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LETTERS

DNA end resection by Dna2–Sgs1–RPA and its stimulation by Top3–Rmi1 and Mre11–Rad50–Xrs2

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The repair of DNA double-strand breaks (DSBs) by homologous recombination requires processing of broken ends. For repair to start, the DSB must first be resected to generate a 3′-single-stranded DNA (ssDNA) overhang, which becomes a substrate for the DNA strand exchange protein, Rad51 (ref. 1). Genetic studies have implicated a multitude of proteins in the process, including helicases, nucleases and topoisomerases[2–4]. Here we biochemically reconstitute elements of the resection process and reveal that it requires the nuclease Dna2, the RecQ-family helicase Sgs1 and the ssDNA-binding protein replication protein-A (RPA). We establish that Dna2, Sgs1 and RPA constitute a minimal protein complex capable of DNA resection *in vitro*. Sgs1 helicase unwinds the DNA to produce an intermediate that is digested by Dna2, and RPA stimulates DNA unwinding by Sgs1 in a species-specific manner. Interestingly, RPA is also required both to direct Dna2 nucleolytic activity to the 5′-terminated strand of the DNA break and to inhibit 3′ to 5′ degradation by Dna2, actions that generate and protect the 3′-ssDNA overhang, respectively. In addition to this core machinery, we establish that both the topoisomerase 3 (Top3) and Rmi1 complex and the Mre11–Rad50–Xrs2 complex (MRX) have important roles as stimulatory components. Stimulation of end resection by the Top3–Rmi1 heterodimer and the MRX proteins is by complex formation with Sgs1 (refs 5, 6), which unexpectedly stimulates DNA unwinding. We suggest that Top3–Rmi1 and MRX are important for recruitment of the Sgs1–Dna2 complex to DSBs. Our experiments provide a mechanistic framework for understanding the initial steps of recombinational DNA repair in eukaryotes.

Recent genetic studies in *Saccharomyces cerevisiae* identified two independent pathways capable of rapid and extensive resection of DNA DSBs: one catalysed by the 5′ to 3′ double-stranded DNA (dsDNA) exonuclease Exo1 (ref. 7), and a second requiring the nuclease/helicase Dna2 (refs 8, 9), and the 3′ to 5′ helicase Sgs1 (refs 2, 4, 5). In addition, the MRX–Sae2 complex (MRXS) mediates a short-range resection[2,4]. In cells deleted for *RAD50* or *MRE11*, the long-range resection by Dna2/Sgs1 or Exo1 occurs at the same rate, but only after an initial delay, demonstrating that the early function of MRXS can be bypassed when chemically clean breaks are generated by HO endonuclease[2,4,5]. Work using point mutations in the nuclease site of *MRE11* showed that processing of HO-induced breaks is not defective[10] and that Dna2 nuclease can replace Mre11-dependent nuclease activity in DSB repair[11], suggesting MRX has a function in end resection independent of nuclease activity[10,11].

To define the roles of Dna2, Sgs1 and other proteins in this intricate *in vivo* process, we examined DNA resection *in vitro* by reconstituting a core reaction using purified proteins (Fig. 1a). Full-length Sgs1 is a vigorous DNA helicase, as recently reported[12], and can fully unwind the 2.7-kilobase-pair (kb) linear dsDNA substrate at nanomolar concentrations (Fig. 1b, lanes 2–4). RPA is essential for resection because it is needed for Sgs1 unwinding at these concentrations (lane 15), and could not be replaced by *Escherichia coli* ssDNA-binding protein (SSB) (lane 16)[12]; this stimulatory effect of RPA on Sgs1 is the consequence of species-specific interaction between Sgs1 and RPA[13] superimposed on a non-specific stimulation due to ssDNA binding[12]. Dna2 showed no detectable nuclease or helicase activity on the dsDNA, as expected owing to its weak unwinding capability[14] (lanes 6–8). However, in the presence of Sgs1, Dna2 degraded the DNA (lanes 10–12), showing that up to 2.7 kb can be readily processed. Consistent with previous findings[4,9], degradation was not observed with nuclease-dead Dna2 (K677R), whereas helicase-dead Dna2 (K1080E) supported resection, albeit with a lower efficiency (Supplementary Fig. 2). A DNA end was required for Dna2-dependent degradation, as no DNA cleavage was observed on a covalently closed circular dsDNA, with or without Sgs1, even if it contained a 450-nucleotide 'bubble' of non-complementary ssDNA (Supplementary Fig. 3). Processing of linear dsDNA (Fig. 1b) required Sgs1 helicase activity (lane 13) and ATP (lane 14). The amount of DNA resected depended on both Sgs1 and Dna2 concentrations (Fig. 1c, d). Thus Sgs1, Dna2 and RPA constitute a minimal set of proteins required for DNA end resection.

To determine whether Dna2 and Sgs1 interact functionally, we replaced Sgs1 with other helicases. Neither *S. cerevisiae* Pif1 nor Srs2 could replace Sgs1, even when at a 1000-fold higher concentration than Sgs1 (Fig. 1f). *E. coli* RecQ could partly replace Sgs1, but a 1000-fold higher concentration (1 μM) was needed to degrade the substrate equivalently (Fig. 1f, lane 23). Collectively, these results imply a specific interaction between Sgs1 and Dna2. To establish whether Dna2 and Sgs1 physically interact, we tested whether partly purified His_6-tagged Dna2 (ref. 15) could pull down MBP-tagged Sgs1 (ref. 12) (Fig. 1e). The results show that recombinant Sgs1 and Dna2 do directly interact, independently of DNA, and that RPA neither blocked nor is required for the interaction. Thus resection catalysed by Dna2–Sgs1–RPA is likely a concerted process where nucleolytic cleavage occurs concomitantly with DNA unwinding by Sgs1.

Resection of a DSB in mitotically growing cells is dependent on the nuclease activity of Dna2 (refs 2, 4, 11) and is largely, but not absolutely, limited to the 5′ strand[16]. This directionality is essential to form 3′-ending ssDNA, which is a primer for DNA synthesis from the joint molecule intermediate[17]. However, it was not clear how this specificity is achieved in the Sgs1/Dna2 pathway, because Dna2 degrades both 5′- and 3′-terminated ssDNA[14]. To determine which strands are resected in our reconstituted system, we designed a set of ^{32}P-labelled oligonucleotides that are complementary to either the 5′- or 3′-terminated strands at positions that are either directly adjacent to,

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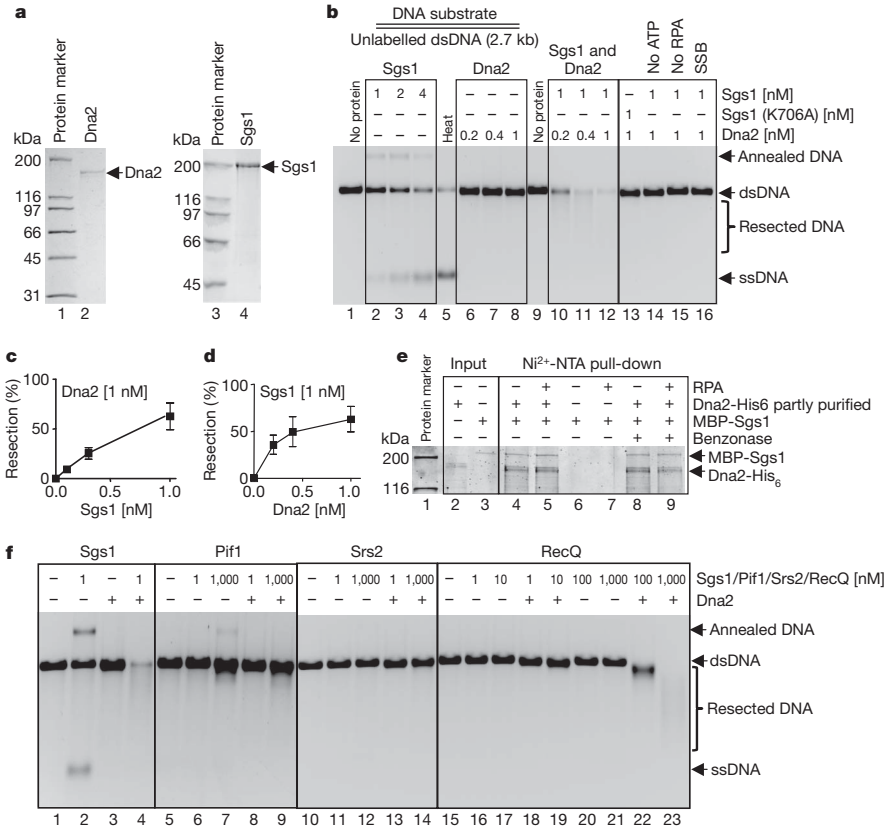

Figure 1 | Sgs1 and Dna2 resect DNA in a reaction dependent on yeast RPA. **a**, Purified Dna2 (80 ng) and Sgs1 (880 ng) stained with Coomassie blue. **b**, Linear pUC19 dsDNA incubated with Sgs1 and/or Dna2, and RPA (3 μM); SSB, SSB substituted for RPA; Heat, heat-denatured dsDNA; Annealed DNA, the result of partial unwinding and annealing of DNA[31]. **c**, **d**, Quantification of experiments as shown in **b**. **e**, Dna2 and Sgs1 physically interact in the absence or presence of RPA (lanes 4 and 5). **f**, Resection by Dna2 (1 nM) is specific for yeast Sgs1 helicase; RPA (3 μM). Error bars, s.e.m.

or 100 nucleotides or 300 nucleotides from, the DNA end (Fig. 2a). These oligonucleotides were used as hybridization probes to determine the DNA strand, and length, exposed by resection. The probes for the 100- and 300-nucleotide positions annealed exclusively to the 3′-terminated strand, indicating that extensive resection is limited to the 5′ strand, leaving the 3′ end largely intact (Fig. 2b). Resection required Dna2 because, without it, both the 3′- and 5′-terminated strands are intact and unpaired, as expected from simple unwinding by Sgs1 helicase (Supplementary Fig. 4a). Unexpectedly, but consistent with the ability of Dna2 to degrade 5′ and 3′ strands, we did not detect hybridization using probes for sequences adjacent to the DNA end (Fig. 2c) suggesting that, although extensive degradation occurs only on the 5′ strand, both strands are degraded in the vicinity of the DNA break. Loss of ssDNA at the 3′ terminus was confirmed independently in assays using dsDNA ^{32}P-labelled at the 3′ end, where the combined action of Dna2–Sgs1–RPA resulted in a rapid loss of signal and the appearance of rapidly migrating degradation products (Supplementary Fig. 4b). Kinetic analysis of resection at the 0-, 100- and 300-nucleotide sites is shown in Fig. 2c; resection to 100 nucleotides is slightly faster than to 300 nucleotides, as expected. We note that, because the enzyme concentrations and incubation time are limiting, resection originating from the opposite DNA end is unlikely

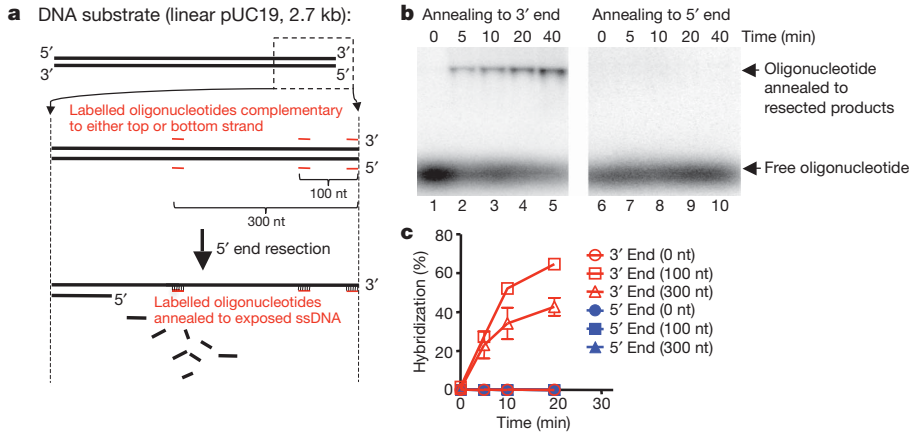

Figure 2 | Sgs1, Dna2 and RPA preferentially resect the 5′-terminated strand of a DNA break. **a**, Assay; nt, nucleotides. **b**, Annealing of oligonucleotide to resection products (0.5 nM Sgs1, 0.5 nM Dna2 and 3 μM RPA) using probe complementary to either top (3′) or bottom (5′) strand, 300 nucleotides from end. **c**, Quantification of DNA end resection at various distances from DSB, based on experiments as shown in **b**. Error bars, s.e.m.

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to reach the end analysed by hybridization and, therefore, does not affect our analysis (Supplementary Fig. 4c, d). In summary, with the exception of the region directly next to the end, resection of DNA by Dna2–Sgs1–RPA is limited to the 5′-terminated DNA strand.

Although consistent with *in vivo* results[1], our findings are surprising because characterization of Dna2 nuclease suggests that both strands should be fully degraded[14]. To determine the basis for the observed strand bias in end resection, we used DNA containing either 5′- or 3′-ssDNA tails that were used previously to study Dna2 (ref. 18). In the absence of RPA, Dna2 does indeed degrade both 5′- and 3′-terminated ssDNA (Fig. 3a, lanes 3 and 9). Unexpectedly, however, RPA blocks degradation of only the 3′-terminated strand; inhibition is concentration dependent and is maximal when RPA exceeds the amount required to saturate the ssDNA (lanes 10–12). In contrast, RPA stimulates the 5′ to 3′ nucleolytic capacity of Dna2 (lanes 4–6). Thus, RPA enforces discrimination of the 3′- and 5′-terminated strands. These results are in agreement with the interpretation of earlier observations using G4-containing DNA[15]. To confirm the observations using a substrate that resembles a DNA end unwound by a helicase, we also used a synthetic DNA duplex containing a Y-structure (Supplementary Fig. 5a, b). In agreement, the results showed that RPA blocked nucleolytic degradation of the 3′-ssDNA arm, whereas it stimulated degradation of the 5′-ssDNA arm (Supplementary Fig. 5a, b). The nuclease activity of Dna2 was limited to the ssDNA region, as reported[14], and Dna2 did not unwind the 31 base-pair (bp) dsDNA (Supplementary Fig. 5a). The effect of RPA is species specific, as SSB completely inhibited Dna2 nuclease activity (Supplementary Fig. 6), consistent with the reported physical interaction between RPA and Dna2 (refs 19, 20). These results collectively show that RPA selectively enhances the 5′ to 3′ degradative capacity of Dna2 while repressing the 3′ to 5′ degradative activity (Fig. 3b). This

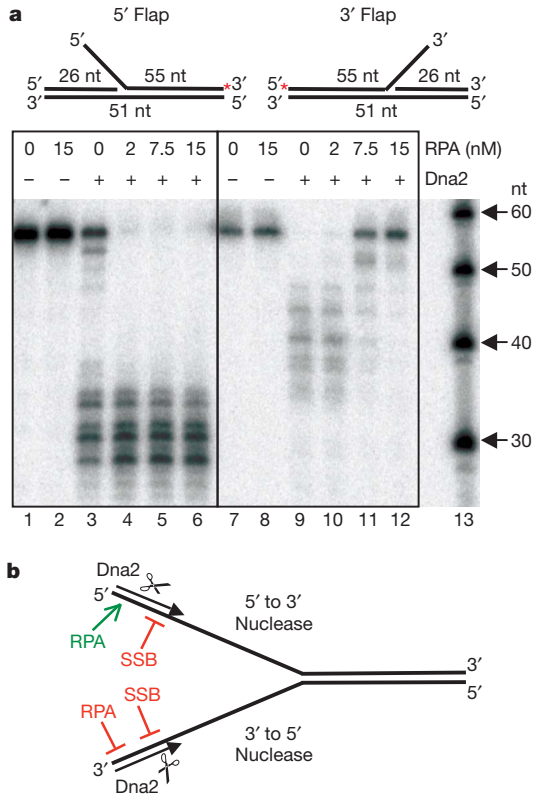

Figure 3 | RPA promotes 5′ to 3′ degradation by Dna2 and inhibits 3′ to 5′ degradation. a, Duplex DNA substrates containing either a 5′- or 3′-ssDNA flap (red asterisk indicates the ^{32}P-label) incubated with Dna2 (15 nM) and indicated RPA. **b**, Illustration summarizing results from **a** and Supplementary Figs 4 and 5 showing modulation of ssDNA nuclease activities of Dna2.

specific interaction with RPA thus alters the functionality of Dna2 and explains the strand bias of DNA end resection.

Sgs1 also physically interacts with Top3 (ref. 5) and Rmi1 (refs 21, 22) to dissolve double Holliday junctions to complete recombination[32]. Surprisingly, *in vivo*, both *top3Δ* and *rmi1Δ* mutants showed similar defects to an *sgs1Δ* mutant in resection, suggesting that the functional unit in resection is an Sgs1–Top3–Rmi1 complex[4], although it remained possible that the proteins are required for Sgs1 protein stability *in vivo*[22]. When examined *in vitro* (Fig. 4a and Supplementary Fig. 7a), Top3–Rmi1 stimulated resection by Dna2–Sgs1–RPA by approximately twofold. A titration with Top3–Rmi1 at a fixed Sgs1 and Dna2 concentration (each 0.5 nM) shows concentration-dependent stimulation with saturation occurring at ~1 nM Top3–Rmi1 (Fig. 4b), suggesting that the proteins function in a nearly equimolar protein complex.

To determine the basis for this stimulation, we examined the effect of Top3–Rmi1 on the activities of Sgs1 and Dna2. The Y-structure oligonucleotide substrate (Supplementary Fig. 5) was used because it allows a quantitative evaluation of either the helicase activity of Sgs1 (ref. 12) or the nuclease activity of Dna2. Sgs1 is the most active RecQ-helicase reported[12], yet unexpectedly we discovered that Top3, Rmi1 and Top3–Rmi1 stimulated the initial rate of DNA unwinding by Sgs1 (Supplementary Fig. 7b). The Sgs1 concentration required for half-maximal unwinding is 68 pM in the absence of Top3–Rmi1, and only 30 pM in the presence of Top3–Rmi1, corresponding to a 2.3-fold increase in apparent affinity (Supplementary Fig. 7c). We found that tenfold more Top3 than Top3–Rmi1 was required to stimulate the helicase activity of Sgs1 (Supplementary Fig. 7e, f). The stimulatory effect of Top3–Rmi1 was completely RPA dependent, as no increase in unwinding by Sgs1 was observed in the absence of RPA (Supplementary Fig. 7g, h). Furthermore, Top3–Rmi1 increased the DNA-dependent ATPase activity of Sgs1 in the presence of RPA ~2.5-fold (Supplementary Fig. 7d). Thus, Top3–Rmi1 promotes the helicase activity of Sgs1 by increasing its affinity to DNA. However, Top3–Rmi1 did not stimulate the nuclease activity of Dna2 (Supplementary Fig. 8, lane 2 compared with lane 4). We therefore conclude that Top3–Rmi1 stimulates DNA end resection by recruiting Sgs1 to DNA, rather than by potentiating the nuclease activity of Dna2.

To further examine the stimulation of Sgs1–Dna2 by Top3–Rmi1, we used elevated concentrations of Mg^{2+} (5 mM magnesium acetate) and Na^{+} (100 mM sodium acetate), which are suboptimal for Sgs1 helicase activity, thereby better revealing substrate specificity[12], and which are more representative of *in vivo* conditions. As expected, higher salt concentrations significantly inhibited DNA end resection by Dna2–Sgs1–RPA (Fig. 4c, d). However, addition of Top3–Rmi1 resulted in a striking restoration of resection (~11-fold stimulation). Top3–Rmi1 also stimulated unwinding of the 2.7-kb DNA substrate by Sgs1 alone (Fig. 4e). Finally, under these conditions, both Top3 and Rmi1 are required for the stimulation of resection, because neither alone is effective (Fig. 4f and Supplementary Fig. 9), in agreement with the *in vivo* resection data[4]. Therefore, the stimulatory role of Top3–Rmi1 in DNA end resection is evident when conditions are more physiological. Collectively, these results show that Top3 and Rmi1 stimulate DNA end resection by promoting the helicase activity of Sgs1 by enhancing its affinity for DNA.

In vivo, DSB resection is delayed in the physical absence of MRX[2,4], whereas both the yield and resection rate in cells expressing nuclease-deficient MRX (*mre11-D56N*) are indistinguishable from wild type[10]. These findings led Llorente and Symington to suggest that MRX is needed as a structural complex to recruit a resection nuclease which is not Exo1 (ref. 10). In agreement, *in vivo* observations by Budd and Campbell revealed that Dna2 nuclease can function in X-ray repair in the absence of Mre11 nuclease, but not in the physical absence of Mre11 (ref. 11). To determine whether MRX has a role in our reconstituted biochemical system, we used the *in vitro* conditions that revealed a nearly essential role for Top3–Rmi1. We found that MRX modestly promoted DNA end resection by Dna2–Sgs1–RPA, but the

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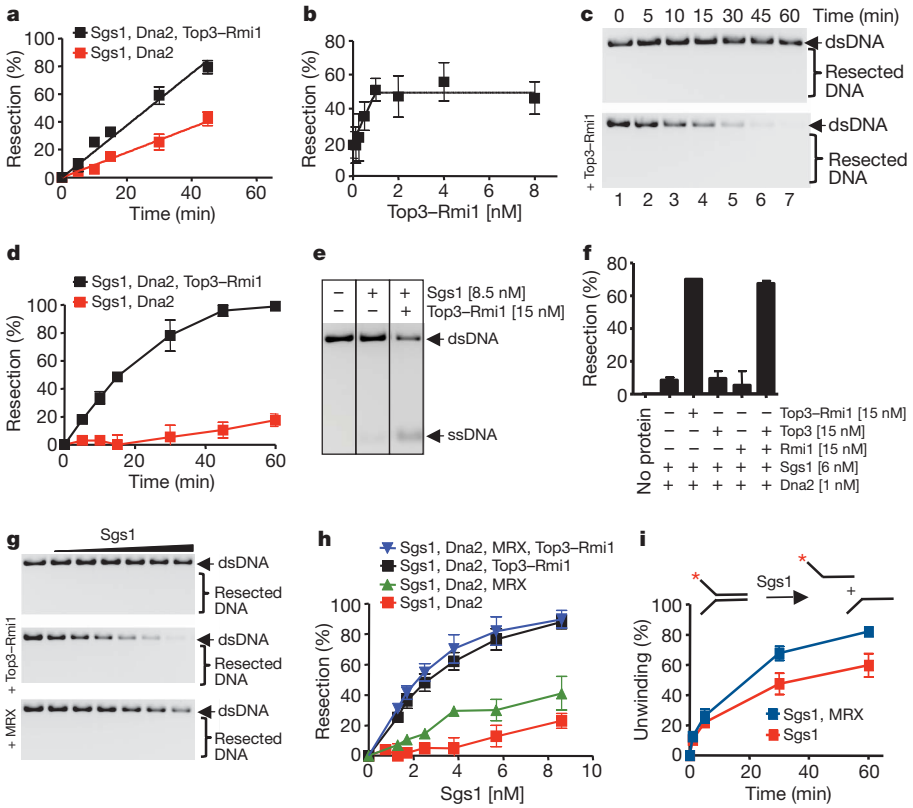

Figure 4 | Top3–Rmi1 and MRX complexes stimulate DNA resection by Dna2–Sgs1–RPA. **a**, Resection kinetics: Sgs1 (0.3 nM), Dna2 (1 nM), RPA (3 μM) and Top3–Rmi1 heterodimer (10 nM). **b**, Top3–Rmi1-dependent stimulation of DNA resection: Sgs1 (0.5 nM), Dna2 (0.5 nM) and RPA (3 μM). **c**, Resection kinetics in high salt buffer (5 mM Mg^{2+} and 100 mM Na^{+}): Sgs1 (7.5 nM), Dna2 (1 nM), RPA (3 μM) and Top3–Rmi1 heterodimer (15 nM). **d**, Quantification of experiments as in **c**. **e**, DNA unwinding in high salt buffer; RPA (3 μM). **f**, Resection in high salt buffer; RPA (3 μM). **g**, Stimulation of resection in high salt buffer by Top3–Rmi1 (15 nM) and MRX (20 nM) using Dna2 (1 nM), RPA (3 μM) and Sgs1 (0, 1.3, 1.7, 2.5, 3.8, 5.7 and 8.6 nM). **h**, Quantification of experiments as in **g**. **i**, Unwinding of Y-structure DNA in high salt buffer: Sgs1 (200 pM); RPA (2.25 nM); MRX (5 nM). Error bars, s.e.m.

magnitude of the stimulation (approximately twofold) was far less than for Top3–Rmi1 (Fig. 4g, h and Supplementary Fig. 10a, b). When Top3–Rmi1 was present, MRX did not further stimulate resection by Dna2–Sgs1–RPA (Fig. 4h). MRX modestly promoted DNA unwinding by Sgs1 (Fig. 4i and Supplementary Fig. 10c, d), whereas it did not affect Dna2 nuclease activity (Supplementary Fig. 8, lane 2 compared with lane 6). These findings are consistent with the previously reported physical interaction between Sgs1 and Mre11 (ref. 6), and we propose that MRX stimulates DNA end resection by recruiting Sgs1 to the DSBs.

In this work, we reconstituted elements of the machinery that processes DSBs in preparation for DNA repair. Our biochemical findings show that Sgs1, Dna2 and RPA are the minimal essential components. By promoting the binding of Sgs1 to DNA, the Top3–Rmi1 complex is stimulatory, and is effectively essential at approximately physiological conditions. The MRX complex can stimulate resection modestly, consistent with a recruitment function in resection *in vivo* that can be bypassed. MRX has a high affinity for DNA ends and is a sensor of DSBs *in vivo*[23]. Mre11 interacts directly with Sgs1 (ref. 6), suggesting that stimulation of resection by MRX might be due to recruitment of Sgs1 to DNA ends, consistent with our biochemical results and *in vivo* interpretations[10,11]. In addition to signalling and recruitment functions, MRXS nuclease is essential during meiosis to remove Spo11 from the 5′-terminated strand of DSBs[24], and in mitotic cells to remove adducts of topoisomerase II covalently linked to DSB ends that arise from drug treatment[3,25]. Thus, the MRXS proteins are especially important for initial processing of DNA ends that contain adducts which could impede processing. In the absence of such adducts, Sgs1–Dna2–RPA in a complex with Top3–Rmi1 compose the core of one of the two major pathways for extensive DNA resection. We show that Top3–Rmi1 and MRX potentiate resection by recruiting Sgs1 to the site of the break, and propose that this recruitment is consistent with both the requirement for Top3–Rmi1 *in vivo* and the delay when MRX is physically absent. Sgs1 interacts with Dna2 to resect DNA in an RPA-regulated manner. Based on the *in vitro* data presented here, we propose a model for DNA end resection by Dna2–Sgs1–RPA (Supplementary Fig. 1). First, the Sgs1–Top3–Rmi1 complex is recruited to a DSB in a step that can be augmented by the physical presence of MRX. Sgs1 unwinds dsDNA, and the single strands of DNA are coated with RPA. Concomitantly, and mediated through direct interaction with Sgs1, Dna2 preferentially degrades the 5′-terminated DNA strand. RPA promotes the degradation of this strand and inhibits the degradation of the 3′-terminated strand. Thus, this reconstituted reaction recapitulates steps required to resect a DSB to produce a 3′-ssDNA overhang. Results that parallel the major findings reported here have been independently obtained by the Sung laboratory[26]. Finally, this eukaryotic resection complex shows intriguing functional parallels to the resection machine of bacteria, RecBCD (or AddAB), where the recombination-promoting complex (post-Chi-recognition) comprises a helicase subunit (RecB) unwinding 3′ to 5′ (equivalent to Sgs1); a slower translocation subunit (RecD) travelling 5′ to 3′ on the complementary strand (equivalent to Dna2); and a nuclease domain that is threaded onto the end of a DNA strand but which acts endonucleolytically to process the 5′-terminated DNA strand to produce 3′-tailed duplex DNA[27]. Furthermore, RecBCD delivers RecA to the 3′-terminated processed ssDNA by an essential interaction with a RecA-loading domain[27,28]; because the carboxy (C) terminus of Sgs1 physically interacts with Rad51 (ref. 29), it is also possible that Sgs1 and its homologues may coordinate processing with DNA pairing in a related manner in eukaryotes[30].

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METHODS SUMMARY

Unless indicated otherwise, the DNA substrate used for end-resection experiments was linear pUC19 dsDNA (2.7 kb). The reaction products were separated by electrophoresis, and visualized by ethidium bromide staining. For clarity, we present all DNA gels as inverted images. The interaction of Sgs1 with Dna2 was determined using Ni^{2+}-nitrilotriacetic acid pulldown assays using Dna2 tagged with His_6 and MBP-tagged Sgs1. The directionality of resection was determined by hybridization using radiolabelled strand-specific oligonucleotide probes. All oligonucleotide-based DNA substrates were ^{32}P-labelled, and visualized by autoradiography. The resection assays with the dsDNA substrates containing either 5′- or 3′-ssDNA flaps were performed as described previously[15]. Helicase assays were performed as described previously[12].

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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METHODS

DNA substrates. The dsDNA substrates containing 5′- or 3′-ssDNA flaps were prepared as described previously[18]. The oligonucleotide-based DNA substrates were described previously[12]. Unless otherwise indicated, the DNA substrate used for resection assays was unlabelled pUC19 dsDNA that had been linearized with HindIII and purified by phenol-chloroform extraction and ethanol precipitation.

Proteins. Sgs1, RPA, SSB and RecQ proteins were expressed and purified as described[12,33–35]. Top3, Rmi1 and Top3–Rmi1 heterodimer were prepared as described[32]. All of the above proteins were free of nuclease contamination. Srs2, Pif1 and MRX proteins were gifts from X. Veaute (Institute of Cellular and Molecular Radiation Biology, France), J.-B. Boulé and A. Nicolas (Institut Curie, Paris), and P. Sung (Yale University), respectively.

Purification of Dna2. Dna2 was expressed from a modified pGAL:DNA2 (ref. 9) vector that contained amino (N)-terminal Flag and HA tags and a C-terminal His_6 tag, in protease-deficient *S. cerevisiae* strain WDH668 (ref. 36). This protocol describes purification from 4 l of cell culture. Yeast cells were grown to an absorbance, $A_{600\,nm}$, of approximately 0.6 in a standard s.d. medium, lacking uracil, and supplemented with both glycerol (3%) and lactic acid (2%). Expression of Dna2 was induced by adding galactose (2%) for 6 h. All subsequent steps were performed on ice or at 4 °C. Pelleted cells were resuspended in 40 ml TBSG-PI buffer (25 mM Tris HCl (pH 7.5), 100 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol, 10 μg ml^{-1} leupeptin, 1 mM phenylmethylsulphonyl fluoride and protease-inhibitor cocktail (Sigma, P8340, diluted 1:1,000)) and lysed in French press. The lysed cells were collected by centrifugation at 58,000*g* for 30 min; the supernatant was transferred to a new centrifuge tube and spun again as above. The cleared extract was then supplemented with imidazole (10 mM) and incubated batch-wise with 5 ml Ni^{2+}-NTA agarose (Qiagen) for 1 h. The resin was washed extensively with TBSG-PI buffer containing imidazole (10 mM) batch-wise and the bound proteins were eluted from the column with 400 mM imidazole in TBSG-PI. The fractions containing proteins were pooled, loaded on a HiTrap Heparin HP column (5 ml; GE Healthcare) at 2.5 ml min^{-1} in HEP buffer A (25 mM Tris HCl (pH 7.5), 100 mM NaCl, 10% glycerol, 1 mM β-mercaptoethanol, 10 μg ml^{-1} leupeptin, 1 mM phenylmethylsulphonyl fluoride and protease-inhibitor cocktail (Sigma, P8340, diluted 1:1,000)) and eluted with HEP buffer B (the same as HEP buffer A, but with 600 mM NaCl) at 2.5 ml min^{-1}. Fractions containing proteins were pooled, diluted 1:1 with TBS buffer (50 mM Tris HCl (pH 7.5), 150 mM NaCl) and incubated batch-wise with M2 anti-FLAG affinity resin (0.5 ml; Sigma) for 30 min. The resin was then washed with TBS buffer, and Dna2 was eluted with TBS supplemented with 3×FLAG Peptide (150 μg ml^{-1}; Sigma). Fractions containing protein were pooled, diluted with ½ volume water and 1 volume of Q buffer A (25 mM Tris HCl (pH 7.5), 100 mM NaCl, 10% glycerol and 5 mM β-mercaptoethanol), then loaded on HiTrapQ column (1 ml; GE Healthcare) at 0.8 ml min^{-1}. The column was washed with Q buffer A, and Dna2 was eluted with Q buffer B (the same as Q buffer A, but with 600 mM NaCl). Fractions containing protein were pooled, small aliquots were frozen in liquid nitrogen, and stored at −80 °C. The final protein concentration was quite low, and thus estimated by densitometry by comparison with dilution series of broad range protein marker (BioRad) on 10% polyacrylamide gel stained with Coomassie blue. The protein yield was ~3.7 μg and concentration ~27 nM. An identical procedure without the FLAG step was used to prepare the enriched Dna2 protein used for the affinity pulldown experiments. This Dna2 preparation was more concentrated but less pure (~20%), as shown previously[15]. The Dna2 protein used in Fig. 3 was prepared as described previously[9].

DNA resection assays. The resection assays with the dsDNA substrates containing either 5′- or 3′-ssDNA flaps were performed as described previously[15]. All other resection assays contained, unless indicated otherwise, 25 mM Tris acetate (pH 7.5), 1 mM dithiothreitol, 2 mM magnesium acetate, 250 μg ml^{-1} BSA, 1 mM ATP, 1 mM phosphoenolpyruvate (Sigma), 80 U ml^{-1} pyruvate kinase (Sigma), 200 ng linear pUC19 DNA substrate (7.6 nM molecules; 41 μM nucleotides) and the indicated proteins. Reactions that were conducted at the 'high salt' condition, where indicated, were in standard buffer containing 100 mM sodium acetate and 5 mM magnesium acetate. Unless otherwise indicated, the reactions were assembled on ice, initiated by adding ATP, and performed for 30 min at 30 °C, in a volume of 15 μl. The reactions were terminated with 5 μl of stop buffer (150 mM EDTA, 2% SDS, 30% glycerol, 0.1% bromphenol blue) and 1 μl of proteinase K (14–22 mg ml^{-1}, Roche) for 30 min at 30 °C, unless otherwise indicated, and analysed by electrophoresis in 1% agarose in the presence of 0.05 μg ml^{-1} ethidium bromide. Gels were analysed using an AlphaImager HP (Alpha Innotech) imaging station, and are presented as the inverted image. Resection was quantified by measuring disappearance of the substrate dsDNA band. All error bars show the standard error from two to five independent experiments as determined by GraphPad Prism 5.0.

To analyse the directionality of resection by hybridization with radiolabelled oligonucleotide probes (as in Fig. 2), a standard reaction was first performed as described above. Upon termination, the reaction was diluted so that the DNA concentration was 1 nM (molecules). The oligonucleotide probe, which was ^{32}P-labelled at the 5′ terminus (2 nM, molecules), was added to the diluted mixture, as well as PNK buffer (New England Biolabs) to final concentrations of 7 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$ and 0.5 mM dithiothreitol. The sequences of the oligonucleotide probes were the following: for 5′ resection at 0 nucleotides (GCATGCCTGCAGGTCGACTC), 100 nucleotides (GGCGTTACCCAACTTA ATCG), 300 nucleotides (AGCCAGCCCCGACACCCGCC), and for 3′ resection at 0 nt (GAGTCGACCTGCAGGCATGC), 100 nucleotides (CGATTAAGTTGG GTAACGCC) and 300 nucleotides (GGCGGGTGTCGGGGCTGGCT). The mixture was then heated to 70 °C for 5 min, and cooled to room temperature over approximately 2 h. The products were then separated by electrophoresis in 1% agarose and analysed by Storm 860 PhosphorImager (GE Healthcare). The percentage of DNA hybridization was calculated from the proportion of annealed versus free oligonucleotides, assuming 100% efficiency of annealing.

Helicase assays. The helicase assays were performed as described previously[12]. The products were separated by native 10% polyacrylamide gel electrophoresis and detected by autoradiography.

Ni^{2+}-nitrilotriacetic acid (NTA) pulldown assays. The Sgs1 protein used for Ni^{2+}-NTA pulldown experiments contained the MBP tag but lacked the His_{10} tag[12]. The Dna2 protein used for the pulldown experiments contained a His_6 tag, and was prepared by a procedure similar to that published previously[15], which provides a higher yield but lower purity of the recombinant polypeptide. The identity of proteins from both purifications was verified by western blotting (data not shown). Purified proteins (1–2 μg in a final volume of 150 μl) were incubated together in 20 mM Tris HCl (pH 7.5), 10% glycerol, 0.1% NP40, 100 mM NaCl and 10 mM imidazole, with or without 12.5 units of Benzonase Nuclease (Novagen) for 20 min at room temperature. Ni^{2+}-NTA agarose (50 μl, Qiagen) was then added, incubated for 30 min, and the resin was washed with buffer. The bound proteins were eluted in the same buffer containing 600 mM imidazole, and 20% of eluate was analysed by electrophoresis in 8% polyacrylamide, stained with Sypro Orange (Invitrogen) and detected by a Storm 860 PhosphorImager (GE Healthcare).

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