaCl, 0.2% CHAPS, 2 mM DTT) and eluted with 1 × SDS-PAGE loading buffer. The reaction was further separated on a 6% SDS-PAGE and transferred onto a PVDF membrane, which was probed with antibody against hExo1 (Genetex: GTX92126) and further visualized with horseradish peroxidase-conjugated anti-mouse secondary antibody by chemiluminescence (Pierce).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: S-HY performed experiments, evaluated the data, and helped in the writing of the manuscript. RZ

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performed experiments and evaluated the data. JC and JC provided essential reagents and contributed to the evaluation of the data. TH evaluated the data and edited the manuscript. TP planned experiments, evaluated the data, and wrote and edited the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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