Live-cell single-molecule tracking highlights requirements for stable Smc5/6 chromatin association *in vivo*

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Abstract

The essential Smc5/6 complex is required in response to replication stress and is best known for ensuring the fidelity of homologous recombination. Using single-molecule tracking in live fission yeast to investigate Smc5/6 chromatin association, we show that Smc5/6 is chromatin associated in unchallenged cells and this depends on the non-SMC protein Nse6. We define a minimum of two Nse6-dependent sub-pathways, one of which requires the BRCT-domain protein Brc1. Using defined mutants in genes encoding the core Smc5/6 complex subunits we show that the Nse3 double-stranded DNA binding activity and the arginine fingers of the two Smc5/6 ATPase binding sites are critical for chromatin association. Interestingly, disrupting the ssDNA binding activity at the hinge region does not prevent chromatin association but leads to elevated levels of gross chromosomal rearrangements during replication restart. This is consistent with a downstream function for ssDNA binding in regulating homologous recombination.

31 **Introduction**

32

33 The structural maintenance of chromosomes (SMC) complexes cohesin, condensin and Smc5/6
34 are critical for the correct organisation of chromosome architecture[1]. Whereas the functions of
35 cohesin and condensin are increasingly well understood, the exact function of Smc5/6 complex
36 remains relatively ambiguous. Smc5/6 is conserved across all eukaryotes and is best known for
37 its role in the cellular response to DNA damage by ensuring the fidelity of homologous
38 recombination repair (HRR)[2,3]. Smc5/6 has been reported to promote replication fork stability[4]
39 and facilitate DNA replication through natural pausing sites[5]. Biochemically, the complex can
40 regulate pro-recombinogenic helicases[6,7]. It has also been proposed to monitor DNA topology[8]
41 and recently been shown to restrict viral transcription[9,10]. Hypomorphic mutants show
42 significant defects in sister-chromatid HRR, display replication fork instability, are sensitive
43 to a wide range of genotoxins and accumulate unresolved recombination intermediates[4,11,12].
44 Intriguingly, complete inactivation of the Smc5/6 complex in a variety of organisms leads to
45 cell death and this essential nature suggests it possesses additional functions beyond HR as
46 deletions of core HR factors are viable.

47

48 Like all SMC complexes, the core of Smc5/6 is composed of two folded proteins, Smc5 and
49 Smc6, which form a heterodimer (Figure 1A). Each subunit comprises a long coiled-coil arm
50 with a hinge region at one end and a globular ATPase head at the other[1]. All three SMC
51 heterodimers interact at the hinge and ATP binding/hydrolysis occurs in two pockets formed
52 between the heads of the two subunits. For all SMC complexes, ATP turnover is essential for
53 cell viability and has been proposed to bring about conformational changes in the arms[13,14,15].
54 The ATPase activity is also key to the interaction of SMC's with DNA: cohesin's ATPase is
55 required for both loading and dissociation from DNA[16], whilst condensin is dependent on its
56 ATPase activity for translocating along DNA and forming loop structures[17,18]. The role of the
57 Smc5/6 ATPase in DNA association has not been studied in detail.

58

59 The Smc5/6 hinge contains specialised interfaces that are important for interacting with single
60 stranded DNA (ssDNA)[19]. Disruption of these regions by mutation results in sensitivity to
61 DNA damaging agents. The Smc5/6 ATPase heads are bridged by a sub-complex of three non-
62 SMC elements (Nse), Nse4 (kleisin) and two kleisin-interacting tandem winged-helix element
63 (KITE) proteins, Nse1 and Nse3. Nse1 has a RING finger and, in association with Nse3, has

been shown to have ubiquitin ligase activity[20]. The winged-helix domain of Nse3 possesses double-stranded DNA (dsDNA) binding activity, which is essential for viability[21]. The dsDNA binding has been predicted to provide the basis for initial chromatin association and loading of the complex[21]. In addition to the Nse1/3/4 subcomplex, Nse2, a SUMO ligase, is associated with the Smc5 coiled-coil arm. DNA association of the Smc5/6 complex is required to activate the Nse2 SUMO ligase, which SUMOylates a range of targets within and outside of the complex[22]. Two further proteins, Nse5 and Nse6, also associate with the Smc5/6 complex in yeasts (both *Saccharomyces cerevisiae* and *Schizosaccharomyces. pombe*). However, unlike the other Nse proteins, Nse5 and Nse6 have not been identified as part of a Smc5/6 holo-complex in human cells[23,24].

Chromatin loading of the structurally related cohesin complex requires accessory proteins, the cohesin–loader complex Scc2–Scc4 (*sp*Mis4–Ssl3)[25]. A loading complex for Smc5/6 has not yet been defined but recent work in fission yeast has shown that its recruitment to sites of replication fork collapse occurs via a multi-BRCT domain protein, Brc1[26]. Brc1 binds to γ-H2A and interacts with the Nse5-Nse6 subcomplex (which associates with Smc5/6 but is not part of the core complex), providing a potential mechanism by which Smc5/6 is recruited and loaded. In *S. cerevisiae* the N-terminal four BRCT domains of the Brc1 homologue, Rtt107, have also been shown to bind Nse6 amongst a number of other proteins in the DNA damage response[27]. In human cells recruitment of Smc5/6 to inter-strand cross-links was shown to depend on interactions between SLF1, another multi-BRCT domain protein, and SLF2 - a distant homologue of Nse6[28]. These observations suggest that recruitment of Smc5/6 through Nse6 and a BRCT-domain mediator protein has been conserved through evolution.

Understanding how Smc5/6 is recruited to, and associates with, the chromatin is an important step in defining how it regulates recombination processes and other potential DNA transactions. To date, the study of Smc5/6 chromatin association has been mostly limited to chromatin immunoprecipitation (ChIP)-based methodologies. Recent studies have shown single-particle tracking (SPT) microscopy can provide robust measurements of chromatin interacting proteins in vivo and offer complementary data to genome-wide approaches.

Here, we perform SPT using photoactivated localisation microscopy (PALM) in live fission yeast cells to monitor chromatin association of Smc5/6. Using a range of *smc* and *nse* mutants

97 we investigated the role of its ATPase activity, DNA interaction sites and protein binding
98 partners in promoting chromatin association. This highlighted that ATPase activity and dsDNA
99 binding are both crucial for chromatin association. In contrast, interaction with ssDNA at the
100 hinge is not required for stable chromatin loading but we show that it is important to prevent
101 gross chromosomal rearrangements at collapsed replication forks. We also establish that the
102 Nse5-Nse6 sub-complex is required for almost all chromatin association, whereas Brc1 is
103 required for only a proportion of the association. These data define the Brc1-Nse6-dependent
104 sub-pathway of chromatin interaction and identify parallel Nse6-dependent but Brc1-
105 independent sub-pathway(s).
106
107 **Results**
108
109 *Smc5/6 is chromatin associated in unchallenged cells*
110
111 To monitor Smc5/6 chromatin association in living yeast cells we used photoactivated
112 localisation microscopy combined with single-particle tracking (SPT)[29]. We created a fission
113 yeast strain that endogenously expressed the kleisin subunit Nse4 fused to the photoconvertible
114 fluorophore mEos3 and verified this allele had no measurable impact on cellular proliferation
115 (Figure S1A). We imaged photoconverted subsets of Nse4-mEos3 in live yeast cells at high
116 temporal resolution (20ms exposure) and created trajectories by localising and tracking
117 individual fluorophores (Figure S2A, B). Nse4-mEos3 localisations and trajectories showed
118 nuclear confinement consistent with previous studies[30] (Figure 1B).
119
120 To evaluate the chromatin association of Smc5/6 from our data we used the recently described
121 'Spot-On' software[31] (see materials and methods). Spot-On implements a bias-aware kinetic
122 modelling framework and robustly extracts diffusion constants and subpopulations from
123 histograms of the molecular displacements that make up each trajectory (Figure 1C). We
124 tracked Nse4-mEos3 in asynchronous live cells and created displacement histograms over 4-
125 time intervals (Figure 1D). The profiles show a clear peak of short displacements (<100nm)
126 indicative of a chromatin-bound fraction of Nse4-mEos3 in unchallenged cells. Spot-On kinetic
127 modelling revealed a fraction bound of about 40% (Figure 1E). The displacement distributions
128 were best described with a 3-state fit which, in addition to bound and freely diffusing species,
129 included an intermediate slow-diffusing population. This may describe transient interactions

130 with chromatin or anomalous diffusion as a result of a crowded molecular environment[32]
131 (Figure S8, materials and methods). Tracking of other core Smc5/6 (Nse2 and Smc6) subunits
132 revealed similar displacement profiles and bound fractions, suggesting the dynamics of the
133 kleisin subunit is indicative of the whole complex (Figure S3A).
134
135 We next compared Smc5/6 chromatin association to the structurally related cohesin complex.
136 As fission yeast cells reside in G2 for the majority of the cell cycle we hypothesised that cohesin
137 would be stably associated with the chromatin[33] and should thus demonstrate a higher fraction
138 bound. As predicted, tracking of Rad21 (kleisin) and Smc1 (arm) fused to mEos3 revealed
139 displacement profiles with greater proportions of short displacements compared to Smc5/6
140 subunits and subsequently resulted in greater bound fractions extracted from Spot-On model
141 fitting (Figure 1D and E, S3B). These observations show that interaction of cohesin and Smc5/6
142 with chromatin are distinct and different and suggest that their association occurs with different
143 dynamics.
144
145 *dsDNA binding is required for efficient chromatin association*
146
147 Smc5/6 has been shown to bind both ds- and ssDNA. The KITE protein Nse3 has a dsDNA
148 binding domain in both humans and fission yeast and is situated at the head end of the complex
149 (Figure 2A). This activity is essential and was predicted to be the initial point of interaction
150 between Smc5/6 and the chromatin required before loading[21]. To assess whether Nse3 dsDNA
151 interaction plays a role in global chromatin association we tracked Nse4-mEos3 in a *nse3-*
152 *R254E* genetic background. This hypomorphic mutation has been shown to disrupt but not fully
153 abolish dsDNA binding by Nse3[21]. When compared to *nse3*$^{+}$, Nse4-mEos3 displacement
154 histograms from asynchronous *nse4-mEos3 nse3-R254E* cells showed a broader profile
155 suggesting the complex had become more dynamic (Figure 2B, C). This resulted in a reduction
156 in the fraction bound value in Spot-On analysis (Figure 2D). This confirms *in vivo* that dsDNA
157 binding by Nse3 underpins the chromatin association of Smc5/6.
158
159 *Smc5/6 ATPase activity is required for efficient chromatin association*
160
161 Each of the SMC complexes possess ATPase activity, with two separate and distinct active
162 sites within juxtaposed 'head' domains, which are generated by bringing together the required
163 signature motifs *in trans* (Figure 3A). Like all SMC complexes the ATPase activity of Smc5/6

164 is essential and inactivating mutations in either of the two Walker motifs are non-viable[34,35].
165 Therefore, to investigate the influence of ATPase activity on chromatin-association of the
166 Smc5/6 complex, we first mutated the 'arginine-finger' of Smc5 (*smc5-R77A*) or Smc6 (*smc6-*
167 *R150A*). Mutation of the equivalent residues in other SMC complexes does not typically affect
168 the basal level of ATP turnover, but instead acts to abolish stimulation of activity by DNA-
169 interaction[36]. Both the *smc5-R77A* and the *smc6-R150A* mutation resulted in sensitivity to
170 replication stress (Figure S4A). Tracking of Nse4-mEos3 in these genetic backgrounds
171 revealed increased single molecule displacements and subsequent decreases in chromatin
172 association of the Smc5/6 complex (Figure 3B, C and S4B). *smc6-R150A* led to a dramatic
173 decrease in chromatin association whereas mutation of the Smc5 arginine was noticeably less
174 detrimental. Interestingly, the reduction in the levels of chromatin association correlated with
175 sensitivity to exogenous genotoxic agents, strongly suggesting that DNA-dependent ATP
176 hydrolysis by the two binding pockets is not equivalent.
177
178 The Smc6 arginine finger mutant was of particular interest to us as the well characterised *smc6-*
179 *74* allele maps to the next residue, A151T[4,34,34,38]. Single particle tracking showed this mutant
180 to have a similar decrease in chromatin association to *smc6-R150A*. Sequence-threaded
181 homology models for the head domain of *S. pombe* Smc6 and comparison to the X-ray crystal
182 structure of the head domain from *Pyrococcus furiosus* SMC in complex with ATP (PfSMC,
183 PDB: 1XEX) allowed us to create specific mutations designed to display a graduated effect on
184 the Smc6 arginine-finger: Thr135 in Smc6 was mutated to a series of hydrophobic amino acids
185 with increasing size, each predicted to produce increasingly severe steric clashes with the
186 arginine-finger when engaged in interaction with bound ATP (Figure 3D).
187
188 Phenotypic analysis of each *smc6* mutant confirmed that the predicted severity of steric clash
189 (Phe>Leu>Val) closely correlated with an increase in sensitivity to a range of genotoxic agents
190 (Figure 3E), culminating with the most severe mutation, T135F, producing a phenotype similar
191 to the well characterised *smc6-74* (A151T) mutant. Single-particle tracking data revealed that
192 increasing the severity of the substitution corresponded with a decrease in the fraction of bound
193 Smc5/6 (Figure 3F, S4C). The *smc6-T135F* strain showed similar levels of bound complex as
194 the *smc6-74* mutation.
195
196 Since mutations in the ATPase domains render cells sensitive to replication stress (Figure 3E)
197 we monitored whether these mutants could recruit the complex to chromatin after treatment

198 with MMS. Acute exposure to 0.03% MMS for 5 hours resulted in a modest increase in the
199 fraction of Nse4-mEos3 bound to the chromatin in cells with a wild type background (Figure
200 3G). However, in contrast both the *smc6-74* and *smc6-T135F* alleles significantly reduced or
201 prevented Smc5/6 from being recruited to chromatin in response to MMS.
202
203 Together these data demonstrate that the ability to stimulate Smc5/6 ATPase activity through
204 the arginine finger is crucial for its stable association with the chromatin. The disparity in
205 phenotype between *smc6* and *smc5* ATPase mutants suggests there could be an underlying
206 asymmetry in the use for the two ATP binding sites, a phenomenon that has been recently
207 described for both condensin and cohesin[16,39].
208
209 *ssDNA binding is dispensable for Smc5/6 chromatin association*
210
211 We recently determined the structure of the *S. pombe* Smc5/6 hinge and demonstrated its
212 preferential binding to ssDNA[19]. Specialised features known as the 'latch' and 'hub' are
213 required for efficient association with ssDNA (Figure 4A). The kinetics of this interaction are
214 biphasic and appear to involve two distinct interaction points. Like mutants compromised for
215 dsDNA binding, mutations in these key regions that weaken the interaction with ssDNA render
216 cells viable but sensitive to replication stress and DNA damaging agents[19]. We tested whether
217 the ability to interact with ssDNA affected the ability of Smc5/6 to associate with chromatin.
218
219 Previously characterised mutations were introduced into the Nse4-mEos3 strain that affect
220 either initial ssDNA interaction (*smc5-R609E R615E*), stable hinge heterodimerisation (*smc5-*
221 *Y612G*) or secondary ssDNA interactions at the Smc6 hub (*smc6-F528A, smc6-R706C*)[19]
222 (Figure 4A, right). Spot-On model fitting to sptPALM data showed that, unlike the dsDNA
223 binding and ATPase mutants, disruption of ssDNA interactions did not alter the bound fraction
224 of Smc5/6 in unchallenged cells (Figure 4B).
225
226 Since these mutations render cells sensitive to replication stress, we monitored recruitment of
227 Smc5/6 complex to chromatin after treatment with MMS. Disruption of ssDNA interactions
228 either reduced, or prevented, further Smc5/6 from being recruited to chromatin in response to
229 MMS (Figure S5). Together, these data show that, while dsDNA binding is required for stable
230 association of the Smc5/6 complex with chromatin, its interactions with ssDNA are not. This
231 is consistent with ssDNA interactions playing a role in processes downstream of loading and

232 we speculate that it may be important for Smc5/6 retention on the DNA during DNA repair-
233 associated processes.
234
235 *ssDNA interaction is required to prevent gross chromosomal rearrangements*
236
237 We hypothesised that the loss of Smc5/6 chromatin association would produce distinct
238 outcomes during HR-dependent processes compared to the loss of ssDNA interaction. To
239 investigate this, we compared the effect of Smc5/6 mutations in the response to replication fork
240 stalling in the previously characterised *'RuraR'* replication fork barrier system[40].
241
242 In fission yeast, binding of Rtf1 to the replication termination sequence, *RTS1*, arrests
243 replication forks in a polar manner[41]. In the *RuraR* system, two copies of *RTS1* are placed in
244 an inverted orientation on either side of the *ura4* marker on chromosome III (Figure 4C). The
245 *RTS1* barrier activity is regulated by placing *rtf1* under the control of the *nmt41* promoter and
246 induction of *rtf1* expression leads to arrest of replication forks converging on both *RTS1*
247 sequences. Replication of the intervening *ura4* requires homologous recombination-dependent
248 replication restart which can result in genome instability via non-allelic homologous
249 recombination (NAHR)[42] or small scale errors by the error prone restarted fork[43]. The loss of
250 *ura4* in the *RuraR* system provides a readout that is particularly useful to characterise NAHR
251 events. In the absence of key HR factors, such as Rad51, induction of arrest leads to viability
252 loss, whereas mis-regulation of HR generates aberrant outcomes[40].
253
254 We introduced the *smc6-R706C* (*smc6-X*) and *smc6-A151T* (*smc6-74*) mutations into the
255 *RuraR* system. There was no loss of viability when stalling was induced at *RTS1* in these
256 backgrounds compared to *rad51*Δ (Figure S6A). This is consistent with Smc5/6 regulating
257 recombination, rather than being core to the recombination process[2]. Induction of replication
258 arrest led to an increase in the loss of *ura4* activity in *smc6*$^{+}$, *smc6*-74 and *smc6*-
259 X backgrounds. There was only a modest change in the ATPase mutant (*smc6*-74) (5.6-fold)
260 compared to *smc6*+ (1.7-fold) suggesting that reduced chromatin association only moderately
261 effects HR fork restart. To confirm this further we introduced the *nse3-R254E* mutation into
262 the *RuraR* strain and found similar results (9-fold) (Figure 4C).
263

264 Introduction of the hinge mutant (*smc6*-X) resulted in a highly elevated induction of *ura4* loss,
265 an 87-fold increase over the uninduced (Figure 4C and Table S1). Analysis of *ura4*$^-$ colonies
266 isolated after replication stalling from *smc6-X* and *smc6-74* mutants showed that most were
267 full deletions of the intervening sequence between the two *RTS1* loci (Figure S6 B and
268 C). Thus, these results highlight the ssDNA binding region of the Smc5/6 hinge as
269 particularly important for the suppression of NAHR and gross chromosomal rearrangements,
270 and that stable recruitment of a defective complex (*smc6*-X) is more detrimental at collapsed
271 replication forks than reduced Smc5/6 chromatin association (*smc6*-74 and *nse3-R254E*).
272
273 *Different requirements for Nse6 and Brc1 for recruitment of Smc5/6*
274
275 Recent work in fission yeast has shown that the Nse6 subunit and the BRCT-containing protein
276 Brc1 are required for the recruitment of Smc5/6 to distinct nuclear foci in response to DNA
277 damage[26] (Figure 5A). To investigate if these factors influence recruitment of the Smc5/6
278 complex to chromatin in unchallenged cells the genes encoding Brc1 and Nse6 were deleted in
279 the Nse4-mEos3 strain and Smc5/6 chromatin association monitored by SPT.
280
281 Deletion of either *brc1* or *nse6* resulted in an altered displacement profile and a concurrent
282 decrease in the fraction of bound molecules (Figure 5B, C). In *brc1*Δ the amount of chromatin
283 associated Smc5/6 decreased by approximately 35% showing that only a proportion of Smc5/6
284 chromatin association is dependent on Brc1. Recruitment of Brc1 to chromatin is reported to
285 be via a specific interaction with γ-H2A[44]. We therefore investigated Smc5/6 complex
286 recruitment in the absence of H2A phosphorylation. Introduction of *nse4-mEos3* into *hta1-SA*
287 *hta2-SA* mutant cells revealed a statistically significant reduction in the fraction bound, similar
288 to that seen in *brc1*Δ cells (Figure S7). These data are consistent with Brc1-dependant loading
289 of Smc5/6 being largely confined to regions of γ-H2A.
290
291 In contrast, deletion of *nse6* showed significant deviation from the wild type data, resulting in
292 an almost complete loss of chromatin associated Nse4 (Figure 5C), strongly supporting a Brc1-
293 independent role for Nse6 in the stable recruitment of Smc5/6 to the chromatin. It should be
294 emphasised that *nse6* deleted *S. pombe* cells are slow growing and very sensitive to genotoxins,
295 whereas deletion of genes encoding proteins in the core complex are inviable. Deletion of *brc1*
296 in an *nse6*Δ background is viable and results in additive sensitivity to DNA damage and

297 replication stress[26]. This suggests that Smc5/6 can still associate with chromatin in the absence
298 of Nse6, albeit at a severely reduced level. We hypothesise that the dsDNA binding activity of
299 Nse3 is sufficient for this residual association with the chromatin. In support of this prediction,
300 we were unable to generate the *nse6*Δ *nse3-R254E* double mutant suggesting it is synthetically
301 lethal. Furthermore, SPT analysis of Nse4-mEos3 in *nse6*Δ *brc1*Δ cells did not lead to further
302 reduction in the fraction of bound complexes (Figure 5D).
303
304 Previous ChIP experiments have shown that Smc5/6 is enriched at repetitive genomic loci
305 following MMS treatment and that this is dependent on Brc1 and Nse6[26]. We tested whether
306 we could detect increased Nse4 chromatin association in response to MMS treatment in *brc1*Δ
307 and *nse6*Δ cells. Both *brc1*Δ and *nse6*Δ cells failed to show any increase above levels detected
308 in untreated cells upon acute exposure to MMS (Figure 5D) supporting the hypothesis that both
309 Brc1 and Nse6 are required for Smc5/6 recruitment to sites of DNA damage[26].
310
311 *The Nse5-Nse6 subcomplex displays different kinetics than the Smc5/6 core complex*
312
313 Intrigued by the significant role of Nse6 even in the absence of DNA damage we investigated
314 the dynamics of the Nse5-Nse6 complex. We tagged both Nse5 and Nse6 with mEos3 (Figure
315 S1B) and compared their behaviour to Nse4. In contrast to Nse2 and Smc6, which show similar
316 chromatin association to Nse4 (Figure S3A), both Nse5 and Nse6 displayed a broader range of
317 displacements and were subsequently less chromatin associated (Figure 6A and B). This
318 suggests Nse6 is more dynamic than other subunits and may indicate its association with the
319 core Smc5/6 complex is transient. To determine whether chromatin association of Nse5-Nse6
320 is affected by that of the core complex we introduced the *nse6-mEos3* allele into a *smc6-74* or
321 *smc6-X* genetic background. We predicted that if Nse5-Nse6 was tightly associated with the
322 core complex then it would display reduced association in a *smc6-74* strain as seen with Nse4,
323 but not in *smc6-X* (Figure 3E and 4B). Tracking of Nse6-mEos3 in both mutants revealed no
324 significant change in the fraction bound (Figure 6C) suggesting Nse5-Nse6 has different
325 chromatin association dynamics to the core Smc5/6 complex. This would be indicative of
326 Nse5-Nse6 acting to transiently stabilise or load Smc5/6 complexes on the chromatin.
327

328 **Discussion**
329
330 The Smc5/6 complex is best known as a component of the DNA repair machinery that ensures
331 the fidelity of homologous recombination (HR). However, the complex is essential in yeast
332 which suggests it possesses additional functions beyond HR as deletions of core HR factors
333 are viable[3]. The recruitment of Smc5/6 to DNA and ATP binding/hydrolysis at both the ATP
334 sites are thought to be essential for each of its cellular roles. Understanding the molecular
335 details of how Smc5/6 associates with DNA and/or chromatin is therefore an important step in
336 elucidating how Smc5/6 regulates recombination and other potential DNA transactions. Here,
337 we have established single-particle tracking as a method to probe Smc5/6 dynamics in live
338 cells and coupled with yeast genetics and structural studies we uncover the key requirements
339 for its association with chromatin.
340
341 *Smc5/6 complex features required for stable chromatin association*
342
343 The Smc5/6 complex contains two separate ATP binding and hydrolysis sites. Both are formed
344 when the Smc5 and Smc6 head domains interact. In common with all SMC complexes, the
345 ATP binding pockets have an arginine finger, which is proposed to regulate DNA-dependent
346 ATP hydrolysis. We show that mutating either of the Smc5 or Smc6 arginine fingers resulted
347 in an increase in sensitivity to DNA damage and replication stress. This correlated with
348 decreases in the fraction of bound Smc5/6 detected in SPT experiments. Interestingly, Smc5
349 and Smc6 arginine fingers were not equivalent as we uncovered an underlying asymmetry in
350 the requirement of the two ATP binding sites for stable chromatin association. This asymmetry
351 is in line with observations made for cohesin and condensin[18,39].
352
353 One of the original *smc6* mutants, *smc6-74* (A151T) maps to the residue adjacent to the
354 arginine residue in the arginine finger domain, suggesting it is compromised in ATP hydrolysis.
355 Using a structural model based on the *Pyrococcus furiosus* SMC head domain, we engineered
356 a series of structurally informed mutations designed to compromise the arginine finger to
357 various degrees. This allowed us to dial in sensitivity to DNA damaging agents that robustly
358 correlated with a reduced ability of Smc5/6 to associate with chromatin. Taken together, these
359 observations strongly suggest that ATPase activity stimulated by DNA binding is pre-requisite
360 for Smc5/6 complex DNA/chromatin association and function.
361

362 Recent structural and biophysical data for the ssDNA-binding activity of the Smc5/6 hinge
363 domain[19] and the dsDNA-binding Nse1/3/4 module[21] allowed an investigation of the role for
364 each of these two functions in promoting Smc5/6 chromatin association. The introduction of
365 defined mutations into fission yeast demonstrated that dsDNA-binding by Nse3 is required for
366 DNA/chromatin association of the Smc5/6 complex, whereas the ability to bind ssDNA at the
367 hinge is dispensable. Since ssDNA-binding mutants are sensitive to a range of genotoxic
368 agents[19], we therefore predicted that ssDNA binding most likely plays a role in downstream
369 processes once the complex has initially bound to dsDNA/chromatin. This would be an
370 analogous situation to cohesin, whereby after initial DNA binding to dsDNA, capture of a
371 second DNA moiety is only achievable for ssDNA[45]. This prediction is supported by results
372 from our site-specific replication stall experiments, which indicate that increased levels of
373 ectopic recombination occur in Smc5/6 mutants that lack the ability to interact with ssDNA
374 correctly. This is much higher than in mutants that fail to stimulate ATPase activity and do not
375 correctly associate with chromatin.
376
377 *Interacting factors influencing Smc5/6 chromatin association*
378
379 Both Brc1 and Nse6 have been implicated in recruiting Smc5/6 to regions of γ-H2A at
380 stalled/collapsed replication forks in fission yeast[26]. We demonstrate here that deletion of either
381 one of these factors reduces the *in vivo* levels of chromatin-associated Smc5/6, in both
382 unchallenged cells and after exposure to MMS. Interestingly, deletion of *brc1* or preventing
383 histone H2A phosphorylation did not generate as severe a defect in chromatin association as
384 deletion of *nse6*. This is in agreement with recent ChIP experiments performed at discreet
385 genomic loci[26] and demonstrates that there is at least one alternative Brc1-independent pathway
386 for recruitment of Smc5/6 to chromatin.
387
388 To explain the data, we consider two possible modes of chromatin association: directed and
389 non-directed association (Figure 6C). Directed association occurs when the complex is
390 recruited to discrete genomic loci via interaction between the Nse5/6 subcomplex and
391 chromatin associated factors. This occurs via Brc1 at sites of γ-H2A but alternative Nse5/6-
392 interacting partners may exist to bring the complex to specific DNA structures, including
393 stalled replication forks, HR intermediates and double strand breaks.

394 Association with the chromatin may also occur in a non-directed manner via Smc5/6's intrinsic
395 ability to associate with DNA through the dsDNA binding site of Nse3. In this scenario,
396 Smc5/6 initially binds DNA structures directly via Nse3 and the Nse5/Nse6 subcomplex acts
397 transiently to stabilise this interaction. This would help explain some important observations.
398 Firstly, while Smc5, Smc6 and Nse1-4 are all essential proteins, fission yeast cells can survive
399 without Nse5/Nse6. In the absence of Nse5/Nse6, the complex still possesses dsDNA binding
400 activity, but the association with the chromatin is unstable. Secondly, deletion of *nse6* is
401 synthetically lethal with the hypomorphic dsDNA binding mutant *nse3-R254E,* suggesting the
402 dsDNA binding activity is sufficient to retain viability in the absence of exogenous DNA
403 damage or replicative stress. If Nse5/6 is required to stabilise DNA/chromatin association after
404 an initial recruitment by dsDNA binding, it would explain both the essential nature of the
405 dsDNA binding activity of Nse3 and the observations that dsDNA binding site is tightly linked
406 to chromatin association.
407
408 These two modes are not mutually exclusive and, in both cases, the Nse5/6 heterodimer may
409 be acting transiently to regulate structural configurations of the complex that promote stable
410 association with the chromatin ('loading'), much like the model for Mis4-Ssl3 being the loader
411 for cohesin[25,46]. Our SPT experiments show that Nse5 and Nse6 are more mobile than
412 components of the core Smc5/6 complex suggesting alternative kinetics. This would be
413 analogous to the cohesin loader Scc2 which displays different dynamics to the cohesin complex
414 and 'hops' between chromatin bound cohesin molecules[47]. Intriguingly, two recent studies
415 have demonstrated that Nse5/6 negatively regulates the ATPase activity of Smc5/6 *in vitro*,
416 and binding to the core complex causes conformational alterations[48,49]. Taken together with
417 our observations that DNA-stimulated ATPase activity is required for stable loading to the
418 chromatin, this provides an Nse5/6-dependent mechanism by which ATPase activity is
419 repressed until a DNA substrate is encountered. We predict that once Nse5/6 inhibition of
420 Smc5/6 ATPase is relieved it is then released from the core complex.
421
422 In summary, by conducting a detailed characterisation of Smc5/6 chromatin association in live
423 cells we demonstrate that SPT is a powerful approach for studying this enigmatic complex.
424 This methodology, when coupled with structure-led mutational analysis and yeast genetics, has
425 provided new insights into Smc5/6 behaviour as well as clarifying previous observations from
426 past genetic and molecular genetic experiments.
427

428 **Materials and Methods**

429

430 *S. pombe strain construction*

431

432 *S. pombe* strains were constructed using Cre-lox mediated cassette exchange (RMCE) as
433 previously described[50]. Strains were created either with essential gene replacement base strains
434 or C-terminal tagging base strains (Supplementary table S2). C-terminal base strains were
435 transformed with plasmid pAW8-mEos3.2-KanMX6 to introduce the mEos3.2 tag at the C-
436 terminal end of the gene.

437

438 *Microscopy sample preparation*

439

440 *S. pombe* cultures were grown to mid-log phase at 30°C in Edinburgh minimal media (EMM)
441 supplemented with leucine, uracil and adenine. Cells were harvested and washed once in
442 phosphate buffered saline (PBS). Cells were then resuspended in PBS and 10µl was deposited
443 on an EMM-agarose pad before being mounted on ozone-cleaned circular coverslips (Thorlabs,
444 #1.5H, ∅25mm) and placed in a metal cell chamber for imaging (Attofluor, Thermofisher).
445 For replicative stress experiments, MMS was added to cultures at a final concentration of
446 0.03% and incubated for 5 hours before being processed for imaging.

447

448 *PALM microscopy*

449

450 Live *S. pombe* cells were imaged with a custom-built microscope similar to that previously
451 described[51]. The microscope is built around an inverted Olympus IX73 body fitted with a
452 motorized stage (Prior H117E1I4) and a heated incubation chamber (Digital Pixel Ltd). Cells
453 were illuminated using a 561-nm imaging laser (Cobolt, Jive) and a 405-nm activation laser
454 (LaserBoxx, Oxxius). Both laser beams were expanded and collimated and were focused to the
455 back focal plane (BFP) of an apochromatic 1.45 NA, 60× TIRF objective (Olympus, UIS2
456 APON 60× OTIRF). Both beams were angled in a highly inclined near-TIRF manner to achieve
457 high signal-to-background. Illumination of the sample was controlled via mechanical shutters
458 and all components were computer-controlled using the Micro-Manager software. The
459 emission fluorescence from the sample was filtered with a band-pass filter (Semrock 593/40)

460 before being expanded to create an optimized image pixel size of 101 nm after projection onto
461 the EMCCD camera (Photometrics Evolve 512 Delta).
462
463 Samples were mounted on microscope stage and incubated at 30°C. Cells were illuminated
464 with continuous 561nm excitation (8.3mW at rear aperture of objective lens) and pulsed with
465 100ms 405nm laser illumination every 10s in order to photoconvert mEos3.2 molecules (max.
466 0.23mW at rear aperture of objective lens). We established the number of nuclei that needed
467 to be assayed for reproducibility empirically. To ensure that single-molecule traces were
468 recorded from a sufficient number of nuclei (>50) each biological repeat consisted of data
469 collection from at least 2 separate fields of view imaged one after the other (technical repeats).
470 Each acquisition consisted of 20,000 frames with a camera exposure time of 20ms.
471
472 *Single particle tracking data analysis*
473
474 Raw sptPALM data was analysed using the 'PeakFit' plugin of the GDSC single-molecule
475 localisation microscopy software package for Fiji (GDSC SMLM -
476 https://github.com/aherbert/gdsc-smlm). Single molecules were identified and localised using
477 a 2D gaussian fitting routine (configuration file available on request). Nuclear localisations
478 consisting of a minimum of 20 photons and localised to a precision of 40nm or better were
479 retained for further analysis. Single molecules were then tracked through time using the 'Trace
480 Diffusion' GDSC SMLM plugin. Localisations appearing in consecutive frames within a
481 threshold distance of 800nm were joined together into a trajectory[51]. Single molecule
482 trajectories were then exported into .csv Spot-On format using the 'Trace Exporter' plugin.
483
484 Track data was uploaded into the Spot-On web interface and was analysed using the following
485 jump length distribution parameters: Bin width (μm) =0.01, number of timepoints =5, Jumps
486 to consider =4, Max jump (μm) =3. For all Smc5/6 components, data sets were fit with a 3-
487 state Spot-On model using the default parameters, except for: D_{slow} min =0.08, localisation
488 error fit from data =yes, dZ (μm) =0.9. The decision on which Spot-On model to fit was based
489 on the Akaike information criterion (AIC) reported by Spot-On (see Figure S8). It is not clear
490 whether this third state describes transient interactions with chromatin or arises from
491 anomalous diffusion as a result of a crowded molecular environment[32]. For cohesin data sets

492 we fit a two-state model with the same parameters, excluding D_{slow}. In all cases, the model was
493 fit to the cumulative distribution function (CDF).
494
495 Probability density function (PDF) histograms and model fit were created using data combined
496 from all three repeats of an experiment and exported from Spot-On before being graphed in
497 Prism (GraphPad). Bar charts were produced by fitting data collected in each repeat (three
498 fields of view) and extracting the fraction of bound molecules. Black circles represent the value
499 derived for each repeat, bars represent the mean and error bars denote standard error of the
500 mean. Two-tailed t-test was performed in Prism software of the Spot-On F_{bound} values from
501 three repeats. Nuclear single molecule traces used for analysis in SpotOn are available via the
502 Open Science Framework (osf.io/myxtr).
503
504 *Structural modelling*
505
506 Sequence-threaded homology models for the head domains of both *S. pombe* Smc5 and Smc6
507 were generated using the PHYRE2 web portal[52]. The potential effects of introducing single
508 point mutations were assessed using PyMOL (v2.32, The PyMOL Molecular Graphics System,
509 Version 2.32, Schrödinger, LLC)
510
511 *Yeast spot test assay*
512
513 Yeast strains were cultured in yeast extract (YE) overnight to mid-log phase. Cells were
514 harvested and resuspended to a concentration of 10^7 cells/ml. Serial dilutions were then spotted
515 onto YE agar plates containing the indicated genotoxic agent.
516
517 *Yeast gross chromosomal rearrangement assay*
518
519 The rate of *ura4*$^+$ loss in the *RuraR* system was measured using a previously described
520 fluctuation test[40]. Colonies growing on YNBA plates lacking uracil (and containing thiamine)
521 were re-streaked onto YNBA plates containing uracil, either in the presence or absence of
522 thiamine. After 5 days, 5 colonies were picked from either condition and each was grown to
523 saturation (~48hrs) in 10ml liquid EMM culture containing uracil, with or without thiamine.
524

525 Each culture was counted and about $1x10^7$ cells were plated in triplicate on YEA plates
526 containing 5'-fluoroorotic acid (5'-FOA; Melford). 100µl of a 1:20000 dilution of each
527 saturated culture (about 200 cells) was plated in duplicate on YEA as titre plates. After 5 to 7
528 days of growth, 5-FOA resistant colonies and colonies on YEA were counted. A proportion of
529 5-FOA resistant colonies were streaked on YNBA lacking uracil to verify *ura4* gene function
530 loss. These *ura4*$^-$ colonies were used in the translocation PCR assay as described previously[40].
531 The rate of *ura4* loss per cell per generation was calculated using the maximum likelihood
532 estimate of the Luria-Delbruck with a correction for inefficient plating[53]. We performed all
533 computations using the R package rSalvador[54].

Author contributions

TJE, AWO, AMC and JMM conceived the experimental approach. TJE and MAO built the custom microscope. TJE acquired and analysed the microscopy data. AH wrote and benchmarked the PeakFit custom single-molecule software. TJE, DVC, AI, HQD and ATW performed strain construction, phenotypic analyses and molecular biology. AI performed translocation PCR assay. ECF performed fluctuation test data analysis. AWO performed structural analysis and designed mutations. TJE, AWO, AMC and JMM wrote the manuscript.

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553 **Figure legends**

554

555 **Figure 1**

556 *Single particle tracking of Smc5/6 to monitor chromatin association in live cells.*

557

558 A. Schematic representation of the Smc5/6 complex in fission yeast.

559

560 B. Nse4-mEos3 tracking shows nuclear localisation of trajectories. SPT trajectories
561 demonstrated confinement within nuclear region (right), that colocalised with the nuclear
562 replication protein Mcm4 fused to GFP. Scale bar = 2μm

563

564 C. Overview of approach to quantifying chromatin association using SPT data and Spot-On
565 kinetic modelling.

566

567 D. Probability density function (PDF) histograms and Spot-On model fitting (dashed line) for
568 Nse4-mEos3 (Smc5/6) and Rad21-mEos3 (cohesin) single-molecule displacements at different
569 time intervals. Displacements are from 3 pooled independent experiments, each with three
570 technical repeats.

571

572 E. Fraction bound values derived from Spot-On model fitting. Mean (+/- S.D). Black dots
573 indicate Spot-On F_{bound} values derived from each technical repeat from 3 independent
574 experiments. Percentages in blue denote fraction bound value from fitting pooled data in D.
575 **** = $p<0.0001$.

576

577 **Figure 2**

578 *Stable Smc5/6 chromatin association requires dsDNA binding activity*

579

580 A. Schematic representation of the region of known dsDNA interaction in *S. pombe* Smc5/6.

581

582 B. Probability density function histogram of pooled Nse4-mEos3 single-molecule in *nse3-*
583 *R254E* background and Spot-On model fitting (dashed line). the resulting fraction of bound
584 molecules compared to wild type data set. Bar chart shows mean +/- S.E.M. Black dots denote
585 independent repeats. *** p=0.0003

586

587 C. Cumulative distribution function (CDF) of pooled Δt = 80ms data from B.

588

589 D. F_{bound} values derived from Spot-On model fitting of Nse4-mEos3 in *nse3-R254E*
590 background. Black dots denote each technical repeat from 3 independent experiments.
591 Percentages in blue denote fraction bound value from fitting pooled data in B. Mean (+/- S.D).
592 **** = p<0.0001.

593

594 **Figure 3**
595
596 *Smc5/6 ATPase activity regulates chromatin association.*
597
598 A. Schematic representation of SMC head engagement upon ATP binding.
599
600 B. CDF of pooled Δt = 80ms single molecule displacements of Nse4-mEos3 in *smc5+ smc6+,*
601 *smc6-R150A* and *smc5-R77A* genetic backgrounds.
602
603 C. Comparison of the fraction of bound molecules from Nse4-mEos3 sptPALM experiments
604 in asynchronous *smc6-R150A* and *smc5-R77A* genetic backgrounds to wild type dataset. Black
605 dots denote each technical repeat from 3 independent experiments. Percentages in blue denote
606 fraction bound value from fitting pooled data. Mean (+/- S.D). *** p=0.0001, **** = p<0.0001.
607
608 D. Secondary structure molecular cartoons of homology models for the head domains of *S.*
609 *pombe* Smc6, highlighting the arginine finger and its interaction with ATP. The X-ray crystal
610 structure for the head domain of *Pyrococcus furiosus SMC* in complex with ATP served as a
611 reference, providing the expected position of bound ATP the homology model. Key amino
612 acids are shown in 'stick representation'. The lower panel shows the predicted increase in
613 severity of steric clashes made with the arginine finger through introduction of each of the
614 indicated mutations.
615
616 E. Yeast spot assay of *S. pombe* strains harbouring different *smc6* ATPase mutations grown at
617 30°C for 3 days.
618
619 F. Fraction bound values in each of the *smc6-T135* mutant backgrounds compared to a wild
620 type data set and *smc6-74* (A151T). Black dots denote each technical repeat from 3 independent
621 experiments. Percentages in blue denote fraction bound value from fitting pooled data. Mean
622 (+/- S.D). * p=0.0158, **** = p<0.0001.
623
624 G. Fraction bound values derived from SPT analysis of MMS treated (0.03%, 5 hours) cells
625 compared to asynchronous untreated data in F. * p=0.0495, *** p=0.0005
626

627 **Figure 4**

628 *ssDNA interactions are required to prevent gross chromosomal re-arrangements but*
629 *dispensable for stable Smc5/6 chromatin association.*

630

631 A. Left: Schematic representation of the hinge region known to interact with ssDNA interaction
632 in Smc5/6. Right: Schematic diagram of the *S. pombe* hinge region adapted from[19]. Residues
633 implicated in ssDNA interaction are highlighted with red filled circles.

634

635 B. Fraction bound values of Nse4-mEos3 derived from SPT experiments in Smc5/6 hinge
636 mutant backgrounds compared to wild type dataset. Mean +/- S.D. Black dots denote
637 independent repeats and percentages in blue denote fraction bound value from fitting pooled
638 data from all repeats.

639

640 C. Diagram of the site-specific replication stall system *RTS1-ura4-RTS1*[40], which consists of
641 two inverted *RTS1* sequences integrated on either sides of the *ura4* gene. Rtf1 binds the *RTS1*
642 sequence and stalls incoming replication forks coming from both centromeric and telomeric
643 sides. Rtf1 is expressed under the control of the *nmt41* promoter which is "off" in the presence
644 of thiamine and "on" upon thiamine removal.

645

646 D. Induction of *rtf1* in cells harbouring *RuraR* construct induces *ura4* marker loss as assayed
647 by 5-FOA resistance. Cells growing in the presence, (Off, arrest repressed) or absence (On,
648 arrest induced) of thiamine were analysed by fluctuation analysis. Mean +/- S.E.M. Black dots
649 denote independent repeats.

650

651

652

653 **Figure 5**

654 *Differential requirements of Nse6 and Brc1 for Smc5/6 chromatin association.*

655

656 A. Schematic diagram of Smc5/6 recruitment to γ-H2A (red dots: H2A phosphorylation) at
657 stalled replication forks. Brc1 binds to γ-H2A and recruits Smc5/6 via an interaction with Nse6.
658 Yellow star indicates a DNA lesion.

659

660 B. Displacement PDF histograms from asynchronous cells expressing Nse4-mEos3 in *brc1*Δ
661 and *nse6*Δ genetic backgrounds. Data are from 3 pooled independent experiments, each with
662 three technical repeats. Spot-On model fit is denoted by dashed line.

663

664 C. Comparison of Nse4-mEos3 F_{bound} values derived from Spot-On fitting of SPT displacement
665 histograms in wild type, *brc1*Δ*, nse6*Δ *and brc1*Δ *nse6*Δ genetic backgrounds. Mean +/- S.D.
666 Black dots values derived from independent technical repeats, percentages in blue denote
667 fraction bound value from fitting pooled data from all repeats. **** = $p<0.0001$, ** $p=0.0043$

668

669 D. F_{Bound} fraction values from *brc1*Δ *and nse6*Δ cells in C compared to parallel experiments
670 where cells were treated with 0.03% MMS for 5 hours. *** = $p<0.005$, *ns* = not significant.

671

672

673 **Figure 6**

674 *Nse5-Nse6 chromatin association is distinct from other Smc5/6 subunits*

675

676 A. CDF histogram of pooled single molecule displacements at Δt = 80ms time interval of Nse4-
677 mEos3, Nse5-mEos3 and Nse6-mEos3.

678

679 B. Fraction of bound molecules extracted from Spot-On model fits from experiment in A.
680 Mean +/- S.D. Black dots denote independent technical repeats, percentages denote fraction
681 bound value from fitting pooled data from all repeats. *** p=0.0003, **** = p<0.0001

682

683 C. Fraction of bound molecules extracted from Spot-On model fits from SPT Nse6-mEos3 in
684 *smc6*-74 or *smc6*-X genetic backgrounds compared to wild type data in B.

685

686 D. Schematic diagram of Smc5/6 DNA interactions and their roles (left) and proposed model
687 of Smc5/6 chromatin association (right). Loading requires dsDNA binding by Nse3 and Smc5
688 and Smc6 ATPase activity. ssDNA binding at the hinge is not required for loading but is
689 required for subsequent functions to regulate homologous recombination, suppress non-allelic
690 recombination and GCRs. Smc5/6 association with chromatin is dependent on Nse5 and Nse6
691 and either directed (e.g. Brc1-dependent recruitment to γ-H2A) (top) or non-directed via
692 dsDNA binding and subsequent loading (bottom). Nse5/6 is required in both instances and may
693 act either to directly load Smc5/6 or may stabilise its association after initial loading by dsDNA
694 interaction.

695

696 **Supplementary Figure 1**

697

698 *Characterisation of mEos3 tagged SMC subunits.*

699

700 Spot assay of *S. pombe* strains expressing A) different SMC components and B) Nse5 or Nse6

701 fused to the mEos3 fluorescent tag. Plates were incubated at 30°C for 3 days.

702

703 **Supplementary Figure 2**

704

705 *Outline of the single particle tracking technique*

706

707 A. Diagram of SPT experimental approach. *Top* - Single mEos3 fluorophores fused to SMC
708 components are stochastically photoconverted and imaged using 405nm and 561nm laser light
709 respectively. In each frame the position of the fluorophore is recorded in each frame it is
710 detected allowing for the creation of a trajectory. Multiple molecules are imaged in each cell
711 over the course of an experiment. *Bottom* – Laser illumination scheme for each experiment.
712 mEos3 is photoconverted using 0.1s pulses of 405nm laser every 10s and photoconverted
713 species are imaged by continuous 561nm illumination.

714

715 B. Raw data processing pipeline for SPT experiments. For specific details see Material and
716 Methods.

717

718 **Supplementary Figure 3**

719

720 *Single-particle tracking of SMC complex subunits*

721

722 A. PDF histograms of single-molecule displacements (left) and fraction bound values
723 calculated from Spot-On model fitting (right) of alternative Smc5/6 subunits tagged with
724 mEos3 compared to Nse4-mEos3. Mean (+/- S.D). Black dots values derived from independent
725 technical repeats, percentages in blue denote fraction bound value from fitting pooled data from
726 all repeats

727

728 B. PDF histograms of single-molecule displacements (left) and fraction bound values
729 calculated from Spot-On model fitting (right) of alternative cohesin subunit, Smc1, tagged with
730 mEos3 compared to Rad21-mEos3. Mean (+/- S.D). Black dots values derived from
731 independent technical repeats, percentages in blue denote fraction bound value from fitting
732 pooled data from all repeats

733

734 **Supplementary Figure 4**

735

736 *Characterisation of mEos3 tagged Smc5/6 ATPase mutants.*

737

738 A. Spot assay of *S. pombe* strains harbouring arginine finger mutations in either *smc5* or *smc6*.
739 Plates were incubated at 30°C for 3 days.

740

741 B. PDF histograms of single-molecule displacements for multiple Δt of Nse4-mEos3 for
742 *smc5* (R77A) or *smc6* (R150A) arginine finger mutants (see Figure 3C and F for fraction
743 bound). Dashed line indicates model derived from CDF fitting in Spot-On. Data are pooled
744 from 3 individual experiments, each with 3 technical repeats.

745

746 C. PDF histograms of single-molecule displacements for multiple Δt of Nse4-mEos3 in the
747 indicated mutants (see figure 4E for fraction bound). Dashed line indicates model derived from
748 CDF fitting in Spot-On. Data are pooled from 3 individual experiments, each with 3 technical
749 repeats.

750

751 **Supplementary Figure 5**

752

753 *Spot-On analysis of MMS treated ssDNA interaction mutants*

754

755 A. Fraction of bound Nse4-mEos3 extracted from Spot-On analysis of Nse4-mEos3 SPT in
756 *smc6* hinge mutants treated with 0.03% MMS for 5 hours. Compared to asynchronous
757 untreated datasets from Figure 4B. Mean +/- S.D. Black dots denote independent technical
758 repeats, percentages denote fraction bound value from fitting pooled data from all repeats.
759 ** = p< 0.005 (*smc6-X* = 0.0034, *smc5-RR* = 0.0025), *** p=0.0005.
760
761

762 **Supplementary Figure 6**

763

764 *Analysis of the consequences of site-specific replication fork stalling on cell viability and gross*
765 *chromosomal re-arrangements.*

766

767 **A.** Yeast spot assay of *S. pombe* strains harbouring the site-specific replication stall system
768 *RuraR*. Replication fork stalling at *RTS1* is induced in the absence of thiamine (on). Plates were
769 incubated at 30°C for 3 days. Unlike the HR-defective *rad51*Δ strain, *smc6* hypomorphs do not
770 lose viability on induction of replication stalling at RTS1

771

772 **B.** PCR-based assay for translocation between *RTS1* at *RuraR* and the native *RTS1* at the mating
773 type locus in *ura4*$^-$ colonies generated in the *ura4* loss of gene function assay[40]. Left: schematic
774 to show the three primer pairs used. One pair (red arrows) amplifies the junction resulting from
775 ectopic recombination between chromosome II and III (TLII/III). The second pair (grey
776 arrows) amplifies the *ura4* locus to distinguish point mutations, truncations (internal deletions)
777 and full-length deletions. *rng3* (blue arrows), an essential gene located between *RuraR* and the
778 telomere, is amplified as positive control. Right: Example of control PCRs (top) and PCRs of
779 5-FOA resistant/*ura4*$^-$ colonies (bottom). The *rng3* product is amplified in all strains, but not
780 in the negative control ("-"). *ura4* is amplified only in a *RuraR* strain, but not in Wild type (wt)
781 (harbours full deletion of *ura4*, *ura4-D18*), the translocation positive control ("+", gift from S.
782 Lambert[40]) or the negative control. Translocation between chromosome II and III can only be
783 detected in the positive control.

784

785 **C.** PCR assay results for *ura*$^-$ colonies of *smc6+, smc6*-74 and *smc6*-X derived from the *RuraR*
786 *ura4* loss assay carried out in the presence (*RuraR* arrest 'Off') or absence (*RuraR* arrest 'On')
787 of thiamine.

788
789

790 **Supplementary Figure 7**

791

792 *Spot-On analysis of Nse4 chromatin association in histone phosphorylation site and brc1γ-*

793 *H2A interaction mutants*

794

795 A. Fraction of bound Nse4-mEos3 extracted from Spot-On analysis of Nse4-mEos3 SPT in

796 *hta1-S128A hta2-S129A* and *brc1-T672A* mutants compared to wild type and *brc1*Δ data sets

797 from Figure 5C. Mean +/- S.D. Black dots denote independent technical repeats, percentages

798 denote fraction bound value from fitting pooled data from all repeats.

799 ** = p< 0.005 (*hta1-SA hta2-SA* = 0.0034, *smc5-RR* = 0.0016), **** p<0.0001.

800

801

802

803 **Supplementary Figure 8**

804

805 *Smc5/6 behaviour fits a 3-state model.*

806

807 A. PDF histograms of single-molecule displacements for Nse4-mEos3 and Rad21-mEos3 over
808 multiple Δt fit with either a 2-state or 3-state Spot-On model. Data are pooled from 3
809 independent experiments, each with three technical repeats. Dashed line indicates model
810 derived from CDF fitting in Spot-On.

811

812 B. Akaike information criterion (AIC) scores from Spot-On model fitting in A. Nse4-mEos3
813 3-state fitting showed a large difference in AIC scores compared to 2-state fitting. This
814 indicates the data are best described by a 3-state model. The difference in AIC scores for
815 Rad21-mEos3 was much smaller and thus a 2-state model was used.

816

817 C. Apparent diffusion coefficients of Spot-On sub-populations of Nse4-mEos3 (3-State) and
818 Rad21-mEos3 (2-State).

819

820 D. Fractions of the total population of molecules observed residing in each kinetic state
821 extracted from Spot-On model fitting data in A.

822

823 E. Comparison of the fractions of Nse4-mEos3 molecules observed residing in each kinetic
824 state extracted from all cells (mostly G2) in the wild type data set or only binuclear cells (S-
825 phase, n=75).

826

827 **Supplementary Tables**

828

829 **Table S1 – Fluctuation experiment data**

830 Data from individual experimental repeats of *ura4* loss assay in Figure 4C

831 **Table S2 - Strain table**

832 Strains used during this study

833

834 **References**
835

836 1. Uhlmann, F. SMC complexes: from DNA to chromosomes. *Nat. Rev. Mol. Cell Biol.* **17**, 399–
837 412 (2016).
838 2. Murray, J. M. & Carr, A. M. Smc5/6: A link between DNA repair and unidirectional
839 replication? *Nat. Rev. Mol. Cell Biol.* **9**, 177–182 (2008).
840 3. Aragón, L. The Smc5/6 Complex: New and Old Functions of the Enigmatic Long-Distance
841 Relative. *Annu. Rev. Genet.* **52**, 89–107 (2018).
842 4. Irmisch, A., Ampatzidou, E., Mizuno, K., O'Connell, M. J. & Murray, J. M. Smc5/6 maintains
843 stalled replication forks in a recombination-competent conformation. *EMBO J.* **28**, 144–155
844 (2009).
845 5. Menolfi, D., Delamarre, A., Lengronne, A., Pasero, P. & Branzei, D. Essential Roles of the
846 Smc5/6 Complex in Replication through Natural Pausing Sites and Endogenous DNA Damage
847 Tolerance. *Mol. Cell* **60**, 835–846 (2015).
848 6. Xue, X. *et al.* Restriction of Replication Fork Regression Activities by a Conserved SMC
849 Complex. *Mol. Cell* **56**, 436–445 (2014).
850 7. Bonner, J. N. *et al.* Smc5/6 Mediated Sumoylation of the Sgs1-Top3-Rmi1 Complex Promotes
851 Removal of Recombination Intermediates. *Cell Rep.* **16**, 368–378 (2016).
852 8. Jeppsson, K. *et al.* The Chromosomal Association of the Smc5/6 Complex Depends on
853 Cohesion and Predicts the Level of Sister Chromatid Entanglement. *PLoS Genet.* **10**,
854 e1004680 (2014).
855 9. Bentley, P., Tan, M. J. A., McBride, A. A., White, E. A. & Howley, P. M. The SMC5/6
856 Complex Interacts with the Papillomavirus E2 Protein and Influences Maintenance of Viral
857 Episomal DNA. *J. Virol.* (2018) doi:10.1128/jvi.00356-18.
858 10. Niu, C. *et al. The Smc5/6 complex restricts HBV when localized to ND10 without inducing an*
859 *innate immune response and is counteracted by the HBV X protein shortly after infection*.
860 *PLoS ONE* vol. 12 (2017).
861 11. De Piccoli, G. *et al.* Smc5-Smc6 mediate DNA double-strand-break repair by promoting
862 sister-chromatid recombination. *Nat. Cell Biol.* **8**, 1032–1034 (2006).
863 12. Bermúdez-López, M. *et al.* The Smc5/6 complex is required for dissolution of DNA-mediated
864 sister chromatid linkages. *Nucleic Acids Res.* **38**, 6502–6512 (2010).
865 13. Hirano, M. & Hirano, T. Opening closed arms: Long-distance activation of SMC ATPase by
866 hinge-DNA interactions. *Mol. Cell* **21**, 175–186 (2006).
867 14. Diebold-Durand, M. L. *et al.* Structure of Full-Length SMC and Rearrangements Required for
868 Chromosome Organization. *Mol. Cell* **67**, 334-347.e5 (2017).
869 15. Muir, K. W., Li, Y., Weis, F. & Panne, D. The structure of the cohesin ATPase elucidates the
870 mechanism of SMC–kleisin ring opening. *Nat. Struct. Mol. Biol.* **27**, 233–239 (2020).

871 16. Elbatsh, A. M. O. *et al.* Cohesin Releases DNA through Asymmetric ATPase-Driven Ring
872 Opening. *Mol. Cell* **61**, 575–588 (2016).
873 17. Terakawa, T. *et al.* The condensin complex is a mechanochemical motor that translocates
874 along DNA. *Science (80-.).* **358**, 672–676 (2017).
875 18. Elbatsh, A. M. O. *et al.* Distinct Roles for Condensin's Two ATPase Sites in Chromosome
876 Condensation. *Mol. Cell* **76**, 724-737.e5 (2019).
877 19. Alt, A. *et al.* Specialized interfaces of Smc5/6 control hinge stability and DNA association.
878 *Nat. Commun.* **8**, 14011 (2017).
879 20. Doyle, J. M., Gao, J., Wang, J., Yang, M. & Potts, P. R. MAGE-RING protein complexes
880 comprise a family of E3 ubiquitin ligases. *Mol. Cell* **39**, 963–974 (2010).
881 21. Zabrady, K. *et al.* Chromatin association of the SMC5/6 complex is dependent on binding of
882 its NSE3 subunit to DNA. *Nucleic Acids Res.* **44**, 1064–1079 (2016).
883 22. Varejão, N. *et al.* DNA activates the Nse2/Mms21 SUMO E3 ligase in the Smc5/6 complex.
884 *EMBO J.* **37**, 1–16 (2018).
885 23. Pebernard, S., Wohlschlegel, J., McDonald, W. H., Yates, J. R. & Boddy, M. N. The Nse5-
886 Nse6 Dimer Mediates DNA Repair Roles of the Smc5-Smc6 Complex. *Mol. Cell. Biol.* **26**,
887 1617–1630 (2006).
888 24. Taylor, E. M., Copsey, A. C., Hudson, J. J. R., Vidot, S. & Lehmann, A. R. Identification of
889 the Proteins, Including MAGEG1, That Make Up the Human SMC5-6 Protein Complex. *Mol.*
890 *Cell. Biol.* **28**, 1197–1206 (2008).
891 25. Ocampo-Hafalla, M. T. & Uhlmann, F. Cohesin loading and sliding. *J. Cell Sci.* **124**, 685–691
892 (2011).
893 26. Oravcová, M. *et al.* Brc1 Promotes the Focal Accumulation and SUMO Ligase Activity of
894 Smc5-Smc6 during Replication Stress. *Mol. Cell. Biol.* **39**, 1–15 (2018).
895 27. Wan, B., Wu, J., Meng, X., Lei, M. & Zhao, X. Molecular Basis for Control of Diverse
896 Genome Stability Factors by the Multi-BRCT Scaffold Rtt107. *Mol. Cell* (2019)
897 doi:10.1016/j.molcel.2019.05.035.
898 28. Räschle, M. *et al.* Proteomics reveals dynamic assembly of Repair complexes during bypass of
899 DNA cross-links. *Science (80-.).* **348**, (2015).
900 29. Manley, S. *et al.* High-density mapping of single-molecule trajectories with photoactivated
901 localization microscopy. *Nat. Methods* **5**, 155–157 (2008).
902 30. Pebernard, S., Schaffer, L., Campbell, D., Head, S. R. & Boddy, M. N. Localization of Smc5/6
903 to centromeres and telomeres requires heterochromatin and SUMO, respectively. *EMBO J.* **27**,
904 3011–3023 (2008).
905 31. Hansen, A. S. *et al.* Robust model-based analysis of single-particle tracking experiments with
906 spot-on. *Elife* **7**, 1–33 (2018).
907 32. Woringer, M., Izeddin, I., Favard, C. & Berry, H. Anomalous Subdiffusion in Living Cells:

908 Bridging the Gap Between Experiments and Realistic Models Through Collaborative
909 Challenges. *Front. Phys.* **8**, 1–9 (2020).
910 33. Bernard, P. *et al.* Cell-cycle regulation of cohesin stability along fission yeast chromosomes.
911 *EMBO J.* (2008) doi:10.1038/sj.emboj.7601955.
912 34. Verkade, H. M., Bugg, S. J., Lindsay, H. D., Carr, A. M. & O'Connell, M. J. Rad18 is required
913 for DNA repair and checkpoint responses in fission yeast. *Mol. Biol. Cell* **10**, 2905–2918
914 (1999).
915 35. Fousteri, M. I. A novel SMC protein complex in Schizosaccharomyces pombe contains the
916 Rad18 DNA repair protein. *EMBO J.* (2000) doi:10.1093/emboj/19.7.1691.
917 36. Lammens, A., Schele, A. & Hopfner, K. P. Structural biochemistry of ATP-driven
918 dimerization and DNA-stimulated activation of SMC ATPases. *Curr. Biol.* (2004)
919 doi:10.1016/j.cub.2004.09.044.
920 37. Ampatzidou, E., Irmisch, A., O'Connell, M. J. & Murray, J. M. Smc5/6 Is Required for Repair
921 at Collapsed Replication Forks ▿ . *Mol. Cell. Biol.* **26**, 9387–9401 (2006).
922 38. Outwin, E. A., Irmisch, A., Murray, J. M. & O'Connell, M. J. Smc5-Smc6-Dependent
923 Removal of Cohesin from Mitotic Chromosomes. *Mol. Cell. Biol.* **29**, 4363–4375 (2009).
924 39. Hassler, M. *et al.* Structural Basis of an Asymmetric Condensin ATPase Cycle. *Mol. Cell* **74**,
925 1175-1188.e9 (2019).
926 40. Lambert, S., Watson, A., Sheedy, D. M., Martin, B. & Carr, A. M. Gross chromosomal
927 rearrangements and elevated recombination at an inducible site-specific replication fork
928 barrier. *Cell* (2005) doi:10.1016/j.cell.2005.03.022.
929 41. Dalgaard, J. Z. & Klar, A. J. S. A DNA replication-arrest site RTS1 regulates imprinting by
930 determining the direction of replication at mat1 in S. pombe. *Genes Dev.* (2001)
931 doi:10.1101/gad.200801.
932 42. Mizuno, K., Miyabe, I., Schalbetter, S. A., Carr, A. M. & Murray, J. M. Recombination-
933 restarted replication makes inverted chromosome fusions at inverted repeats. *Nature* **493**, 246–
934 249 (2013).
935 43. Iraqui, I. *et al.* Recovery of Arrested Replication Forks by Homologous Recombination Is
936 Error-Prone. *PLoS Genet.* (2012) doi:10.1371/journal.pgen.1002976.
937 44. Williams, J. S. *et al.* γh2A binds Brc1 to maintain genome integrity during S-phase. *EMBO J.*
938 (2010) doi:10.1038/emboj.2009.413.
939 45. Murayama, Y., Samora, C. P., Kurokawa, Y., Iwasaki, H. & Uhlmann, F. Establishment of
940 DNA-DNA Interactions by the Cohesin Ring. *Cell* **172**, 465-477.e15 (2018).
941 46. Furuya, K., Takahashi, K. & Yanagida, M. Faithful anaphase is ensured by Mis4, a sister
942 chromatid cohesion molecule required in S phase and not destroyed in G1 phase. *Genes Dev.*
943 (1998) doi:10.1101/gad.12.21.3408.

47. Rhodes, J., Mazza, D., Nasmyth, K. & Uphoff, S. Scc2/Nipbl hops between chromosomal cohesin rings after loading. *Elife* **6**, 1–20 (2017).
48. Hallett, S. T. *et al.* Nse5 / 6 is a negative regulator of the ATPase activity of the Smc5 / 6 complex. (2021).
49. Steigenberger, B., Schaefer, I., Soh, Y., Scheltema, R. A. & Gruber, S. Nse5 / 6 inhibits the Smc5 / 6 ATPase to facilitate DNA substrate selection. (2021).
50. Watson, A. T., Garcia, V., Bone, N., Carr, A. M. & Armstrong, J. Gene tagging and gene replacement using recombinase-mediated cassette exchange in Schizosaccharomyces pombe. *Gene* **407**, 63–74 (2008).
51. Etheridge, T. J. *et al.* Quantification of DNA-associated proteins inside eukaryotic cells using single-molecule localization microscopy. *Nucleic Acids Res.* **42**, (2014).
52. Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N. & Sternberg, M. J. E. The Phyre2 web portal for protein modeling, prediction and analysis. *Nat. Protoc.* (2015) doi:10.1038/nprot.2015.053.
53. Zheng, Q. A note on plating efficiency in fluctuation experiments. *Math. Biosci.* **216**, (2008).
54. Zheng, Q. rSalvador: An R Package for the Fluctuation Experiment. *G3* **7**, (2017).

Figure 1

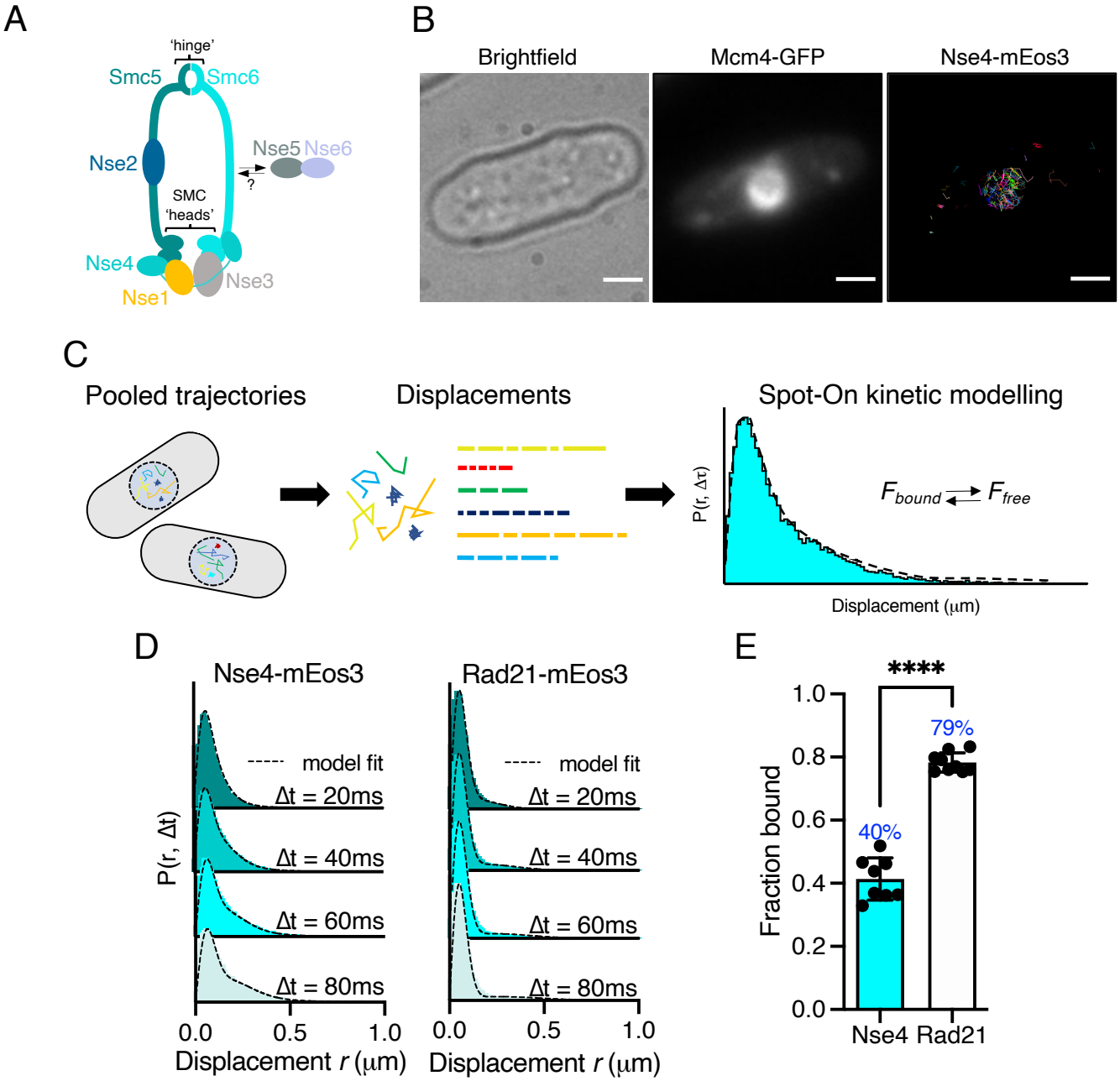

A
'hinge'
Smc5
Smc6
Nse2
Nse5
Nse6
?
SMC
'heads'
Nse4
Nse3
Nse1
B
Brightfield
Mcm4-GFP
Nse4-mEos3
C
Pooled trajectories
Displacements
Spot-On kinetic modelling
P(r, Δτ)
F bound
F free
Displacement (μm)
D
Nse4-mEos3
Rad21-mEos3
model fit
Δt = 20ms
Δt = 40ms
Δt = 60ms
Δt = 80ms
P(r, Δt)
0.0
0.5
1.0
Displacement r (μm)
E
****
79%
40%
Fraction bound
1.0
0.8
0.6
0.4
0.2
0.0
Nse4
Rad21

Figure 2

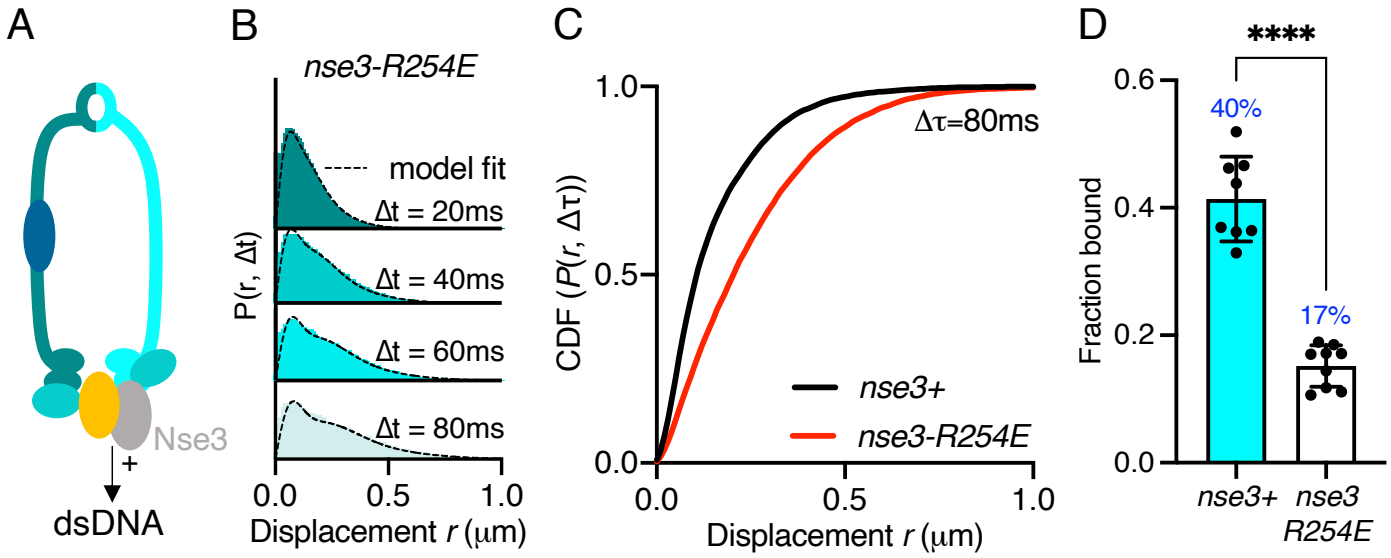

A
Nse3
+
dsDNA
B
nse3-R254E
model fit
Δt = 20ms
Δt = 40ms
Δt = 60ms
Δt = 80ms
P(r, Δt)
0.0
0.5
1.0
Displacement r (μm)
C
1.0
0.5
0.0
CDF (P(r, Δτ))
Δτ=80ms
nse3+
nse3-R254E
0.0
0.5
1.0
Displacement r (μm)
D
****
0.6
0.4
0.2
0.0
Fraction bound
40%
17%
nse3+
nse3 R254E

Figure 3

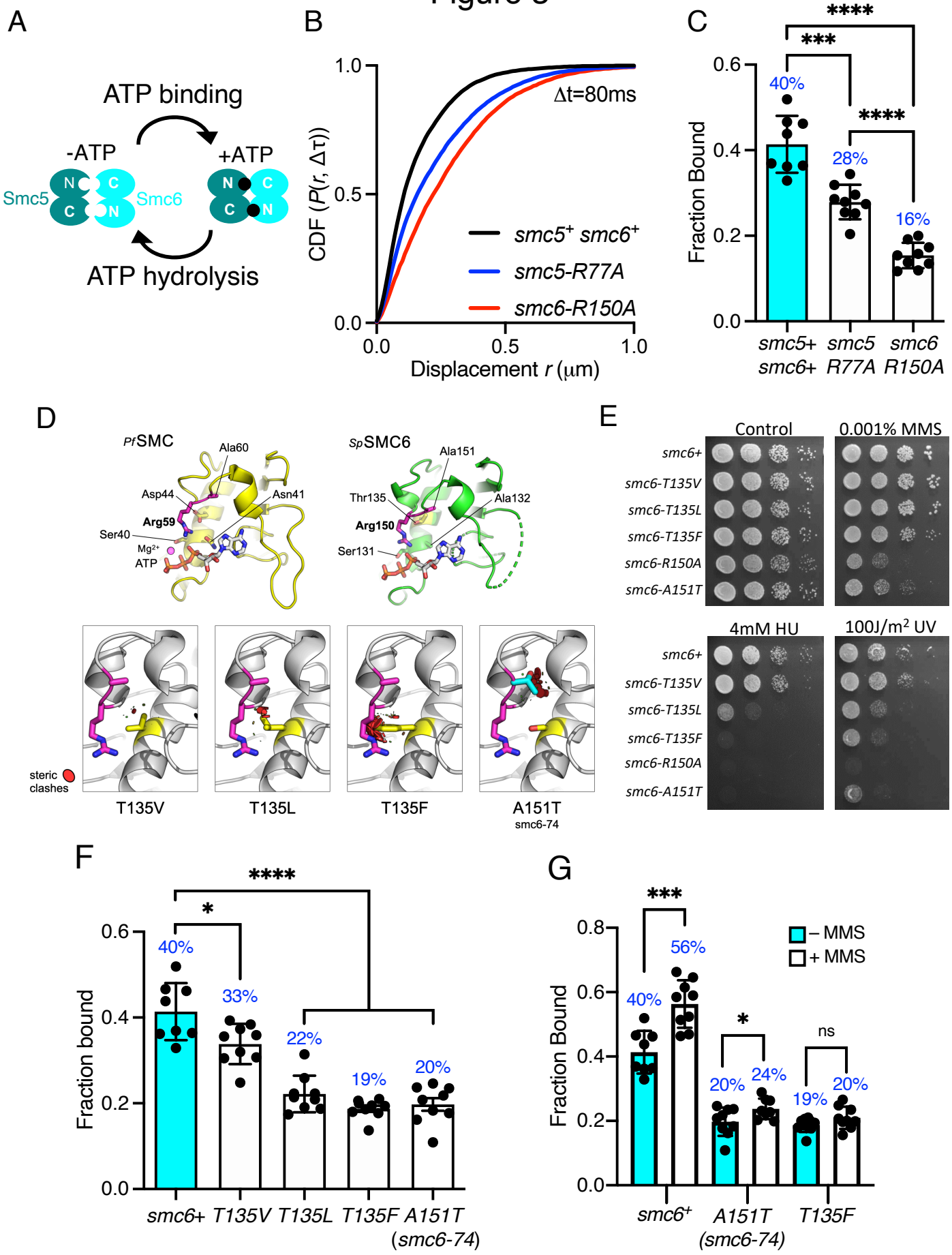

A
ATP binding
-ATP
+ATP
Smc5
Smc6
N
C
ATP hydrolysis
B
1.0
0.5
0.0
CDF (P(r, Δτ))
Δt=80ms
smc5+ smc6+
smc5-R77A
smc6-R150A
0.0
0.5
1.0
Displacement r (μm)
C
****
***
****
0.6
0.4
0.2
0.0
Fraction Bound
40%
28%
16%
smc5+ smc6+
smc5 R77A
smc6 R150A
D
PfSMC
Ala60
Asp44
Asn41
Arg59
Ser40
Mg2+
ATP
SpSMC6
Ala151
Thr135
Ala132
Arg150
Ser131
steric clashes
T135V
T135L
T135F
A151T
smc6-74
E
Control
0.001% MMS
smc6+
smc6-T135V
smc6-T135L
smc6-T135F
smc6-R150A
smc6-A151T
4mM HU
100J/m2 UV
F
****
*
0.6
0.4
0.2
0.0
Fraction bound
40%
33%
22%
19%
20%
smc6+ T135V T135L T135F A151T
(smc6-74)
G
***
0.8
0.6
0.4
0.2
0.0
Fraction Bound
– MMS
+ MMS
40%
56%
*
ns
20%
24%
19%
20%
smc6+
A151T
(smc6-74)
T135F

Figure 4

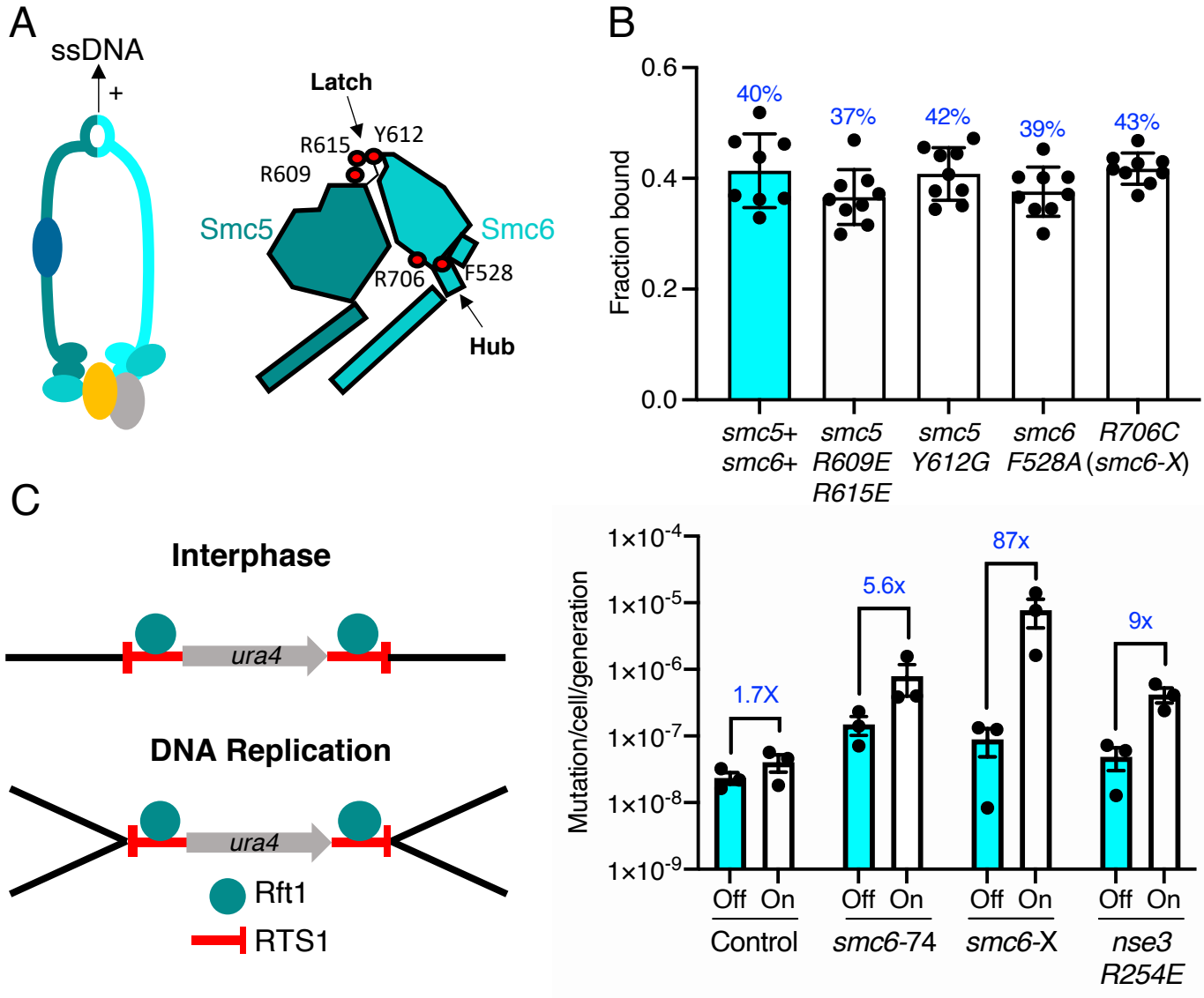

A
ssDNA
+
Latch
R615
Y612
R609
Smc5
Smc6
R706
F528
Hub
B
Fraction bound
0.6
0.4
0.2
0.0
40%
37%
42%
39%
43%
smc5+ smc6+
smc5 R609E R615E
smc5 Y612G
smc6 F528A
R706C (smc6-X)
C
Interphase
ura4
DNA Replication
ura4
Rft1
RTS1
Mutation/cell/generation
1×10-4
1×10-5
1×10-6
1×10-7
1×10-8
1×10-9
1.7X
5.6x
87x
9x
Off On
Control
smc6-74
smc6-X
nse3 R254E

Figure 5

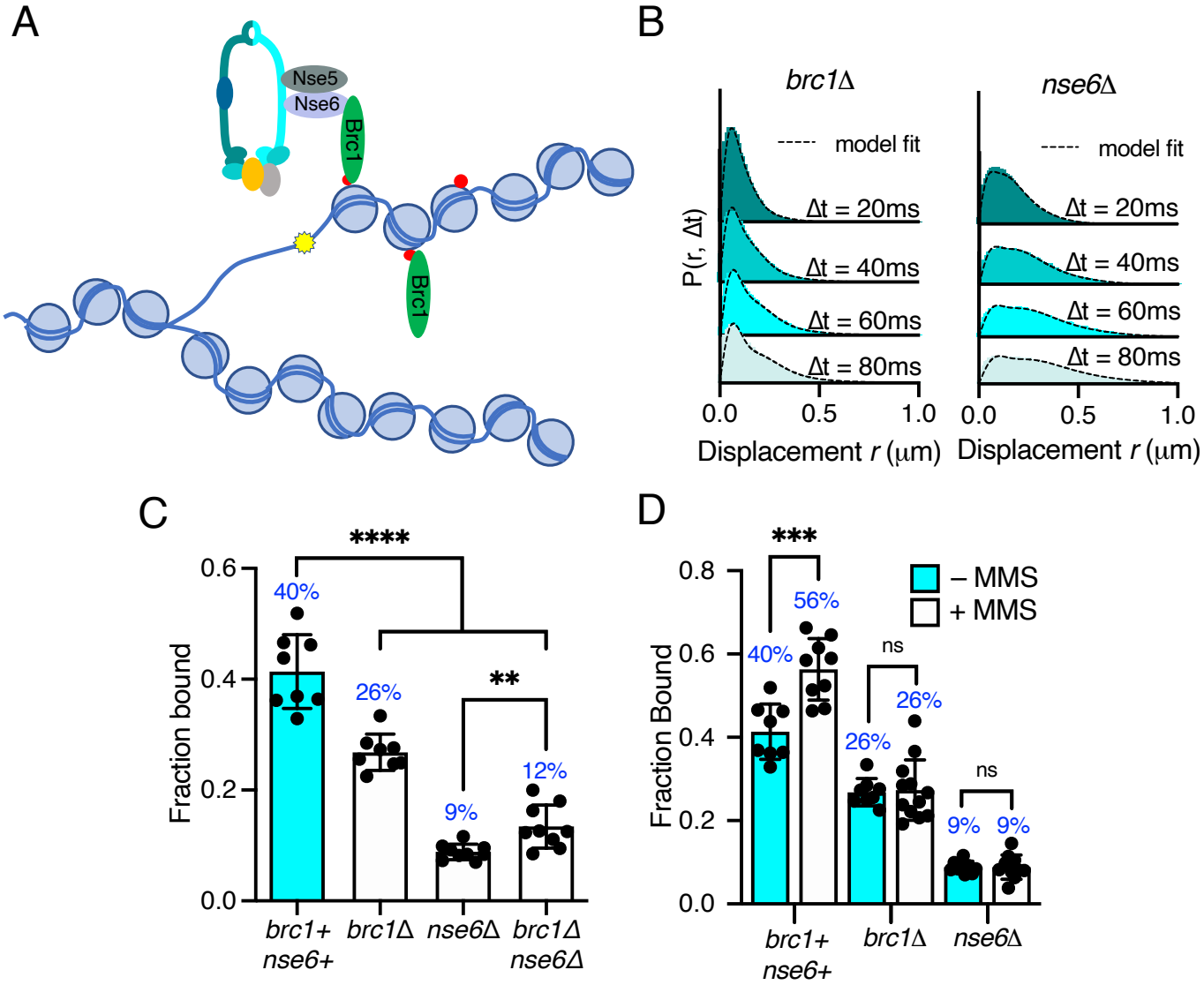
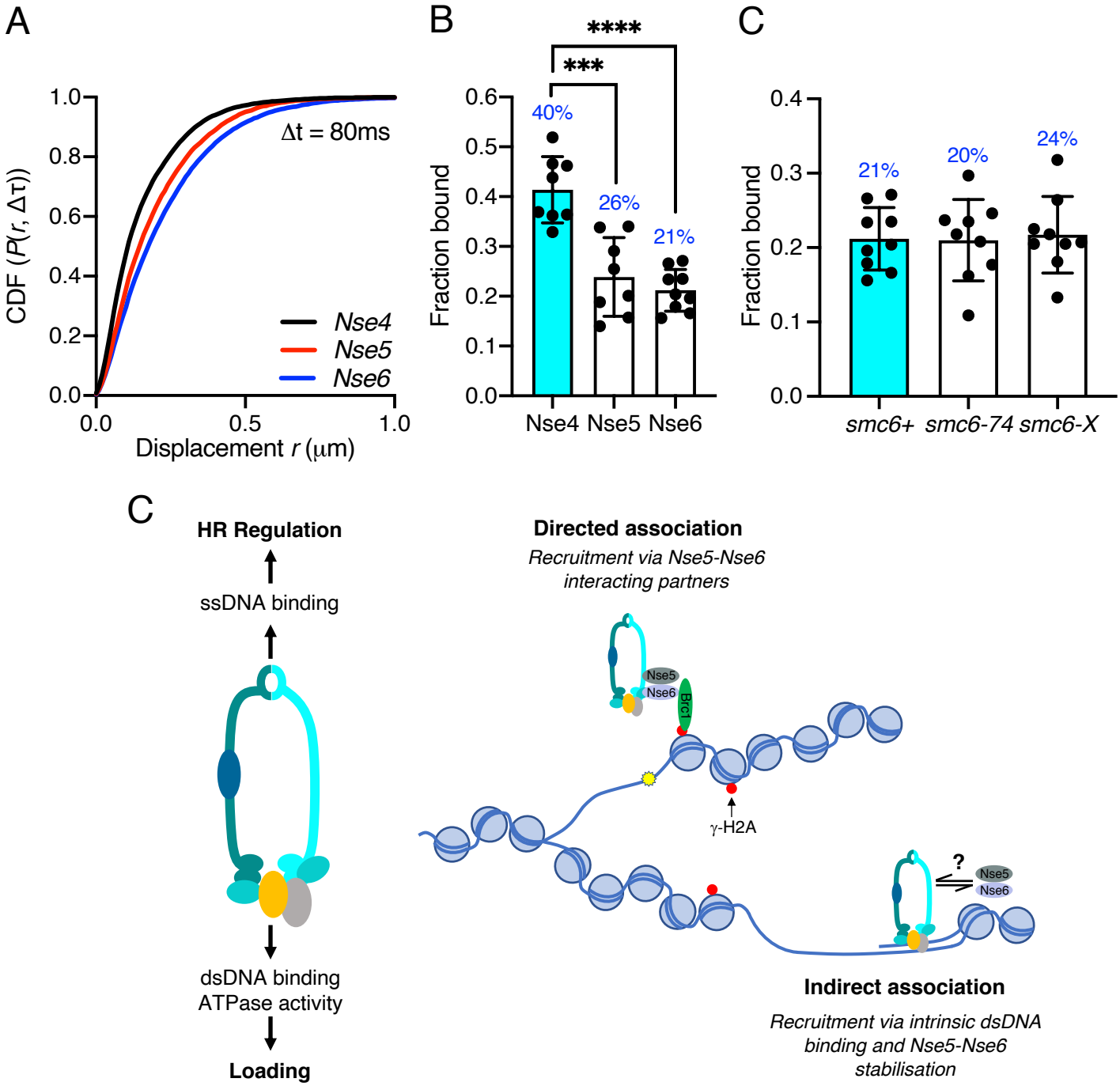
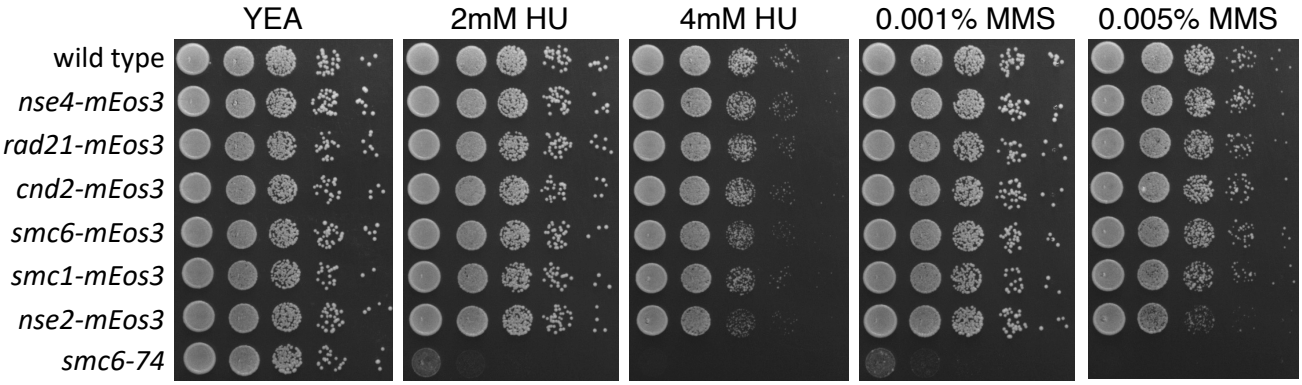

A
Nse5
Nse6
Brc1
Brc1
B
brc1Δ
nse6Δ
model fit
model fit
Δt = 20ms
Δt = 40ms
Δt = 60ms
Δt = 80ms
Δt = 20ms
Δt = 40ms
Δt = 60ms
Δt = 80ms
P(r, Δt)
0.0
0.5
1.0
0.0
0.5
1.0
Displacement r (μm)
Displacement r (μm)
C
****
**
Fraction bound
0.0
0.2
0.4
0.6
40%
26%
9%
12%
brc1+ nse6+
brc1Δ
nse6Δ
brc1Δ nse6Δ
D
***
ns
ns
– MMS
+ MMS
Fraction Bound
0.0
0.2
0.4
0.6
0.8
40%
56%
26%
26%
9%
9%
brc1+ nse6+
brc1Δ
nse6Δ

Figure 6

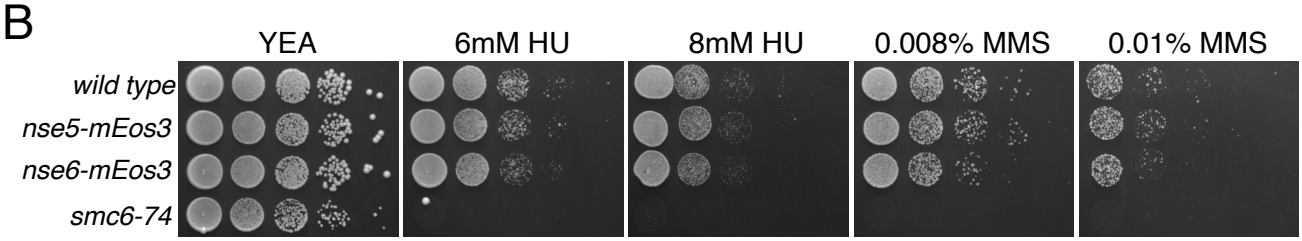

A
1.0
0.8
0.6
0.4
0.2
0.0
CDF (P(r, Δτ))
Δt = 80ms
Nse4
Nse5
Nse6
0.0
0.5
1.0
Displacement r (μm)
B
****
***
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Fraction bound
40%
26%
21%
Nse4 Nse5 Nse6
C
0.4
0.3
0.2
0.1
0.0
Fraction bound
21%
20%
24%
smc6+ smc6-74 smc6-X
C
HR Regulation
ssDNA binding
dsDNA binding
ATPase activity
Loading
Directed association
Recruitment via Nse5-Nse6
interacting partners
Nse5
Nse6
Brc1
γ-H2A
?
Nse5
Nse6
Indirect association
Recruitment via intrinsic dsDNA
binding and Nse5-Nse6
stabilisation

Supplementary Figure 1

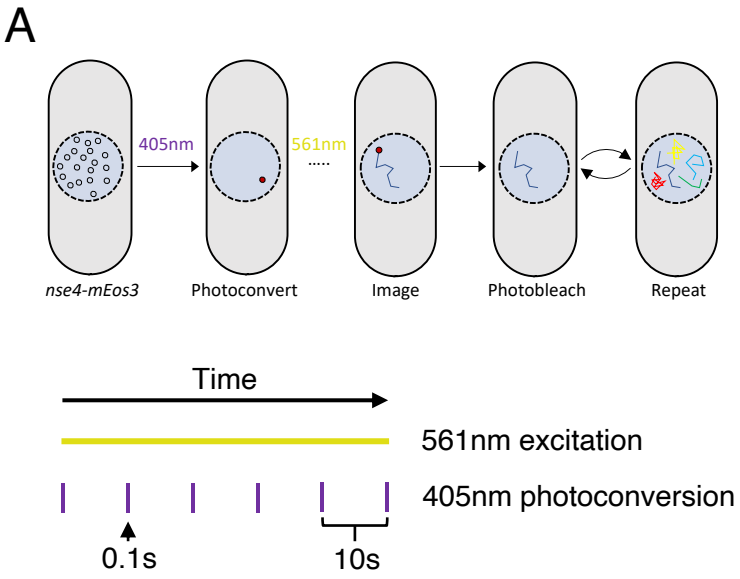
A

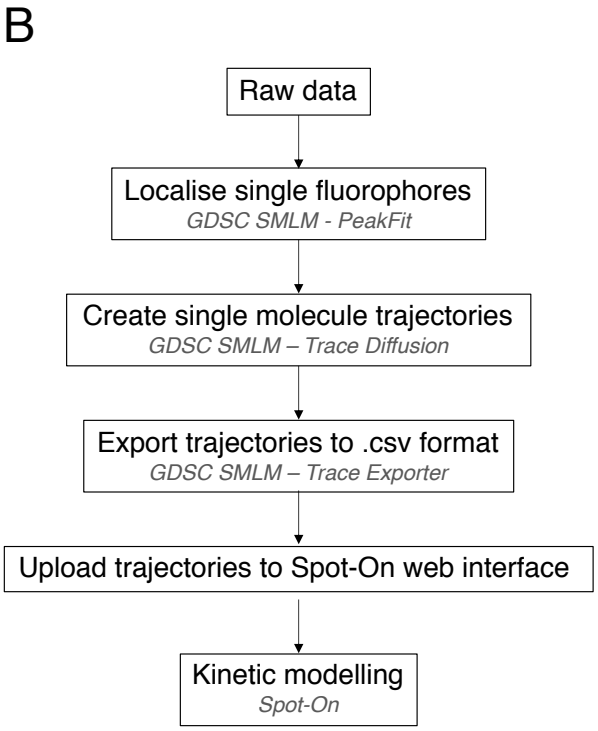


B

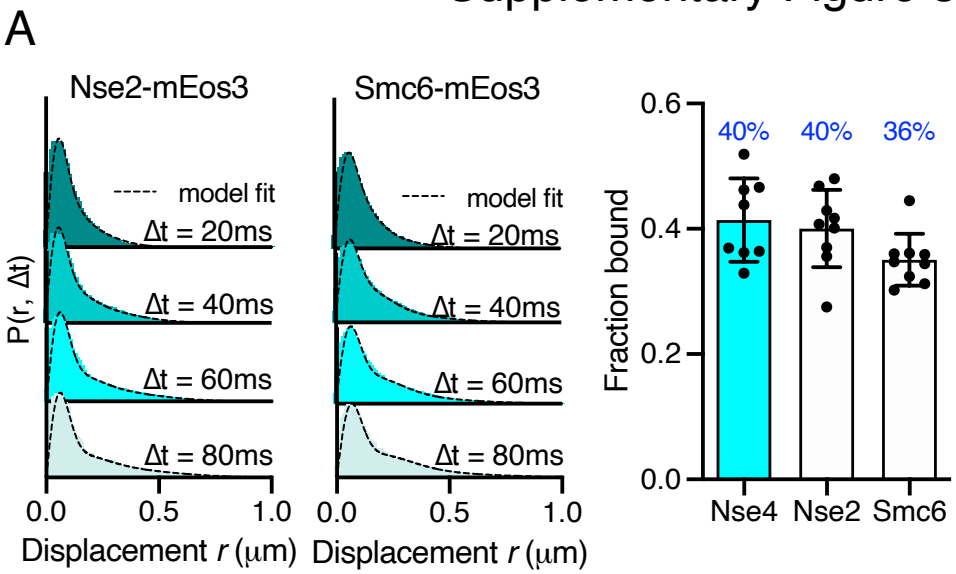


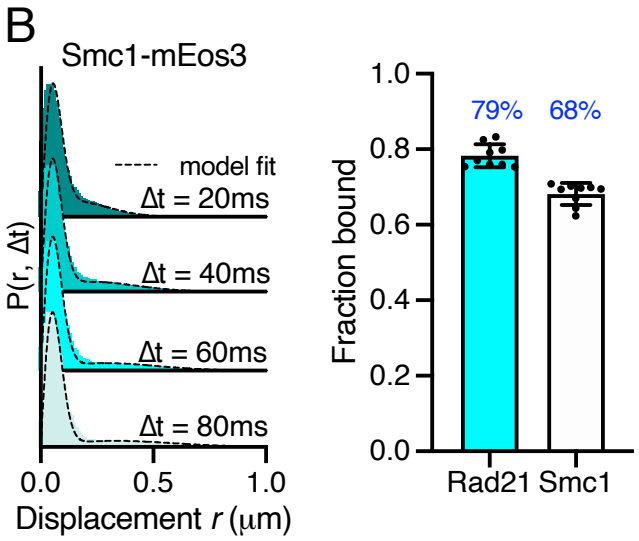
Supplementary Figure 2



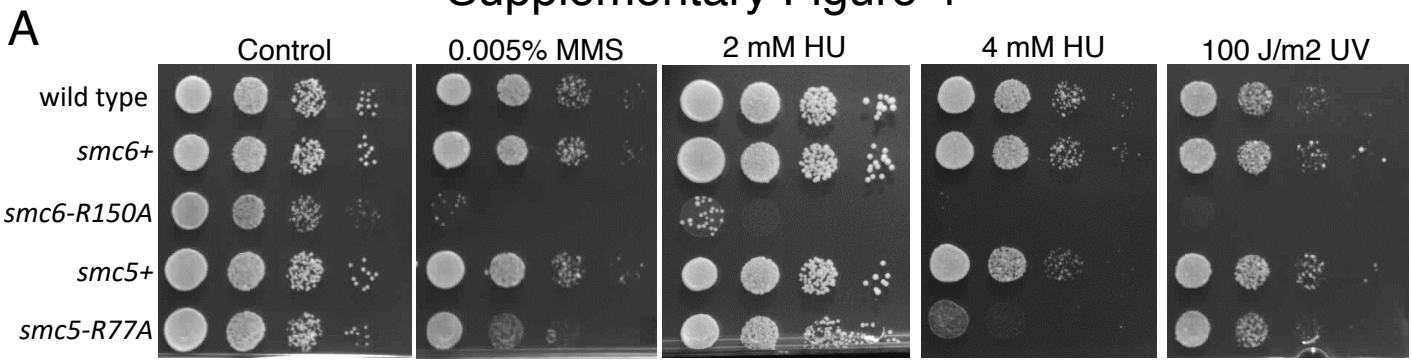


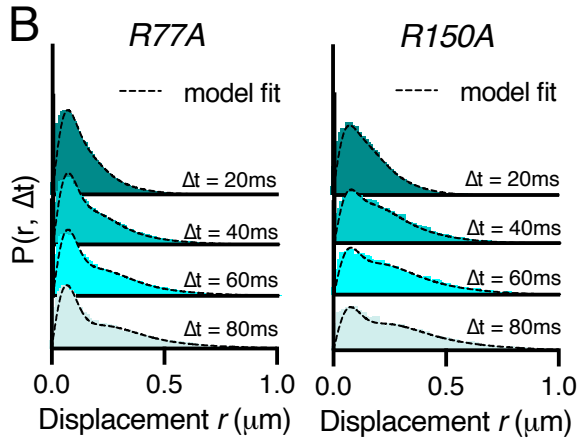
Supplementary Figure 3

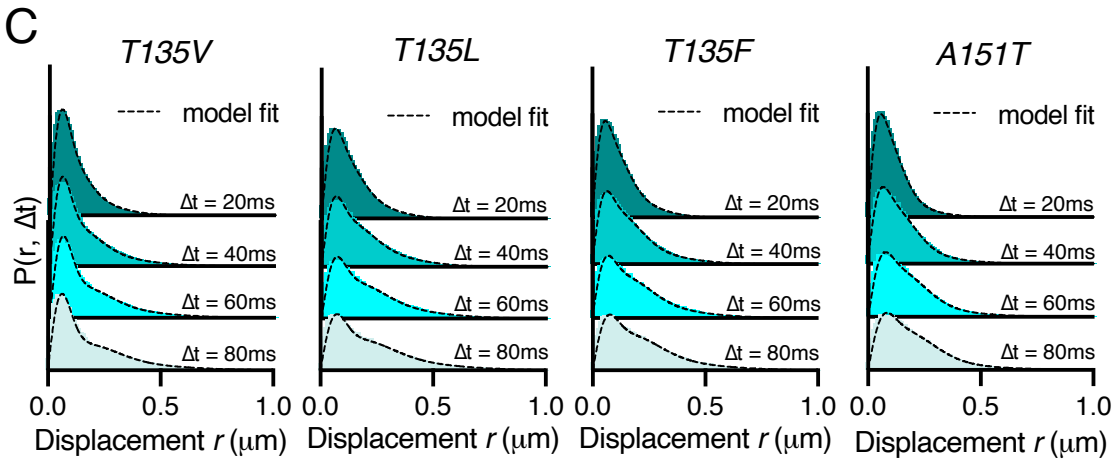




Supplementary Figure 4

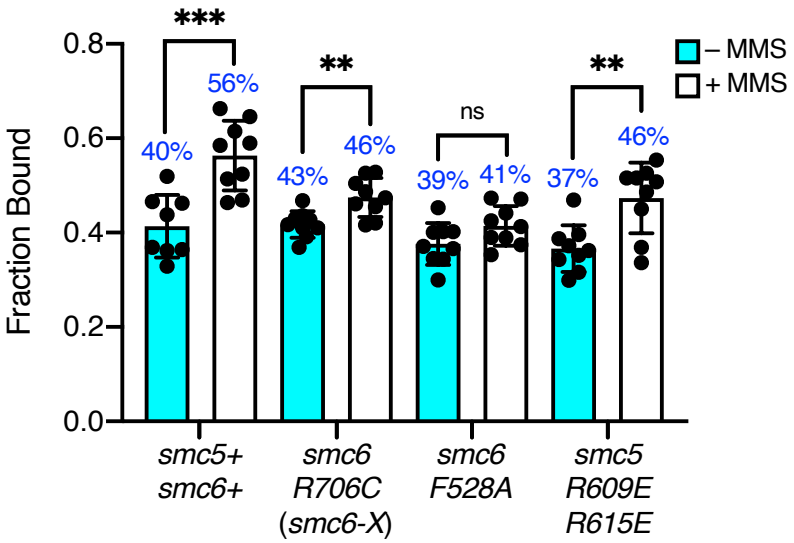






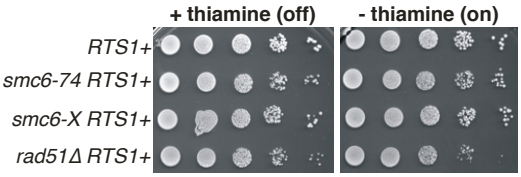
Supplementary Figure 5

A

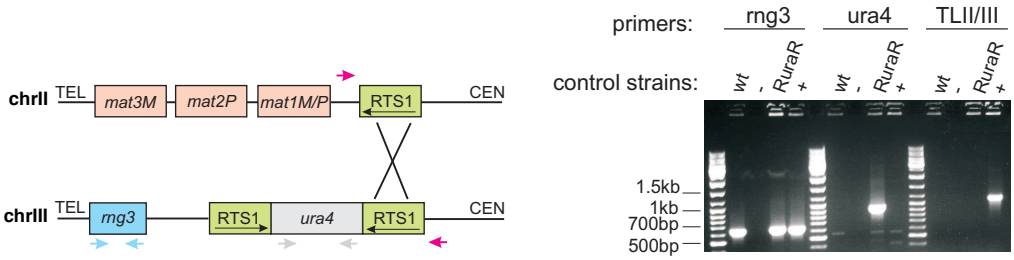


Supplementary Figure 6

A



B

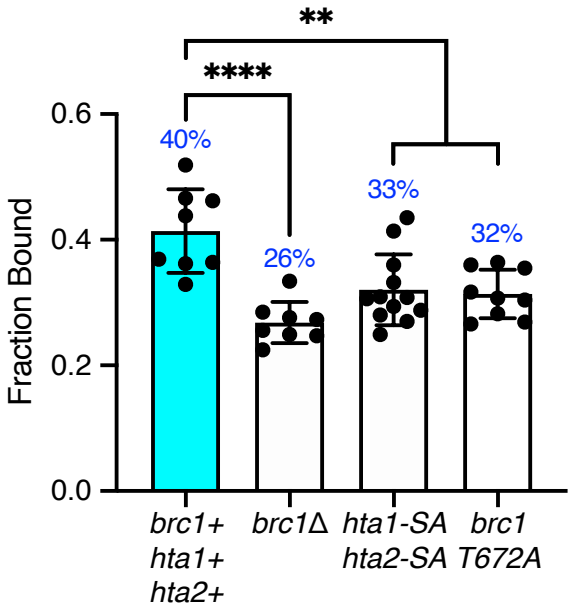


C

Strain	Translocations		*ura4* deletion	*ura4* point mutation	*ura4* truncation	*ura4* deletion	*ura4* point mutation	*ura4* truncation
	Off	**On**	**Off**			**On**		
smc6⁺	0/36 (0%)	1/36 (3%)	9/36 (25%)	27/36 (75%)	0/36 (0%)	26/36 (72%)	8/36 (22%)	2/36 (6%)
smc6-74	2/36 (6%)	3/36 (8%)	15/36 (42%)	21/36 (58%)	0/36 (0%)	26/36 (72%)	9/36 (25%)	1/36 (3%)
smc6-X	2/36 (6%)	6/36 (17%)	14/36 (39%)	19/36 (53%)	3/36 (8%)	31/36 (86%)	5/36 (14%)	0/36 (0%)

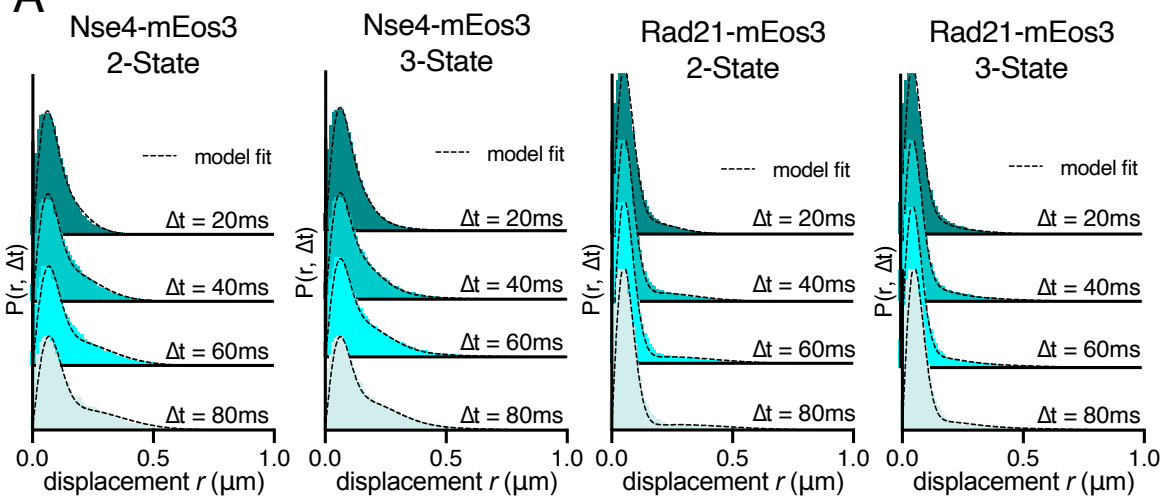
Supplementary Figure 7

A



Supplementary Figure 8

A



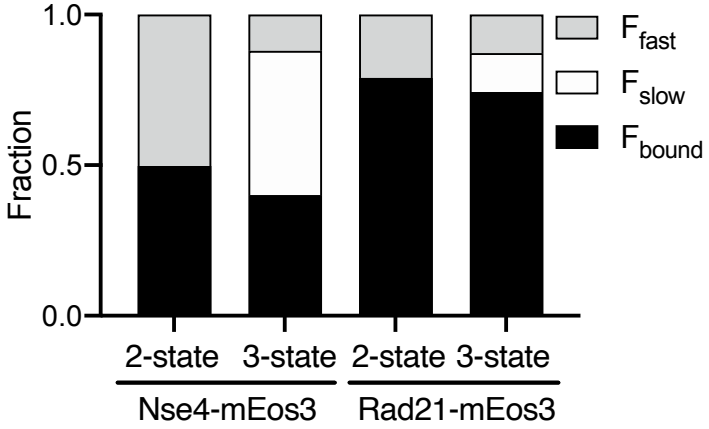
B

	Akaike information criterion (AIC)	
Strain	**2-State**	**3-State**
Nse4-mEos3	-130,870.50	-144,755.61
Rad21-mEos3	-130,529.27	-135,461.01

C

	Apparent diffusion coefficient (µm²/s)		
Strain	D_{bound}	D_{slow}	D_{fast}
Nse4-mEos3	0.005 ± 0.001	0.153 ± 0.0008	0.694 ± 0.0068
Rad21-mEos3	0.001 ± 0.0001	n/a	0.465 ± 0.0018

D



E

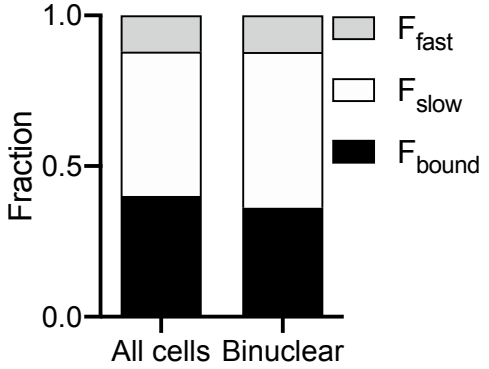


Table S1

Experiment 1	Rate of *ura4* loss per cell per generation		
Strain	**Off (+thiamine)**	**On (-thiamine)**	**Relative to Off**
smc6^{+}	2.2×10^{-8}	4.6×10^{-8}	2.09
smc6-74 (A151T)	1.4×10^{-7}	3.83×10^{-7}	2.71
smc6-X (R706C)	8.4×10^{-9}	1.6×10^{-6}	194.00
nse3-R254E	6.0×10^{-8}	2.4×10^{-7}	3.99

Experiment 2	Rate of *ura4* loss per cell per generation		
Strain	**Off (+thiamine)**	**On (-thiamine)**	**Relative to Off**
smc6^{+}	3.2×10^{-8}	5.7×10^{-8}	1.77
smc6-74 (A151T)	2.3×10^{-7}	1.56×10^{-6}	6.78
smc6-X (R706C)	1.3×10^{-7}	7.7×10^{-6}	61.2
nse3-R254E	7.2×10^{-8}	6.0×10^{-7}	8.36

Experiment 3	Rate of *ura4* loss per cell per generation		
Strain	**Off (+thiamine)**	**On (-thiamine)**	**Relative to Off**
smc6^{+}	1.6×10^{-8}	1.8×10^{-8}	1.12
smc6-74 (A151T)	7.09×10^{-8}	3.98×10^{-7}	5.61
smc6-X (R706C)	1.3×10^{-7}	1.4×10^{-5}	105.70
nse3-R254E	1.3×10^{-8}	4.1×10^{-7}	32.20

Table S2

Strain No.	Genotype	Reference
TJE323	*loxP-nse4-mEos3.2-loxM3 ura4-D18 leu1-32 ade6-704*	This study
TJE350	*loxP-smc6-mEos3.2-loxM3 ura4-D18 leu1-32 ade6-704*	This study
TJE496	*smc1-loxP-mEos3.2:kanMX6-loxM3 ura4-D18 leu1-32*	This study
TJE480	*loxP-nse4-mEos3.2-loxM3 loxP-smc6-T135V-loxM3 ura4-D18 leu1-32 ade6-704*	This study
TJE477	*loxP-nse4-mEos3.2-loxM3 loxP-smc6-T135L-loxM3 ura4-D18 leu1-32 ade6-704*	This study
TJE475	*loxP-nse4-mEos3.2-loxM3 loxP-smc6-T135F-loxM3 ura4-D18 leu1-32 ade6-704*	This study
TJE410	*loxP-nse4-mEos3.2-loxM3 smc6-A151T ura4-D18 leu1-32 ade6-704*	This study
TJE719	*loxP-nse4-mEos3.2-loxM3 loxP-smc6-R150A-loxM3 ura4-D18 leu1-32 ade6-704*	This study
TJE711	*loxP-nse4-mEos3.2-loxM3 loxP-smc5-R77A-loxM3 ura4-D18 leu1-32 ade6-704*	This study
TJE509	*loxP-nse4-mEos3.2-loxM3 smc6-F528A ura4-D18 leu1-32 ade6-704*	This study
TJE483	*loxP-nse4-mEos3.2-loxM3 smc5-R609E R615E ura4-D18 leu1-32 ade6-704*	This study
TJE671	*loxP-nse4-mEos3.2-loxM3 smc5-Y612G ura4-D18 leu1-32 ade6-704*	This study
TJE418	*loxP-nse4-mEos3.2-loxM3 smc6-R706C ura4-D18 leu1-32 ade6-704*	This study
TJE492	*loxP-nse4-mEos3.2-loxM3 loxP-nse3-R254E-loxM3 ura4-D18 leu1-32 ade6-704*	This study
TJE730	*loxP-nse4-mEos3.2-loxM3 brc1::hphMX6 ura4-D18 leu1-32 ade6-704*	This study
TJE734	*loxP-nse4-mEos3.2-loxM3 nse6::kanMX6 ura4-D18 leu1-32 ade6-704*	This study
TJE796	*nse6-loxP-mEos3.2-loxM3 ura4-D18 leu1-32*	This study
TJE816	*loxP-nse4-mEos3.2-loxM3 hta1-S129A:ura4 hta2-S128A:his3 his3-D1 ura4-D18 leu1-32*	This study
TJE393	*rad21-loxP-mEos3.2:kanMX6-loxM3 ura4-D18 leu1-32*	This study
TJE586	*nse2-loxP-mEos3.2:kanMX6-loxM3 ura4-D18 leu1-32*	This study
TJE522	*cnd2-loxP-mEos3.2:kanMX6-loxM3 ura4-D18 leu1-32*	This study
TJE886	*loxP-nse4-mEos3.2-loxM3 mcm4-loxP-yEGFP:KanMX6-loxM3 ura4-D18 leu1-32 ade6-704*	This study
THE 884	*loxP-nse4-mEos3.2-loxM3 loxP-brc1-T672A-loxM3 ura4-D18 leu1-32*	This study
TJE828	*nse6-loxP-mEos3.2-loxM3 smc6-74 ura4-D18 leu1-32*	This study
TJE830	*nse6-loxP-mEos3.2-loxM3 smc6-X ura4-D18 leu1-32*	This study
TJE888	*loxP-nse4-mEos3.2-loxM3 brc1::hphMX6 nse6::kanMX6 ura4-D18 leu1-32 ade6-704*	This study
HQD87	*loxP-smc5+-ura4-loxM3 ura4-D18 leu1-32 ade6-704*	This study
DE297	*loxP-smc6+-ura4-loxM3 ura4-D18 leu1-32 ade6-704*	This study
DE285	*loxP-smc6-T135V-loxM3 ura4-D18 leu1-32 ade6-704*	This study
DE283	*loxP-smc6-T135L-loxM3 ura4-D18 leu1-32 ade6-704*	This study
DE281	*loxP-smc6-T135F-loxM3 ura4-D18 leu1-32 ade6-704*	This study
DE279	*loxP-smc6-R150A-loxM3 ura4-D18 leu1-32 ade6-704*	This study
DE342	*loxP-smc5-R77A-loxM3 ura4-D18 leu1-32 ade6-704*	This study
DE273	*smc6-A151T ura4-D18 leu1-32 ade6-704*	Lab strain
JMM1188	*ura4-D18 leu1-32 ade6-704*	Lab strain
JMM1162	*nmt41:rtf1:sup35 RTS1-ura4-RTS1 ade6-704 leu1-32*	*Lambert et al 2005*
JMM1171	*rhp51::NAT nmt41:rtf1:sup35 RTS1-ura4-RTS1 ade6-704 leu1-32*	*Lambert et al 2005*
JMM1371	*smc6-A151T nmt41:rtf1:sup35 RTS1-ura4-RTS1 ade6-704 leu1-32*	This study
JMM1375	*smc6-R706C nmt41:rtf1:sup35 RTS1-ura4-RTS1 ade6-704 leu1-32*	This study
DE331	*nse3-R254E nmt41:rtf1:sup35 RTS1-ura4-RTS1 ade6-704 leu1-32*	This study