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► **To cite this version:**

Hugo Dorison, Ibtissam Talhaoui, Gerard Mazón. Sumo-mediated recruitment allows timely function of the Yen1 nuclease in mitotic cells. 2021. hal-03419397

**HAL Id: hal-03419397**

**<https://hal.science/hal-03419397>**

Preprint submitted on 17 Nov 2021

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1 **Sumo-mediated recruitment allows timely function of the Yen1**  
2 **nuclease in mitotic cells**

3

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5

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8

9 **Author Contributions:** GM obtained funding, designed experiments, contributed to Figures 1, 2,  
10 6, S1, S3, S4 and wrote the manuscript. HD edited the manuscript and performed experiments for  
11 Figures 1, 2, 4, 5, 6, S1, S2 and S5. IT edited the manuscript and performed experiments for  
12 figures 1, 3, S3 and S4.

13 **Competing Interest Statement:** No competing interests.

14 **Keywords:** Homologous Recombination. Structure-selective endonucleases. Sumoylation.

15 **The Manuscript consist in a PDF file including:**

16 Main Text

17 Figures 1 to 5

18 Supplementary information includes 5 figures and 11 tables that can be found in a  
19 separate document.

20

21 **Abstract**

22 The modification of DNA damage response proteins with Sumo is an important mechanism to  
23 orchestrate a timely and orderly recruitment of repair factors to damaged sites. After replication  
24 stress and double-strand break formation a number of repair factors are Sumoylated and interact  
25 with other Sumoylated factors, including the nuclease Yen1. Yen1 plays a critical role to ensure  
26 genome stability and unperturbed chromosome segregation by removing covalently linked DNA  
27 intermediates that are formed by homologous recombination. Here we show how this important  
28 role of Yen1 is dependent on interactions mediated by non-covalent binding to Sumoylated  
29 partners. Mutations in the motifs that allow Sumo-mediated recruitment of Yen1 impair its ability  
30 to resolve DNA intermediates and result in increased genome instability and chromosome mis-  
31 segregation.

32 **Introduction**

33

34 The DNA integrity of the genomes is constantly exposed to multiple challenges either from  
35 endogenous or exogenous sources of DNA damage. Cells have evolved multiple DNA repair  
36 pathways to ensure genome stability, Homologous Recombination (HR) being one of the most  
37 critical pathways to specifically counter the deleterious DNA double-strand breaks and other  
38 problems in the DNA integrity arising during replication. As the HR pathway operates, different  
39 DNA substrates and intermediates are formed that physically inter-connect distinct DNA  
40 molecules creating a joint-molecule (JM) intermediate. These intermediates are a threat to the  
41 successful segregation of chromosomes and are to be dismantled during mitosis by different  
42 specialized proteins acting in concert to prevent segregation defects and genome  
43 rearrangements. In yeast, the dissolution pathway mediated by the complex of Sgs1-Top3-Rmi1  
44 (STR) ensures the disentanglement and release of double Holliday Junctions (dHJs) and two  
45 other helicases, Mph1 and Srs2, act early on the pathway preferentially over D-loop intermediates  
46 to reduce the number of JM intermediates and ensure the completion of the recombinational  
47 repair without crossing-over between the involved DNA templates. Opposed to these non-  
48 crossover (NCO) pathways, the nucleolytic processing of these JM intermediates can result in  
49 reciprocal crossovers (COs), with the risk of genome rearrangements and loss of heterozygosity  
50 (LOH) events (1, 2).

51 Given the risk for genome stability of a nucleolytic processing of HR intermediates, the different  
52 actors able to cleave these intermediates are strictly controlled and used as an option of last  
53 resort in DNA substrates not previously dismantled by the action of helicases (3, 4). Two major  
54 nucleases are involved in the nucleolytic processing of recombination intermediates in the yeast  
55 model, Mus81-Mms4 and Yen1 (5). The Mus81-Mms4 nuclease plays different roles at replication  
56 forks, and is gradually hyper-activated by Cdc5- and Cdc28/CDK1-dependent phosphorylation of  
57 Mms4 to peak its activity in late G2/M (6-8) where it associates with the Slx4-Dpb11 scaffold (9).  
58 Its broad substrate recognition enables Mus81-Mms4 to cleave 3'-flap containing DNA substrates  
59 and HJs, preferentially when they are still nicked or not completely ligated (10). Its hyper-  
60 activation in late G2 and its broad substrate specificity positions Mus81-Mms4 in a critical role to  
61 cleave both captured D-loop and early HJ intermediates, possibly targeting these intermediates  
62 that can't complete full conversion to a dHJ and thus remain inaccessible to processing by the  
63 STR complex (3). As mitosis progresses, the Cdc14 phosphatase will trigger the reversal of the  
64 inhibitory Cdc28-mediated phosphorylation of Yen1 in turn allowing its nuclear localization and its  
65 proper substrate recognition (11, 12). This late activation of Yen1 at the anaphase entry ensures  
66 that all remaining recombination intermediates, especially those that escaped dissolution by STR  
67 or cleavage by Mus81-Mms4, are resolved before mitotic exit (11, 12). To ensure the clearing of

68 Yen1 nuclease from the nucleus in the subsequent S-phase, and prevent off-targeted activity  
69 directed to 5'-flap containing DNA intermediates, Yen1 is additionally controlled by a Sumo-  
70 targeted degradation mediated by the Slx5-Slx8 ubiquitin ligase, further limiting the potential of  
71 crossover formation (13).

72 Protein covalent modification with the small ubiquitin-like modifier (Sumo) (14) is an important  
73 mechanism to fine tune DNA-mediated transactions during the DNA damage and repair  
74 responses (15-18). In *Saccharomyces cerevisiae* Sumoylation occurs in a multi-step reaction  
75 involving the E1 Aos1-Uba2 activating enzyme dimer, the E2 conjugating enzyme Ubc9, and  
76 three possible E3 ligases (Siz1, Siz2 and Mms21), with some redundancy of Siz1 and Siz2 for its  
77 substrates (19-21). Several players of the HR pathway, besides the nuclease Yen1, are also  
78 found among the Sumoylated DNA repair targets, including Rad52, PCNA, RPA and Sgs1 (17,  
79 22-26). Sumoylation is able to influence biological processes in different ways. Proteins can be  
80 mono-Sumoylated, multi-Sumoylated or poly-Sumoylated, and the modification will re-design the  
81 protein surfaces allowing changes in protein activity, or in its way it can interact with other  
82 proteins. One of the best-described effects of protein Sumoylation is the enabling of interaction  
83 with other protein partners in a bait-to-prey fashion using Sumo as the moiety that is recognized  
84 by a specific domain in the partner protein, called a Sumo Interacting Motif (SIM). These motifs  
85 are found throughout species and according to its amino acid composition can be classified in  
86 several families of consensus sequences (27). Most SIMs can be defined as a core stretch of four  
87 amino acids with a majority of hydrophobic residues (typically rich in V/I/L). This hydrophobic core  
88 fits into the hydrophobic groove on the Sumo surface and is often flanked by a stretch of 3-4  
89 acidic or polar residues in the SIM sequence that interact with basic residues on the surface of  
90 Sumo (28-31). SIM types showing the flanking stretch of acidic residues present thus a similar  
91 architecture to that of ubiquitin interacting motifs (UIM) that also show a key stretch of polar  
92 residues flanking the hydrophobic core (32, 33). SIM motifs are able to interact with mono- or  
93 poly-Sumoylated proteins and can be usually present in tandem dispositions, probably helping  
94 interact with multiple Sumoylated lysines or with a poly-Sumoylated lysine in the interacting  
95 protein (28, 34, 35). Interactions by Sumo-SIM partnerships are extremely labile and can be  
96 easily induced and curbed down by altering the Sumoylation status of the involved proteins. This  
97 flexibility allows a quick building of protein complexes in response to changing stress conditions in  
98 the cell (17, 35). The build up of these protein complexes by Sumo-mediated recruitment *via*  
99 SIMs is generally associated to the actual Sumoylation of the two involved proteins (36, 37).

100 Then, it was thus not surprising to identify in Yen1, which is Sumoylated (13), several putative  
101 Sumo interacting motifs. In the present study we define two functional SIMs in the Yen1 C-  
102 terminal region that play important roles in the nuclease sub-nuclear localization and its function

103 alleviating the persistence of chromosome-segregation challenging JMs throughout the end of  
104 mitosis.

105

## 106 **Results**

107

108 **Yen1 contains two functional Sumo-binding motifs (SIM) in the C-terminal region.** As  
109 stressed before, Sumoylated proteins are able to transiently interact with a variable strength to  
110 other proteins containing SIMs. These motifs consist in a stretch of amino-acids with a core of  
111 aliphatic residues, often flanked by three or more amino-acids with a negative charge or  
112 susceptible to become phosphorylated (27). We inspected the Yen1 sequence through available  
113 algorithms (27) to detect SIM motifs and we identified several interesting hits in the primary  
114 sequence of Yen1 (Figure 1A). To validate the presence of such motifs in Yen1 we performed a  
115 two-hybrid analysis with Smt3 as bait and either wild type full-length or truncated versions of  
116 Yen1 as a prey (Figure 1B). The ability to interact with Sumo in the two-hybrid assay was only  
117 retained by the C-terminal part of Yen1 (amino-acids 354 to 759) and mutations in two putative  
118 SIMs present in that half of the protein completely abolished the interaction. A mutation in the first  
119 SIM, a type r motif (27) with a core at amino-acids 636 to 642 and a flanking stretch of acidic  
120 residues was indeed sufficient to almost completely impair the interaction to Smt3 in the two-  
121 hybrid assay (Figure 1B). Interestingly, this motif is highly conserved across Yen1 in other fungi  
122 and is very reminiscent of SIMs found in the Slx5, Rad18 and Elg1 proteins (36, 38, 39) (Figure  
123 S1).

124

125 <Figure 1>

126

127 While mutation on the consensus SIM sites abolishes the interaction in the two-hybrid assay, this  
128 assay was suggested to reflect a covalent modification of Yen1 (40) and thus the effect detected  
129 in this test might be an indirect effect reflecting the loss of direct sumoylation of Yen1 related to  
130 the absence of non-covalent interaction of Yen1 to SUMO through its SIMs. Moreover, a possible  
131 SIM-mediated interaction would be difficult to be confirmed in this test if polymerization of Smt3 is  
132 needed or a Sumo covalent modification of a bridging partner is required for the growth read-out  
133 of the test. To better confirm the nature of the interaction lost in our mutants and thus validate the  
134 SIM sites, we used a pull-down approach (36). GST-Smt3 was over-expressed and purified from  
135 bacteria, and bound to a Glutathione resin. Purified Yen1 or its mutant SIM variants were then  
136 allowed to bind to the pre-bound GST-Smt3, and after several washes, the column content was  
137 eluted in denaturing conditions and inspected by western blotting (Figure 1C). Yen1 was detected  
138 in the eluates thus confirming its ability to interact non-covalently to Smt3, but was much less

139 retained (30% compared to wild-type) when bearing mutations in both of its SIMs, while  
140 inactivation of only one of the two motifs did not significantly altered retention suggesting that at  
141 least *in vitro*, the presence of one single SIM at the Yen1 C-terminal region confers it ability to  
142 bind Smt3 (Figure 1C). While the GST-Smt3 column may indicate the ability of Yen1 to bind to  
143 monomers of Smt3 through its SIMs, the packed dispositions of GST-Smt3 in the column can  
144 mimic a poly-Sumoylated chain and contact Yen1 simultaneously in multiple SIM sites. To  
145 further evaluate the non-covalent binding of Yen1 to poly-Smt3 we decided to generate an affinity  
146 column containing poly-Sumo chains to test whether they have the ability to non-covalently bind  
147 to Yen1 and thus capture the protein. We generated Smt3 chains by adding Aos1-Uba2 and  
148 Ubc9 in a reaction with purified 6xHis-Smt3 (13), the resulting poly-Sumo chains were dialyzed  
149 and used to bind to a Cobalt HisPur Superflow agarose matrix, the poly-Smt3 coated matrix was  
150 used to test retention of Yen1-1xHA (Figure 1D). While the column retained the wild-type Yen1,  
151 the recovery of the protein mutated in both SIMs (Yen1<sup>SIM1-2ΔΔ</sup>) was greatly decreased (Figure  
152 1E), thus confirming that Yen1 binds non-covalently to poly-Sumo chains, a binding that depends  
153 on the presence of the two identified Yen1's SIMs.

154

155 **Strains carrying SIM-defective variants of Yen1 display increased sensitivity to DNA**  
156 **damage.** We next aimed to understand the effect of the mutations in the SIM motifs on the ability  
157 of Yen1 to be normally regulated by Cdk1/Cdc14 and shuttled timely to the nucleus. Given the  
158 proximity of Yen1's SIMs to its NLS, a C-terminal GFP fusion of the Yen1 mutants was monitored  
159 to see if any gross defect occurred for its nuclear shuttling (Figure 2). Both single and double SIM  
160 mutants presented nuclear exclusion in S-phase as the wild-type and were nuclear in late mitosis  
161 and G1 and the relative distribution of GFP intensity detected in the nucleus or in the cytoplasm  
162 at the different cell cycle phases was not changed between the different Yen1 variants (Figure  
163 2B, Figure S2). We also analyzed the pattern of cyclic phosphorylation by synchronizing cells in  
164 G1 and analysing the mobility of Yen1 at different time points after its release (Figure 2C, Figure  
165 S2A). All the mutants displayed a normal cycle of phosphorylation in S-phase followed by gradual  
166 de-phosphorylation with only slight variations in the total amount of the protein all across cell-  
167 cycle phases. Next we asked whether the presence of an endogenous copy of the SIM mutants  
168 would compromise the ability of Yen1 to back-up for the functions of Mus81-Mms4 (5). The  
169 mutants were introduced into a *mus81Δ* background and tested for its sensitivity to an array of  
170 DNA damaging agents (Figure 2D and E). Cells with a double mutation *mus81Δ yen1Δ* are  
171 extremely sensitive to MMS at low doses, and they also present a moderate sensitivity to the  
172 radiomimetic drug Zeocin and to the replication stalling drug Hydroxyurea (HU) (13), both a  
173 mutation in the first SIM and the double SIM mutant significantly increased the sensitivity of a  
174 *mus81Δ* strain to MMS, while increased sensitivity to MMS after mutation in the second SIM

175 alone was not significant (Figure 2D, 2E, Table S3). Mutation in both SIMs was necessary to see  
176 a moderate increase in sensitivity to Zeocin (Figure 2D, 2E). Mutation in both SIMs was also  
177 necessary to sensitize cells at 20mM HU and while individual SIM mutants sensitized cells to 40  
178 mM HU, at such dose survival was already compromised by a two log difference in cells bearing  
179 both SIM mutations or lacking *YEN1* (Figure 2E). Yen1 has also been described to be essential in  
180 cells with a deletion on the *DNA2* helicase-nuclease in the presence of its suppressor *pif1Δ* (41-  
181 44). Similarly, cells carrying the *dna2-2* helicase-deficient allele rely on the activity of *YEN1* to  
182 process DNA intermediates in this background (41). Similar to what was already observed in  
183 *dna2-2* cells in the W303 background (41) we found that a *dna2-2* mutation is partially unviable  
184 with *yen1Δ* (Figure S3). The *dna2-2* cells, while viable, present a strong heterogeneity of  
185 phenotypes likely associated to spontaneous accumulation of suppressors, as described (41)  
186 (Figure S3). Nonetheless, the introduction of the more severe of the SIM alleles, carrying  
187 simultaneous mutations in both SIMs, was viable in a *dna2-2* background, suggesting a minor  
188 role of non-covalent Sumo binding for the Yen1's functions required in a *dna2-2* context. In  
189 agreement to the observation that *dna2Δ pif1Δ* strains do not significantly accumulate more  
190 Sumoylated Yen1 compared to the wild-type strain (13), we found that in a *dna2Δ pif1Δ*  
191 background the SIM defective allele does not increase the already strong sensitivity of cells to  
192 DNA damaging agents like MMS or HU (Figure S3).

193

194 <Figure 2>

195

196 **Mutation of the SIMs induces a sumo-less Yen1 phenotype *in vivo*.** In other Sumoylated  
197 DNA repair proteins containing functional SIMs, the mutation of these motifs has an impact in the  
198 ability of the protein to be directly Sumoylated (36, 37). The results in the two-hybrid experiments  
199 suggested such an effect for Yen1 (Figure 1), which we have previously characterized to be  
200 Sumoylated in a Siz1/Siz2-dependent manner (13). To further confirm the absence of covalent  
201 sumoylation after impairment of Yen1's SIMs we compared the Sumoylation levels of the wild-  
202 type and the SIM-defective Yen1 mutants by performing denaturing pull-downs of His-tagged  
203 Smt3 (Figure 3A). Yen1 Sumoylation peaks when cells are exposed to high MMS doses (13) and  
204 we reproduced Yen1 Sumoylation in these conditions for the wild-type protein (Figure 3A).  
205 Nonetheless, the fraction of Sumoylated Yen1 in the mutant in either the first SIM motif or the  
206 double mutant in the two SIM motifs was greatly reduced in conditions with similar input levels to  
207 5% and 1% of the wild-type levels respectively (Figure 3A), mutation of SIM2 had a milder effect  
208 reducing the recovery of Sumoylated forms to ≈20% of the forms recovered in the wild-type  
209 (Figure 3A). The gradual effects detected for individual or combined mutations of both SIMs  
210 points to a concerted action of both motifs to promote Yen1 Sumoylation by allowing Yen1 non-

211 covalent binding to Smt3. Despite its sensitivity to very low doses of MMS, cells carrying *dna2-2*  
212 did not spontaneously increase the yield of Sumoylated forms of Yen1 while we detected in such  
213 conditions under spontaneous damage a minor yield of Sumoylation in the presence of mutations  
214 on both Yen1 SIMs in either a wild-type or a *dna2-2* background (Figure S3).

215

216 <Figure 3>

217

218 The mutations introduced to inactivate the SIM sites do not contain any lysine substitution, and  
219 SIM1 is not directly flanked by lysines in the immediate vicinity. To further confirm that the lack of  
220 Sumoylation was not due to un-adverted absence or un-accessibility of Sumoylation-target  
221 lysines in Yen1, we decided to test the mutant proteins in an *in vitro* Sumoylation reaction. After a  
222 reaction of the SIM mutants of Yen1 with Aos1-Uba2 and the conjugating enzyme Ubc9, a normal  
223 Sumoylation pattern was detected with the same ladder of bands of increasing sizes for all Yen1  
224 variants (Figure 3B). We also performed a complete ligation reaction containing Siz2 as E3,  
225 increasing the yield of the reaction. Comparing the ligation reactions over time we couldn't detect  
226 significant differences in the amount or timing of accumulation of the Sumoylated forms (Figure  
227 3B, Figure S4) that in all the Yen1 variants achieved complete Sumoylation of the substrates at  
228 similar time points. We conclude that the presence of the SIM mutations does not preclude  
229 modification of any of the Yen1's lysines targeted by the Sumoylation machinery.

230 The c-terminal domain of Yen1 containing the two SIMs is dispensable for a complete nuclease  
231 activity (45). Nonetheless, we verified that the mutation of both SIMs does not impair Yen1's  
232 nuclease activity *in vitro* by using a synthetic Holliday Junction (46) as a substrate. Immuno-  
233 precipitated Yen1 was added to cleavage reactions, and we compared the yield of HJ cutting for  
234 either the wild-type Yen1 and the SIM defective mutant Yen1<sup>SIM1-2ΔΔ</sup>. The nuclease activity was  
235 undistinguishable for both the wild-type and the mutant, that were able to linearize the HJ  
236 substrate at similar rates (Figure 3C). Alteration of the SIM motifs at the C-terminal part of the  
237 protein seems thus not to alter the cutting efficiency of Yen1, whose nuclease and conserved  
238 XPG domains are present at the N-terminal part of the protein (Figure 1A). Nonetheless, the test  
239 did not take into account the disposition of the junction in a chromatin context in the cell that could  
240 influence the ability to cut HJ *in vivo*.

241

242 **Localization to spontaneous and induced sites of activity is impaired by inactivation of**  
243 **the Yen1's SIMs.** Sumoylation and interaction with SIMs has been proposed as a way to enforce  
244 a cascade of interactions to foster recruitment of factors to specific subcellular locations (35). We  
245 decided to determine if the impairment of the Yen1 SIMs was altering its ability to cluster in foci  
246 that are observed to occur either spontaneously or induced by DNA damage (4, 13). C-terminal

247 GFP tagged versions of Yen1 were compared for its foci distribution (Figure 4, Figure S5, Table  
248 S4 and S5), the number of cells showing spontaneous foci in a wild-type strain ranges around  
249 10%, while only 3% of the cells in the double SIM mutant displayed foci in strains with a functional  
250 Mus81-Mms4 (Figure 4B). The effect was more marked when observing the spontaneous foci in  
251 a *mus81Δ* strain, where cells displaying foci decreased from 30% to 3-4% in the SIM mutant  
252 (Figure 4B). These foci were equally decreased in the presence of exogenous damages,  
253 suggesting that Yen1 SIMs are equally important to properly localize the protein to spontaneous  
254 damaged sites and exogenous damages sites (Figure 4B, Table S6 and S7).

255

256 <Figure 4>

257

258 **Absence of Yen1's SIMs prevent accumulation of the Yen1 fraction targeted by Slx5-Slx8.**

259 We have demonstrated in a previous work a role for the Slx5-Slx8 Sumo-targeted ubiquitin ligase  
260 in the removal of a subset of Yen1 from the nucleus during the transition from G1 to S phase (13).  
261 As a result, cells defective in Slx8 show a persistence of Yen1 foci, which are detected in large  
262 numbers even in the presence of functional Mus81-Mms4 (13). We wondered if the absence of  
263 proper localization in the SIM mutant would prevent Yen1 accumulation in the absence of Slx5-  
264 Slx8. According to our expectations, a deletion of *slx8Δ* in the strain bearing the mutations in the  
265 Yen1's SIMs did not increase the number of Yen1 foci, thus suggesting mis-localization of Yen1  
266 in the mutant strain prevents the need for Slx5-Slx8 targeted removal (Figure 5A, Table S8). The  
267 persistence of the Slx5-Slx8 targeted Yen1 fraction can also be detected by performing a  
268 cycloheximide chase during a synchronous release from G1, and observing the degradation of  
269 Yen1 under inhibition of *de novo* protein synthesis in this cell-cycle interval (13). In a *slx8Δ*  
270 background, the double SIM mutant protein was degraded during such cycloheximide chase  
271 faster than the wild-type protein (Figure 5B), indicating that the lack of accumulation of Yen1 at  
272 nuclear sites does also dispense for a targeted removal of this subset of the nuclease, that is  
273 removed timely even in the absence of Sumo-targeted ubiquitination by Slx5-Slx8.

274

275 <Figure 5>

276

277 **Unpaired Sumo-directed localization induces an increase in untimely chromosome**

278 **segregation.** The presence of both *mus81Δ* and *yen1Δ* deletions makes cells synergistically  
279 sensitive to drugs like MMS, as stated before, and also increase their spontaneous number of  
280 chromosome mis-segregations monitored either by dedicated genetic systems (5, 47) or by a  
281 direct observation of fluorescent-tagged chromosomes during mitotic divisions (13) (Figure 5C).  
282 We compared the mitoses of single *mus81Δ* cells to that of cells carrying *mus81Δ* and the allele

283 with the two mutated SIMs. Similar to what we could observe in a *mus81Δ yen1Δ* control strain,  
284 *mus81Δ* cells with the mutated Yen1 SIMs displayed an increased number of segregation issues.  
285 As it can be observed in the violin plots displaying the time that individual monitored cells spent to  
286 fully segregate the fluorescent tag, the Yen1 double SIM mutant does segregate chromosomes in  
287 a similar average time than the wild type for those cells completing full segregation (Figure 5D)  
288 but about 30% of the cells carrying these Yen1 SIM mutations in the *mus81Δ* background were  
289 unable to resolve its segregation within the time of video-microscopy observation (2 h), and were  
290 classified as non-disjunctions (Figure 5D, Table S9). The high number of chromosome mis-  
291 segregation detected for the Yen1 mutant variant in a *mus81Δ* background is in line with that  
292 observed in cells completely lacking Yen1 clearly pointing to a faulty function of Yen1 when SIMs  
293 are absent.

294

295 **Mutation in the SIMs of Yen1 reduces the formation of crossing-over after a single DSB.** To  
296 further browse the implications of the presence of a defective Sumo-interacting Yen1 allele for the  
297 actual resolution of recombination intermediates, we decided to analyse the level of crossing-over  
298 (CO) formation in two widely used tests that estimate the CO levels after a single DSB formation  
299 (3, 5, 48) (Figure 6A and D). In accordance with the increased sensitivity to different DNA  
300 damaging agents observed for the Yen1 allele carrying the SIM mutations when combined with a  
301 *mus81Δ* background (Figure 2), we detected a decreased formation of crossovers in this  
302 genotype after the induction of a single DSB in a diploid tester strain (5), half-way to the  
303 phenotype observed with a double mutant carrying both deletions in *mus81Δ* and *yen1Δ* (Figure  
304 6B and C, Table S10). The decrease in crossover formation was paralleled by an increase in  
305 Break-Induced Replication (BIR) events (Figure 6B and C, Table S10). Using an ectopic  
306 recombination assay (48) (Figure 6D and E), we detected a decrease in viability after the  
307 induction of an HO cut site in chromosome II in the *mus81Δ yen1<sup>SIM1-2ΔΔ</sup>* strain, already signalling  
308 a defective crossover resolution resulting in a number of unviable events (Figure 6F). This  
309 survival decrease probably reflects a BIR increase that in this test leads to lethality by the loss of  
310 essential genes in the chromosome II distal arm. The number of crossovers quantified by  
311 southern blotting analysis of the survivors showed a nearly 50% reduction in the crossover yields  
312 in *mus81Δ yen1<sup>SIM1-2ΔΔ</sup>* cells (Figure 6E and F, Table S11), not significantly different from the  
313 levels detected in *mus81Δ yen1Δ* cells.

314

315 <Figure 6>

316

317

318

319 **Discussion**

320

321 In the present work we aimed to understand whether the Yen1 nuclease depends on interactions  
322 with Sumoylated partners to be able to act accurately and promptly on its substrates. We have  
323 demonstrated that in addition to being Sumoylated, Yen1 is also able to interact non-covalently to  
324 Sumoylated chains and Sumo monomers through at least two Sumo-interacting motifs in its C-  
325 terminal region (Figure 1). Previous reports suggested covalent Sumoylation occurring in the C-  
326 terminal region of Yen1 was responsible for the Smt3-interaction detected in a two-hybrid assay  
327 (40), our results are in agreement with the original observation of a C-terminal motif mediating  
328 such Smt3-interaction but we conclude this results are compatible with a non-covalent interaction  
329 mediated by the two SIMs, leading to a covalent modification of Yen1. We have demonstrated  
330 such non-covalent interaction with dedicated retention assays using either immobilized GST-  
331 Smt3 or pre-polymerized poly-(6HIS)-Smt3 coupled to a Cobalt HisPur Superflow agarose matrix  
332 (Figure 1) in a strategy similar to that used to validate other sumoylated protein's SIMs (36, 49-  
333 51). Moreover, we further confirmed the nearly complete loss of *in vivo* Sumo covalent  
334 modification in the SIM defective Yen1 using 6His-Smt3 denaturing pull-downs (Figure 3A). Our  
335 Yen1 SIM mutant acts thus as an *in vivo* Sumo-less variant without requiring a large number of  
336 Lysine substitutions, which can sometimes result in the protein's destabilization. While direct  
337 Yen1 sumoylation depends strongly in the presence of the identified SIM motifs, we conclude  
338 those are not required to mediate interaction to the sumoylation machinery *per se*, as we can  
339 observe full sumoylation patterns after an *in vitro* sumoylation reaction (Figure 3B) and we also  
340 detect residual forms of sumoylated Yen1 in our denaturing pull-down assays (Figure 3A)  
341 displaying the regular band pattern of *in vivo* sumoylated Yen1, and not the absence of these  
342 forms that is obtained when Siz1 and Siz2 are removed (13). However, we conclude that this  
343 direct sumoylation of Yen1 is largely prevented in the cells by a faulty localization *via* SIMs to  
344 specific nuclear sites as suggested by the inability of the SIM mutant to accumulate in foci in sub-  
345 nuclear localizations previously characterized (13) (Figure 4). Accordingly, while the absence of  
346 the SIMs in Yen1 has no effect on its catalytic activity (Figure 3C), we have detected a sub-  
347 optimal function of these mutants in the cells, leading to phenotypes of chromosome mis-  
348 segregation and DNA damage sensitivity similar to those observed for a null mutant in  
349 combination with a deletion of the partially redundant cell's major resolvase activity mediated by  
350 the heterodimer Mus81-Mms4 (Figures 2, 4 and 5) (5). The impaired localization not only  
351 correlates with a sub-optimal function of Yen1 in response to spontaneous damages under  
352 normal growth conditions and exogenous treatments with genotoxic agents, but also decreases  
353 the number of crossing-over that can be observed after a single DSB induction in two different  
354 settings (Figure 6). While inactivation of both SIMs identified in the C-terminal region is necessary

355 to impair non-covalent binding to Sumo, the mutation we introduced in SIM1 seems to achieve a  
356 stronger phenotype alone than the one in SIM2. Nonetheless, we did not perform an optimized  
357 serial mutation of each motif and thus we can't exclude that both SIM motifs contribute with equal  
358 importance to Sumo binding in the cells.

359

360 Our results are in line with group modification (35) and would suggest a local enrichment of  
361 multiple Sumoylated proteins together with free Smt3 and the Sumoylation machinery when  
362 persistent recombination intermediates are revealed during anaphase. While Sumoylation has  
363 been previously shown to play important roles in the fine-tuning of DNA repair processes, our  
364 study highlights the importance of Sumoylation for genome maintenance processes occurring in  
365 anaphase, and probably disconnected to previous Sumoylation cascades influencing HR  
366 proteins. Several key proteins acting in anaphase, like Condensin subunits and chromosomal  
367 passenger complex (CPC) components, have been described to be Sumoylated (20, 52, 53). It is  
368 thus of great interest to continue studying Yen1 functional interactions in conditions that are  
369 greatly transient and ephemeral, and determine which other factors ensure prompt Yen1  
370 recruitment to its activity sites during its anaphase activity window, thus influencing the delicate  
371 balance between chromosome segregation and genome integrity.

372

373

## 374 **Materials and Methods**

375

376 **Yeast Strains and Growth Conditions.** *S. cerevisiae* strains used in this study are derivatives of  
377 the W303c background and are listed in Table S1. The Yen1-FX-GFP allele was made by  
378 inserting a Factor X site and the GFP epitope from pGAD-Yen1-GFP(54) between amino acids  
379 D753 and S754 at the C-terminus of Yen1 using dedicated oligonucleotides and was cloned into  
380 TOPO-pYES2 (Invitrogen) to allow controlled expression by Galactose induction, all plasmid  
381 derivatives are listed in Table S2. Mutants in the different designated loci were either obtained  
382 by crossing or by gene replacement with the indicated selective cassettes. Cells were typically  
383 grown in YP (1% yeast extract; 2% peptone) or SC media with alternatively 2% glucose, 2%  
384 raffinose or 2% galactose in strains under inducible conditions. A modified medium (SC with  
385 0.17% YNB without ammonium sulfate, 0.1% proline and 0.003% SDS) was used for the Smt3  
386 pull-down assays.

387

388 **Western Blot analyses.** If not stated otherwise, proteins were extracted by the TCA  
389 (Trichloroacetic acid) method. For routine monitoring, samples were loaded into 7.5% Tris-  
390 Glycine stain-free pre-casted gels (BioRad). Samples from pull-downs analyses were loaded into

391 3-8% gradient NuPAGE Tris-Acetate gels (ThermoFisher). Gels were transferred using a semi-  
392 dry transfer machine (BioRad) to PVDF membranes and hybridized with the appropriate  
393 antibodies in 5% w/v nonfat dry milk, 1X TBST buffer. Antibodies for anti-HA-HRP (3F10, Roche),  
394 anti-Smt3 (B. Palancade), anti-Pgk1-HRP (22C5D8, Abcam) were used at the suggested dilutions  
395 and revealed using an ECL reagent (Advansta). When required, HRP-conjugated secondary  
396 antibodies from Cell Signaling were used at 1/10000 dilution.

397

398 **Microscopy and Cell Biology Methods.** Live cell imaging was performed with a Spinning Disk  
399 Confocal Microscope (CSU-W1, Yokogawa), with an electron multiplying charge device camera  
400 (ANDOR Zyla sCMOS) and a  $\times 60/1.35$  numerical aperture objective at 30 °C. Cells were  
401 centrifuged and plated as a droplet between an SC agarose pad and a glass slice (55). Images  
402 were recorded with 17 z-sections with 0.5  $\mu\text{m}$  spacing for each wavelength at a time. Video  
403 recordings were built with images taken every 2 minutes. Metamorph was used for image  
404 acquisition, and analysis was performed using Image J-Fiji (56).

405 For Yen1 foci observations, cells were grown in SC medium without uracil (SC-URA 2%  
406 raffinose), GFP-Yen1 was induced in a short burst of 30 min with Galactose at 2%, followed by  
407 addition of Glucose at 2%. For acute exposure to DNA damage, cells were treated with MMS  
408 0.01% for 15 min at room temperature and were washed once with fresh SC-URA 2% glucose  
409 before continuing the experiment. Aliquots were taken at the indicated times. Cells showing an  
410 accumulation of spots were measured at maximum projection of the GFP channel. Statistical  
411 significance was determined by the  $\chi^2$  test using contingency tables with the number of cells  
412 observed in each different category.

413 For segregation monitoring using strains with the lacO/GFP-LacI array, all cells were recorded for  
414 a duration of 2 h minimum in their agarose pads. Individual cells were identified with an ongoing  
415 chromosome segregation. To determine segregation duration a start point was determined as the  
416 signature S-phase bud was the smallest yet discernable. At this point, only one foci of GFP-  
417 tagged chromosome fluorescent markers is visible. The cell is followed until the dot separates in  
418 two and resides durably in the daughter cells. The ending time point is taken at the last frame of  
419 definitive separation of the fluorescent foci. The duration of the movement of the two separate  
420 dots was reported for each individual cell under monitoring, cells with dots moving together for the  
421 whole duration of the time-lapse were assigned as non-disjunction and their segregation time was  
422 not used to establish the average segregation time.

423

424 **Sumoylation assays and Smt3-bound retention assay.** In *ex vivo* Sumoylation assays, the  
425 wild-type or mutant Yen1-HA was produced from a pYES2 vector and immuno-precipitated from

426 cell lysates as described (13). Eluates were subjected to Sumo conjugation and ligation as  
427 described (57).

428 For Smt3-retention assays, 6x-His-Smt3 was purified from BL21 *E.coli* cells using a Ni-NTA  
429 affinity column (Qiagen) following manufacturer indications. Smt3 protein was eluted with 250 mM  
430 imidazole before being dialyzed using a G2 Slide-a-Lyzer cassette (Thermo Fisher) with a 10 kDa  
431 cut-off. Purified Smt3 was subjected to a self-conjugation reaction by adding Aos1-Uba2 and  
432 Ubc9 and ATP as described (57) and the reaction was subjected to a second purification in a  
433 Cobalt HisPur Superflow agarose matrix (Thermo Fisher) to generate poly-Smt3 retention  
434 column. Equal amounts of Yen1 or its mutant were added to the non eluted matrix containing  
435 poly-Smt3 bound in E buffer (20 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 5 mM Imidazole, pH 7.4) and  
436 binding was allowed for 60 min at 4°C. Mini-Columns were then centrifuged to remove the buffer  
437 and non-retained proteins, washed 5 times in washing buffer (E buffer 12.5 mM Imidazole) and  
438 eluted in denaturing conditions with Laemmli buffer at 95°C. The eluates were loaded into 4-15%  
439 SDS-PAGE gradient gels and immunoblotted. GST-Smt3 retention assays were performed as  
440 described (36) with purified GST-Smt3 obtained by expression of pGEX-4T1-Smt3 into BL21  
441 *E.coli* cells. Briefly, purified GST-Smt3 was incubated with glutathione matrix during 40 min at 4°C  
442 in GST buffer (25 mM Tris-HCl (pH8.0), 150mM NaCl, 1mM DTT). Then matrix was washed  
443 before to load purified wild-type or mutated Yen1. Mixtures were incubated 3 min at 4°C before to  
444 wash the glutathione matrix and elute retained proteins for HA and anti-GST immuno-bolt  
445 analysis.

446

447 **Cycloheximide chase experiments.** Cycloheximide chase experiments were essentially done  
448 as reported (13). Cultures grown in SC complete modified media (0.1% proline 0.017% YNB  
449 without ammonium sulfate, 0.0003% SDS) were diluted to OD<sub>600</sub>=0.2 and synchronized with  
450 alpha factor (3 μM) for 2 h. Once synchronized, cells were treated with cycloheximide (250 μg/ml)  
451 in fresh media, to inhibit proteins new synthesis, and released from the G1 arrest. Samples were  
452 taken at indicated time points and analyzed by TCA extraction and western blotting.

453

454 **Denaturing Histidine pull-downs.** For 6xHIS-Smt3 pull-downs, strains containing the  
455 expression vectors or the control empty plasmid were grown in SC-LEU modified medium (0.1%  
456 proline, 0.017% YNB without ammonium sulfate). Cells were allowed to grow to OD<sub>600</sub>=0.3 when  
457 CuSO<sub>4</sub> was added at 100 μM final concentration in a volume of 100 ml. After 1 h, MMS was  
458 added to 0.3% and cells were collected 3 h later. Cells where lysed under denaturing conditions  
459 and Sumo-conjugated proteins where isolated and analyzed by western blot using a Nu-PAGE  
460 Tris-acetate 3-8% gradient gel, basically as previously described (13). In *dna2-2* strains (LEU2), a

461 plasmid expressing under Galactose induction Flag-6His-Smt3 (URA3) was used instead of the  
462 Cu-inducible.

463

464 **Synthetic DNA substrates and Yen1 resolvase activity assays.** The synthetic HJ-X0 was  
465 prepared by annealing the Cy5-X0-1, X0-2, X0-3 and X0-4 oligonucleotides (Sigma-Aldrich) in a  
466 buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 100 mM NaCl as described (46). The  
467 annealing product was analyzed in a native PAGE to verify the presence of a HJ structure. To  
468 test Yen1 activity, an enzymatic reaction was performed in 10 µl cleavage buffer (50 mM Tris-HCl  
469 (pH 7.5), 1 mM MgCl<sub>2</sub>, 1 mM DTT) containing 25 nM of Cy5-labeled HJ X0 substrate, and equal  
470 amounts of immunoprecipitated Yen1 or its SIM-defective mutant. After incubation at 30°C for 1  
471 h, the reaction was stopped by adding 2.5 µl of stop buffer (100 mM Tris-HCl (7.5), 50 mM EDTA,  
472 2.5% SDS, 10 mg/ml proteinase K) and further incubated for 30 min at 37°C. Cleavage products  
473 were migrated in 10% native PAGE, scanned using a Typhoon FLA 9500 Biomolecular Imager  
474 and the images were analyzed with ImageQuant (GE Healthcare).

475

476 **DSB-induced recombination assays.** The diploid recombination assays were performed as  
477 described previously (for a detailed protocol see (47)). The reporter diploid strains that contain 2  
478 *ade2* hetero-alleles were cleaved by induction of I-SceI in its *ade2-I* allele and allowed to repair  
479 with its *ade2-n* allele under non-selective conditions to give rise to either ADE2 or *ade2-n* repair  
480 products in three types of colonies (red, white and sectored). Each recombinant colony was  
481 scored with its two recombination events (for each repaired sister chromatid) and considering the  
482 possible segregation patterns in daughter cells. Frequencies of the recombination events were  
483 normalized to the galactose vs glucose plating efficiency. The distribution of CO and NCO in the  
484 ectopic recombination assay based in chromosomes V and II (48) were addressed by southern  
485 blot hybridization of ApaLI-PvuII digested genomic DNA from cell populations growing in YP-  
486 Raffinose after galactose induction of HO. Membranes were hybridized with a *URA3* radiolabeled  
487 probe and results were normalized relative to the galactose versus glucose plating efficiency of  
488 the strains as described (3).

489

490

491

492

#### 493 **Acknowledgments**

494

495 We thank L.S. Symington, J. Campbell, M. E. Budd, K. Dubrana and S. Marcand for generous  
496 gifts of yeast strains and plasmids. We thank B. Palancade and S. Brill for supplying reagents for

497 the Sumoylation assays. We thank the members of the UMR9019 CNRS for the useful  
498 discussions and suggestions. We thank Fondation ARC pour la Recherche sur le Cancer and  
499 donors from Natixis for their support of our research. GM is a full-time INSERM researcher at  
500 the CNRS. IT is a full-time CNRS researcher. HD has benefited from a doctoral fellowship from  
501 the French Education Ministry.

502

### 503 **Funding**

504

505 This study was supported by grants to GM from the Gustave Roussy Foundation (FGR-JC6,  
506 funded in part from donations from Natixis) and from Fondation ARC pour la Recherche sur le  
507 Cancer (PJA 20181208087).

508

### 509 **Data Availability**

510 The data that support the findings of this study are available from the corresponding author upon  
511 reasonable request

512

513 **Figure Legends**

514

515 **Figure 1. Yen1 contains two Sumo Interacting Motifs (SIMs) in its C-terminal domain**

516 **(A)** Diagram showing the conserved domains of Yen1 and the positions of the regulatory Cdk1-  
517 phosphorylation sites. Amino acid 354 shows the cut-off point for truncated forms of Yen1 in Two-  
518 Hybrid assays. The two identified candidate SIMs are shown near the Nuclear localization  
519 sequence (NLS). **(B)** A Two-hybrid assay was performed with strains carrying the indicated  
520 Activator Domain (AD) and DNA Binding Domain (BD) fusions to test interaction between Yen1  
521 and Smt3 (SUMO), and the Yen1 critical domains for such interaction. Mutations D635A D636A  
522 D637A for SIM1 $\Delta$  and V675A E677A for SIM2 $\Delta$  were introduced to test for the putative SIMs.  
523 SIM1-2 $\Delta\Delta$  is used for the combined mutations. Strains were grown on selective media lacking  
524 Leucine and Tryptophan and spotted in selective media also lacking Histidine to reveal interaction  
525 of the proteins fused to the AD and BD domains. Non-specific interactions were minimized by the  
526 addition of 3-aminotriazole (AT). **(C)** Purified GST-Smt3 was bound to a Glutathione resin and  
527 either purified wild-type or the SIM mutant Yen1 proteins were then loaded to the resin. The  
528 retained fractions of Yen1 were eluted after several washes and detected by immuno-blotting. **(D)**  
529 SUMO-retention assay using *in vitro* generated poly-Smt3 immobilized into a Cobalt HisPur  
530 Superflow agarose matrix. Purified Yen1 was added to the pre-bound Smt3 and after a binding-  
531 time and washing, columns were eluted in denaturing conditions and the eluates inspected by  
532 western blot for the presence of Yen1 (anti-HA, left panel) and the pre-bound Smt3 chains (right  
533 panel). **(E)** Immuno-blotting of the inputs and eluates of the retention assay (as in D) comparing  
534 the ability of wild-type or SIM-mutant Yen1 to bind poly-Smt3.

535

536 **Figure 2. Mutation in Yen1 SIMs has no impact to its CDK1 regulation and nuclear shuttling**  
537 **but sensitizes cells to DNA damage**

538 **(A)** Cells carrying an endogenous histone Hta2-mCherry marker and chromosomally –HA tagged  
539 versions of Yen1 wild-type and the different SIM mutants were transformed with a plasmid  
540 carrying an equivalent version of Yen1 fused with GFP at its C-terminal region. Cells were grown  
541 on selective media and observed using a spinning-disk microscope after a brief induction with  
542 galactose. Shuttling of the protein from cytoplasm to the nucleus can be observed in  
543 representative fields displaying cells with nuclear excluded Yen1 (S-phase and early G2-M) and  
544 nuclear localized Yen1 (anaphase to G1) for the indicated GFP-tagged proteins. **(B)**  
545 Quantification of the relative amount of GFP signal detected into the nucleus over the overall

546 signal for the indicated strains in cells classified by their cell-cycle status **(C)** Strains with a  
547 chromosomally inserted copy of –HA tagged wild-type Yen1 or its double SIM mutant (Yen1<sup>SIM1-</sup>  
548 <sup>2ΔΔ</sup>) were synchronized with alpha factor and released into fresh medium to monitor the  
549 modification of the protein through the cell cycle by immunoblot (left). Both unmodified and  
550 phosphorylated Yen1 are indicated. Average levels of endogenous Yen1 were normalized with  
551 PGK1 protein in triplicate experiments (right). **(D)** Sensitivity to different DNA damaging agents  
552 and drugs was determined by spotting serial dilutions of strains carrying different Yen1 mutants in  
553 its SIM in a *MUS81* deleted background for the indicated media. **(E)** Survival curves to the agents  
554 tested in (C) were established by counting colony forming units of the different strains after plating  
555 in YPD containing the indicated doses of drugs in replicate trials. Survival was normalized per trial  
556 with its respective control YPD counts and the average % survival is plotted in the graphs (+/-  
557 SEM). Significance was estimated by the student T-test at P<0.05 (\*) and P<0.01 (\*\*), see Table  
558 S3. Additional data related to this figure is found in Figure S2.

559

560 **Figure 3. Mutation in Yen1's SIMs does not alter its activity or its sumoylation *in vitro* but**  
561 **prevents sumoylation *in vivo*.**

562 **(A)** Strains carrying endogenous copies of –HA tagged wild type Yen1, Yen1<sup>SIM1Δ</sup>, Yen1<sup>SIM2Δ</sup> and  
563 Yen1<sup>SIM1-2ΔΔ</sup> mutants, with (+) or without (-) the plasmid pCUP-6xHIS-Smt3, were grown in the  
564 presence of MMS 0.3%. Cells were lysed and lysates subjected to a denaturing Ni-NTA pull-down  
565 followed by immunoblot analysis. Yen1 was detected by anti-HA. Membranes were subsequently  
566 probed with anti-Smt3. Prior to Ni-NTA pull-down, input samples were taken from the lysates and  
567 were analysed by immunoblotting for the levels of Smt3 induction (Anti-Smt3) and relative protein  
568 amounts (Anti-PGK1, Anti-HA) of each lysate (input panels). **(B)** Purified Yen1-HA and Yen1 SIM-  
569 mutant variants were subjected to an *in vitro* sumoylation reaction containing Siz2, Aos1-Uba2,  
570 Ubc9 and Smt3-3KR and ATP and subjected to Tris-Acetate PAGE for comparison of their  
571 sumoylation patterns after immunoblotting with anti-HA. The reaction without the ligase Siz2 is  
572 shown at the right of each panel. Quantification of the relative % of sumoylation forms (R.I.) and  
573 unmodified forms is shown at the bottom and more in detail in Figure S4 **(C)** Activity of Immuno-  
574 precipitated Yen1 was tested in a cleavage reaction using synthetic Holliday Junctions (HJ) made  
575 with oligonucleotides and labeled with Cy5. The DNA products were run in non-denaturing PAGE  
576 and revealed by the fluorescence of the Cy5 labeled oligonucleotide.

577

578

579 **Figure 4. Mutation in the SIMs of Yen1 prevents foci accumulation in G2/M.**

580 **(A)** Cells with an endogenous copy of Hta2-mCherry and YEN1-HA expressing Yen1-GFP from  
581 an inducible vector were observed under a spinning-disk microscope after a brief induction with  
582 galactose. The white triangles denote the presence of Yen1-GFP foci. **(B)** Chromosomally tagged  
583 Yen1-HA wild-type and SIM mutant in the indicated genetic backgrounds and carrying its  
584 corresponding Yen1-GFP expressing plasmid (blue graphs for WT copy, red for SIM mutant)  
585 were observed under the microscope after a brief induction. Cells from the indicated conditions  
586 were classified according to its cell cycle phase and the presence or absence of Yen1 foci. Violin  
587 Plots display the distribution of G2/M cells showing no foci, 1-2 foci or more than 2 foci for each  
588 strain. Counting was performed for over 400 distinct G2/M cells for each strain over several  
589 independent trials. Asterisks represent statistical significance in a  $\chi^2$  test  $p < 0.0001$  (\*\*\*), see  
590 tables S4, S5, S6 and S7 for details.

591

592 **Figure 5. Mutation in the SIMs of Yen1 prevents foci accumulation in *slx8* $\Delta$  cells during**  
593 **G2/M and impacts chromosome segregation in a *mus81* $\Delta$  background.**

594 **(A)** Cells containing a deletion on *SLX8* were observed for their distribution of foci of the different  
595 variants of Yen1-GFP. Violin plots display the distribution of cells and asterisks denote statistical  
596 significance in a Chi-square test at  $P < 0.0001$  (\*\*\*), see table S8. **(B)** Cells from the indicated  
597 genotypes were arrested in G1 and released in the presence of cycloheximide with samples  
598 being taken at the indicated time points. Total protein extracts were inspected by immunoblot for  
599 the presence of Yen1-HA and their intensity quantified relative to the loading control obtained by  
600 stain-free imaging of the gels (BioRad). Relative amounts of Yen1 are plotted in the graph to  
601 facilitate comparison (+/- SD). **(C)** Diagram showing chromosome segregation in cells harboring  
602 a lacO/GFP-LacI array tag on chromosome VII. To discriminate cells with timely chromosome  
603 segregation from those presenting aberrant segregation (delayed segregation or non-disjunction)  
604 a 2 h limit of observation was implemented. Two sets of representative actual images of a normal  
605 segregation pattern and a non-disjunction pattern are shown below the diagram. **(D)** Over 400  
606 cells per strain were individually counted and are represented in violin plots according to the time  
607 spent to segregate the lacO/lacI array. The median segregation time is indicated excluding cells  
608 with non-disjunctions. Statistical relevance of the differences observed between the number of  
609 non-disjunctions of the different strains was determined by the Chi-square test at  $P < 0.0001$ , see  
610 table S9.

611

612 **Figure 6. Crossover formation is impaired in cells containing the mutant version of Yen1**  
613 **inactivating both SIMs in a *mus81*Δ background.**

614 **(A)** Diagram showing the chromosome XV based DSB-induced recombination reporter. **(B)**  
615 Recombination outcomes in red-white (*ade2/ADE2*) sectored colonies of the indicated strains,  
616 normalized to their Plating Efficiency (PE) in Galactose compared to Glucose. **(C)** Recombination  
617 outcomes combining the results obtained for all types of colonies (full red, full white and sectored)  
618 of the indicated strains, normalized to their Plating Efficiency (PE) in Galactose compared to  
619 Glucose. Statistical significance for B and C was determined by the Chi-square test at  $P < 0.05$ ,  
620 see table S10. **(D)** Diagram showing the chromosome II-V based ectopic DSB-induced  
621 recombination reporter and its expected outcomes during physical analysis. **(E)** Representative  
622 southern blot analysis of the indicated strains after genomic DNA digestion with the restriction  
623 enzymes as highlighted in diagram D and hybridization with a radiolabeled probe against the  
624 *URA3* locus. **(F)** Quantification of at least three independent southern blot analyses is plotted  
625 relative to PE (Galactose vs Glucose). Statistical significance was determined by the Student T-  
626 test at  $P < 0.05$ , see table S11.

627

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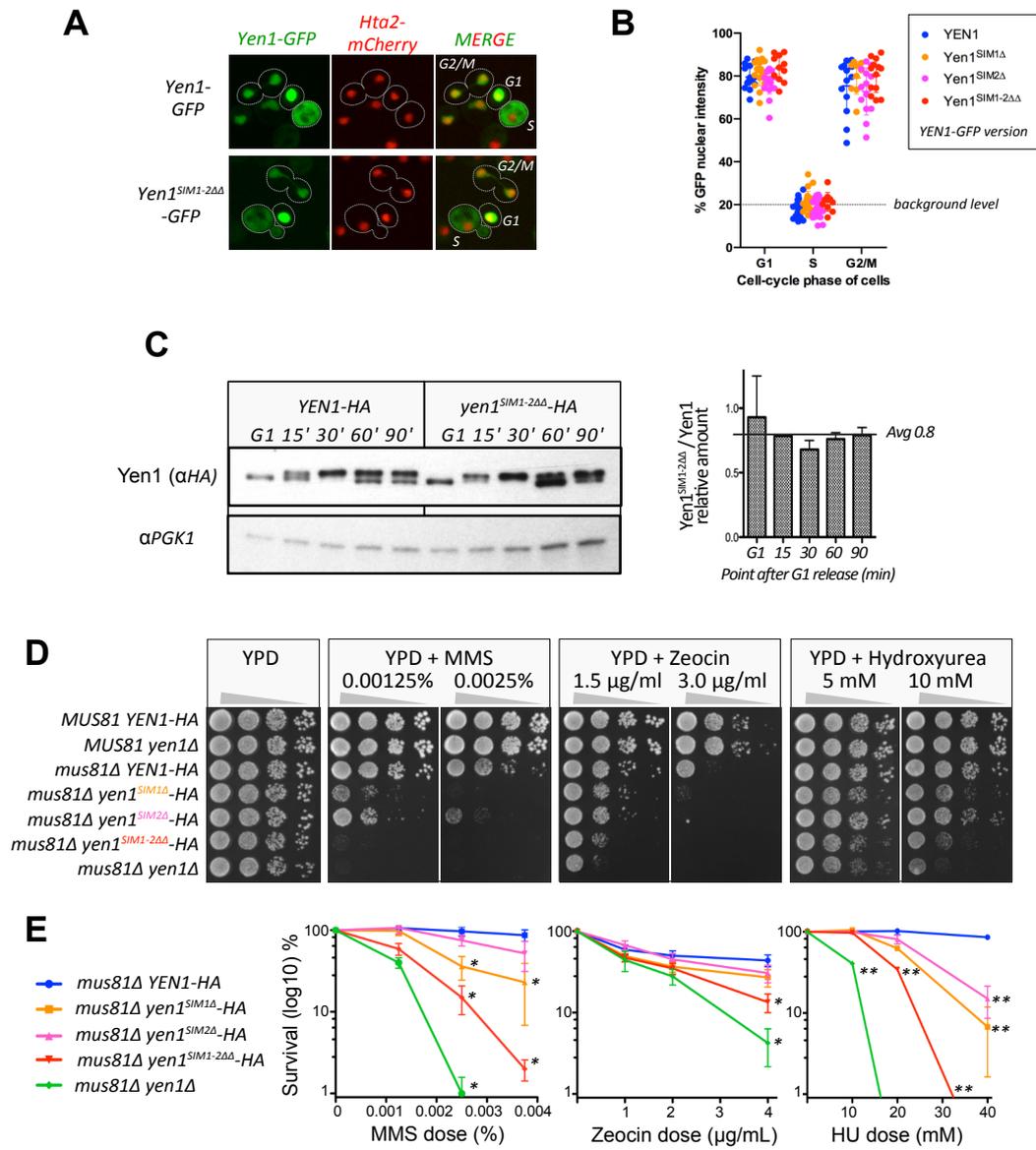
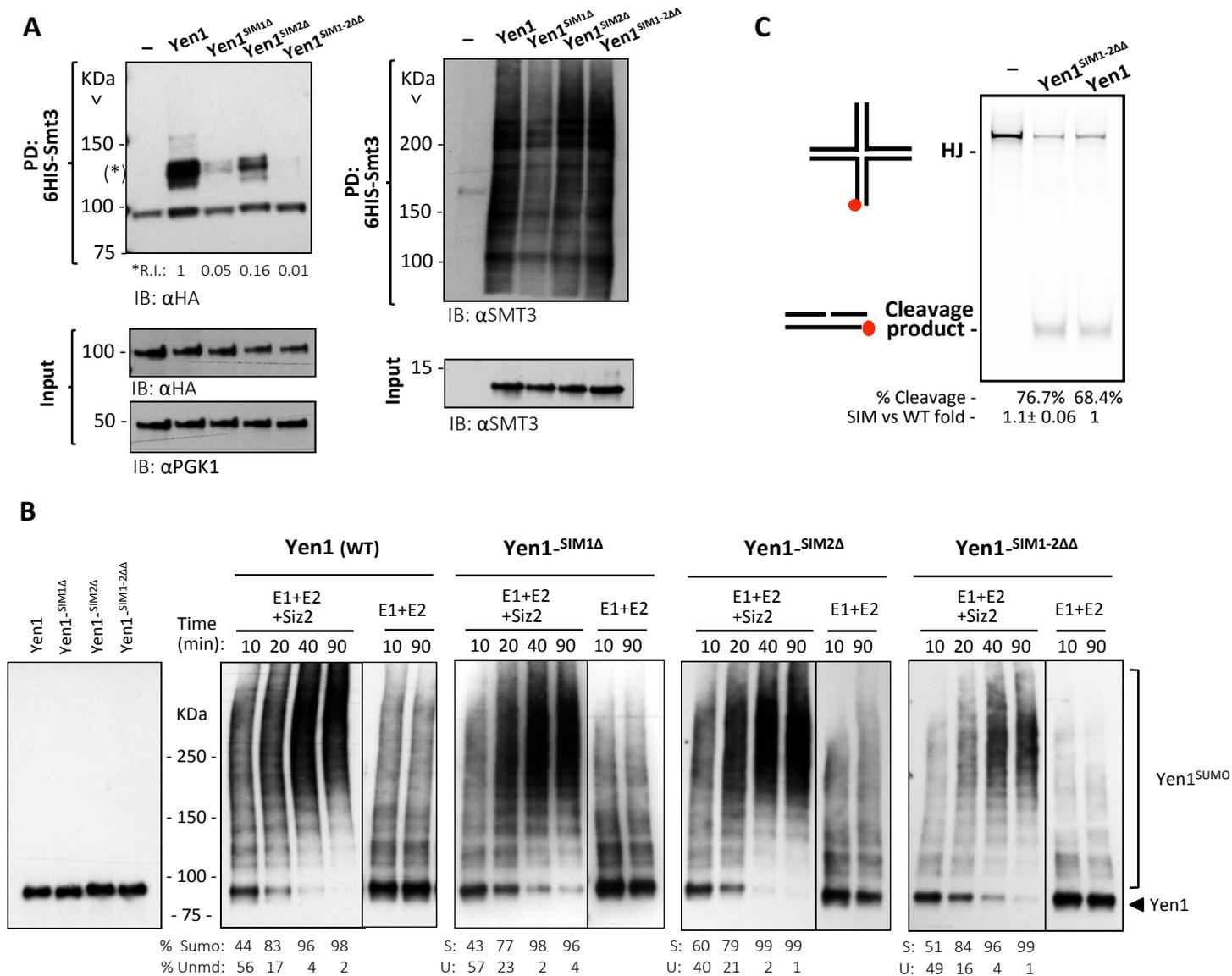


Figure 2



**Figure 3**

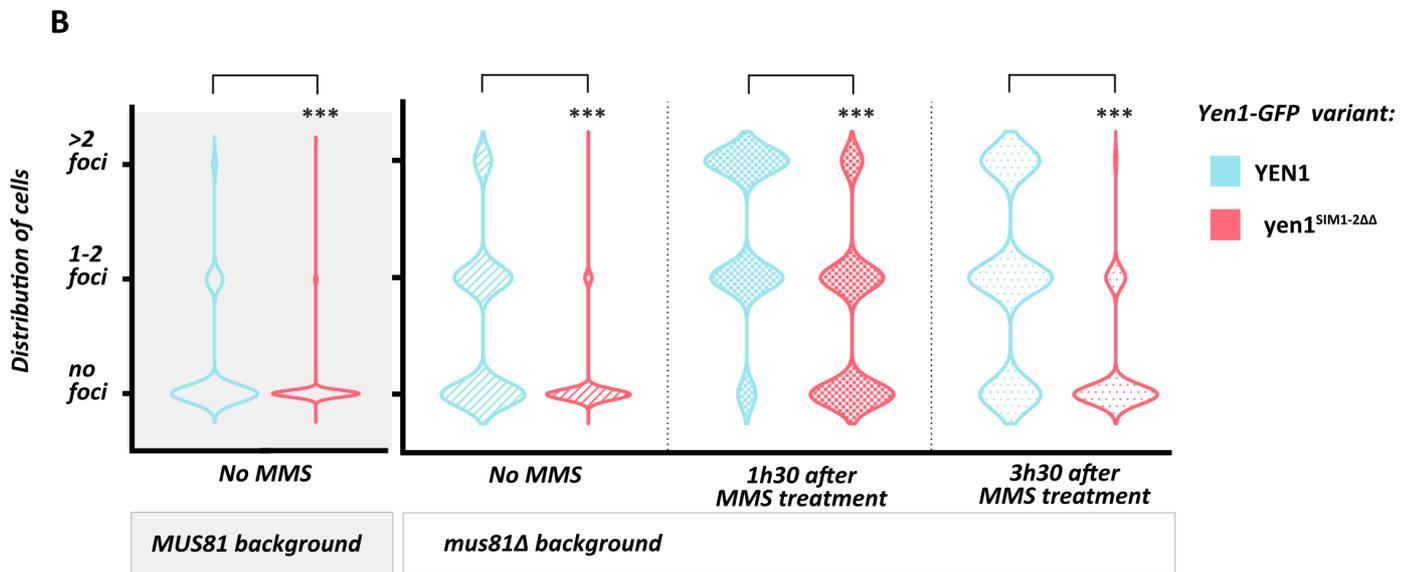
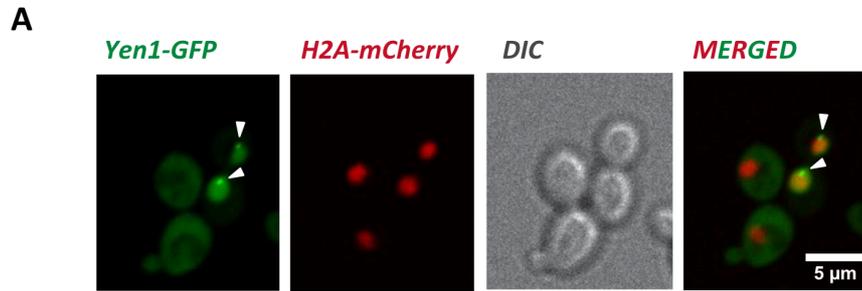
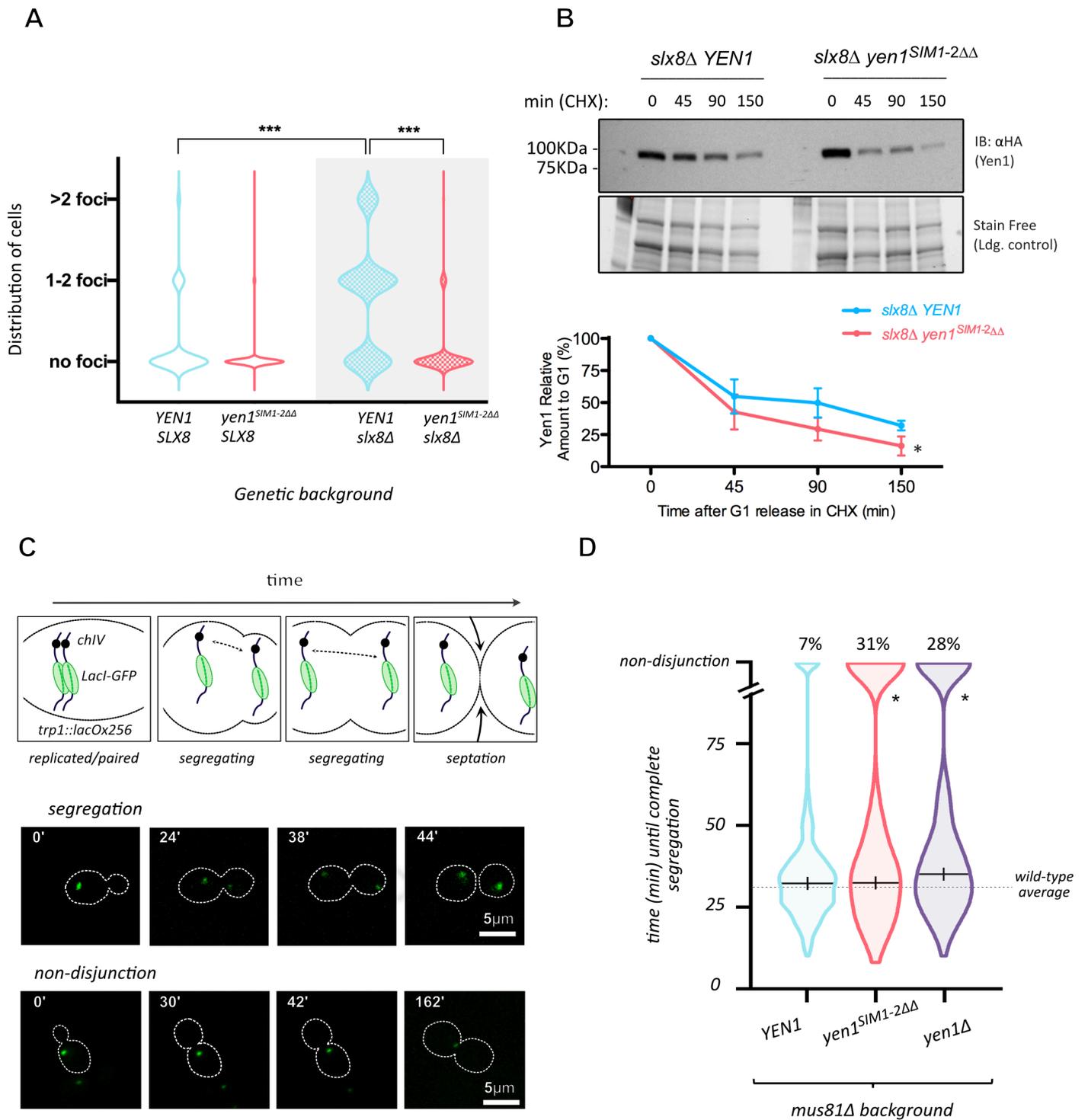


Figure 4



**Figure 5**

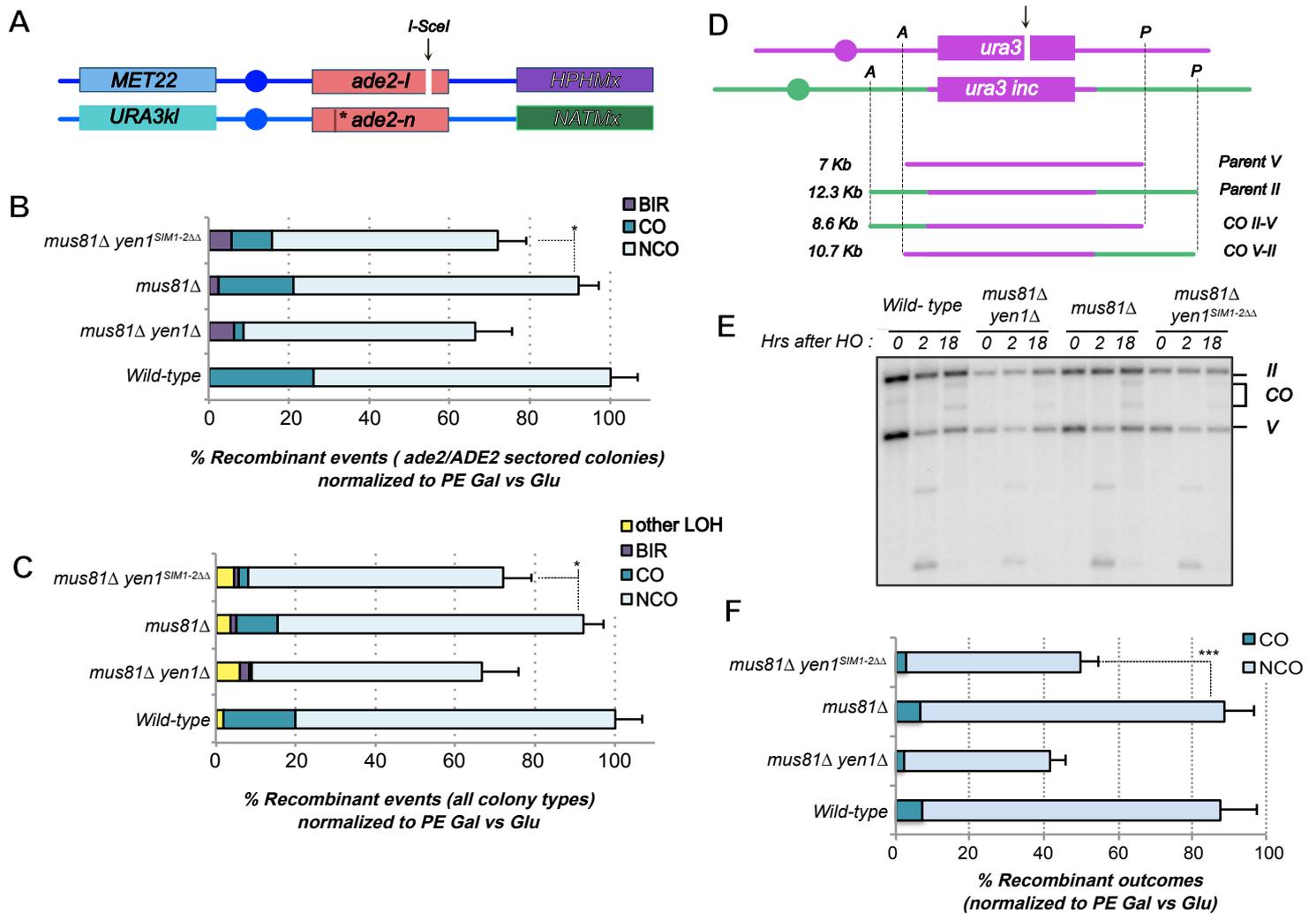


Figure 6