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Regulation of cohesin-mediated chromosome folding by Eco1 and other partners

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22 **SUMMARY**

23

24 Cohesin, a member of the SMC complex family, holds sister chromatids together but also
25 shapes chromosomes by promoting the formation of long-range intra-chromatid loops, a
26 process proposed to be mediated by DNA loop extrusion. Here we describe the roles of three
27 cohesin partners Pds5, Wpl1 and Eco1 in loop formation along either unreplicated or mitotic
28 *Saccharomyces cerevisiae* chromosomes. Pds5 limits the size of DNA loops *via* two different
29 pathways: the canonical Wpl1-mediated releasing activity, and an Eco1-dependent
30 mechanism. In the absence of Pds5, the main barrier to DNA loop expansion appears to be the
31 centromere. Our data also show that Eco1 acetyl-transferase inhibits the translocase activity
32 that powers loop formation and contributes to the positioning of loops, through a mechanism
33 that is distinguishable from its role in cohesion establishment. This study reveals that the
34 mechanisms regulating cohesin-dependent chromatin loops are conserved among eukaryotes,
35 while promoting different functions.

36

37 INTRODUCTION

38 Recent advances in imaging and chromosome conformation capture techniques (3C,
39 Hi-C; (Dekker et al., 2002)) have unveiled a hierarchy of chromatin structural arrangements
40 (Cavalli and Misteli, 2013; Dekker and Mirny, 2016; Yu and Ren, 2017). A variety of
41 megabase (Mb) and sub-Mb structures have been proposed to organize the chromosomes of
42 bacteria (Le et al., 2013; Lioy et al., 2018; Marbouty et al., 2015), yeasts (Lazar-Stefanita et
43 al., 2017; Mizuguchi et al., 2014; Muller et al., 2018; Schalbetter et al., 2017) and mammals
44 (Dekker and Mirny, 2016; Yu and Ren, 2017) and are presumably a feature of most if not all
45 organisms. During mitosis, this organization undergoes its most radical change when
46 chromatin is transformed in condensed thread-like structures that facilitate chromosome
47 segregation (Liang et al., 2015).

48

49 In many species, the cohesin and condensin SMC protein complexes play important
50 roles in chromosome folding. They consist of a pair of SMC proteins that associate with a
51 Kleisin protein to form a large tripartite ring capable of embracing one or two DNA
52 molecules (Nasmyth and Haering, 2009). In addition to mediating sister chromatid cohesion
53 during G2/M phases of the cell cycle (Gligoris et al., 2014; Haering et al., 2008), cohesin
54 shares with condensin the ability to convert DNA fibers into chromatid thread-like structures
55 through a mechanism that remains poorly understood (Kagey et al., 2010; Parelho et al., 2008;
56 Wutz et al., 2017). SMC rings may organize DNA within chromatids by a DNA loop
57 extrusion process, which consists in capturing small chromatin loops and progressively
58 catalyzing their enlargement into large, megabase-sized structures (Fudenberg et al., 2016;
59 Gibcus et al., 2018; Nasmyth, 2001). The precise mechanisms driving the generation of loops
60 remain however to be fully characterized.

61

62 In mammals, condensin-dependent loops represent basic units for the folding of
63 mitotic chromosomes into compact, higher-order chromatin structures. Cohesin-dependent
64 loops, on the other hand, appear to contribute to long-range gene regulation in interphase, and
65 to the segmentation of interphase chromosomes into topologically associating domains
66 (TADs) defined as sub-megabase self-interacting regions. The organization of interphasic
67 chromosomes into self-interacting domains has been proposed to insulate enhancers from
68 non-cognate promoters by blocking cohesin-dependent loop progression when cohesin
69 reaches the insulator protein CTCF bound to DNA (Nora et al., 2017). Interphasic self-
70 interacting domains are chromosomal features found in many studied living organisms but
71 their dependency on cohesin and CTCF activities is not automatic. Besides mammals, the
72 involvement of cohesin in domain formation has for instance been reported in fission yeast
73 (Mizuguchi et al., 2014). In budding yeast *S. cerevisiae* however, no clear TADs-like
74 structure nor DNA loops have been identified along interphase chromosomes. Nevertheless,
75 the fact that cohesin is essential to convert the rDNA locus into a thread-like structure (Guacci
76 et al., 1994) during mitosis raises the idea that cohesin may also mediate cis-DNA looping
77 along the yeast chromosome. Recently, we and others showed using Hi-C that meiotic and
78 mitotic compaction in budding yeast is dependent on cohesin (Lazar-Stefanita et al., 2017;
79 Muller et al., 2018; Schalbetter et al., 2017). Surprisingly, we also found that condensin is
80 dispensable for mitotic chromosome arm compaction. Instead, condensin is required for
81 higher-order chromatin structuring at the rDNA locus during anaphase and for promoting
82 resolution at peri-centromeric regions.

83

84 Cohesin is composed of two SMC ATPases (Smc1 and Smc3) and an α -kleisin subunit
85 called Scc1/Rad21. This complex binds to DNA in a dynamic manner throughout the cell
86 cycle. Cohesin loading requires ATP hydrolysis mediated by Scc2/NIPBL, that is thought to

87 result in entrapment of DNA inside the cohesin tripartite ring (Chapard et al., 2019; Ciosk et
88 al., 2000; Gligoris et al., 2014; Haering et al., 2002; Hu et al., 2011). Cohesin may dissociate
89 from DNA by separase-mediated cleavage of Scc1 at anaphase onset (Uhlmann et al., 1999)
90 and in a manner that involves a “releasing activity” at other stages of the cell cycle. This
91 separase-independent releasing activity relies on two cohesin-associated regulatory subunits:
92 Wpl1/Wapl and Pds5 (Gandhi et al., 2006; Kueng et al., 2006; Rolef Ben-Shahar et al., 2008;
93 Rowland et al., 2009). Pds5 binds within the N-terminal part of Scc1 and recruits Wpl1 (Chan
94 et al., 2013) which opens the gate located between Scc1 and Smc3, triggering cohesin
95 dissociation from DNA (Beckouët et al., 2016; Chan et al., 2012; Murayama and Uhlmann,
96 2015). When replication takes place, the releasing activity is repressed by Eco1-mediated
97 acetylation of two conserved lysine residues (K112/113) within the Smc3 ATPase head (Rolef
98 Ben-Shahar et al., 2008; Unal et al., 2008). This results in stabilization of cohesin on DNA
99 and establishment of sister chromatid cohesion. It has been suggested that acetylation of
100 K112/113 is in fact stimulated by both DNA replication fork progression and the Pds5 subunit
101 (Beckouët et al., 2010; Chan et al., 2013; Rolef Ben-Shahar et al., 2008; Vaur et al., 2012).
102 Smc3 acetylation is then maintained throughout G2 and M phases and only removed after
103 cleavage of Scc1 and subsequent deacetylation by Hos1 (Beckouët et al., 2010).

104

105 If cohesin-mediated loops are established by loop extrusion, their lengths should
106 depend on several parameters, notably cohesin residence time on DNA and an extrusion
107 driving force. This prediction is supported by the observation that the length of chromatin
108 loops in mammalian interphasic nuclei increases once cohesin turnover is abolished after
109 inactivation of Wpl1 and Pds5 (Haarhuis and Rowland, 2017; Wutz et al., 2017). However,
110 the mechanism(s) leading to loop enlargement remains a matter of debate. ATP hydrolysis
111 may be the driving force for chromatin loop extrusion, as observed for the purified condensin

112 complex (Ganji et al., 2018). Scc2, being essential for cohesin's ATPase activity could thus
113 play a key role in stimulating the translocation process and promoting DNA loop expansion
114 (Petela et al., 2018). Alternatively, transcribing RNA polymerases may promote loop
115 enlargement by pushing cohesin along the DNA (Busslinger et al., 2017; Lengronne et al.,
116 2004). Finally, the frequency of cohesin injection along chromosomes may also influence
117 their structure.

118

119 In the present study, we show that mitotic yeast chromosomes are compacted by
120 cohesin dependent loops. By dissecting the roles of cohesin regulatory partners in DNA loop
121 expansion, we also show that the mechanisms regulating loop positioning are highly
122 conserved between yeast and mammals. Moreover, we find that in addition to promoting
123 sister chromatid cohesion Eco1 inhibits the translocation process that extends DNA loops. In
124 the absence of negative regulation, the main barrier to loop expansion is the centromere,
125 which remains physically connected to the microtubule-organizing center (MTOC) during the
126 entire yeast cell cycle.

127

128 **RESULTS**

129 *Yeast mitotic chromosome arms are organized by cohesin-dependent loops*

130 To characterize *S. cerevisiae* mitotic chromosome organization we took advantage of
131 yeast strains in which the cyclosome activator Cdc20 is under the control of the repressible
132 MET3 promoter. To arrest the cells in mitosis, we released them from a G1 alpha-factor arrest
133 by growing them in rich media supplemented with methionine (Figure 1A; Methods). Once
134 arrested in metaphase, genome-wide chromatin contacts were quantified by Hi-C. Normalized
135 contact maps (1 kb resolution) of mitotic chromosomes differed from G1 pattern in several
136 ways (Figure 1B, left panels). First, the broad and thicker diagonal observed in metaphase

137 compared to G1 revealed increased contacts at short/medium range, blurring the boundaries
138 of the small microdomains visible in G1. Second, discrete dots of variable intensities
139 corresponding to enriched contacts between pairs of loci, consistent with DNA loops, were
140 also often visible along mitotic chromosomes (Figure 1B, black arrows). Not all chromosomal
141 regions were covered by a loop signal, but whether this results from the resolution of our
142 experiment or a real feature of chromosome structure remains to be characterized. To better
143 characterize chromosome compaction we computed the contact probability curve $P_c(s)$ for
144 each condition (Lazar-Stefanita et al., 2017; Schalbetter et al., 2017) (Figure S1A; Methods).
145 The derivative of the $P_c(s)$ curve in a log-log space can be used to facilitate the visualization
146 of local differences (Methods). In mice, the maximum of the derivative curve also matches
147 with the average length of extruded loops (Gassler et al., 2017). The same representation in
148 yeast showed a net enrichment in mitosis in the contacts between loci separated by 30 kb or
149 less, compared to G1 (Figure 1C, blue vs. orange curve). It also suggested a loop size in
150 metaphase arrested cells of ~15 kb on average.

151 To directly test the role of cohesin in the maintenance of these structures, we depleted
152 Scc1 in synchronized cells using an auxin degron strategy (Figure 1A right panel, Methods).
153 Efficient Scc1 depletion was quantified by a split-dots assay measuring cohesion loss (Figure
154 1D). Preventing cohesin loading during S phase suppressed establishment of intra-
155 chromosomal contacts (Figure S1B) and loop formation (Figure 1B, right panel) and the
156 overall contacts displayed little changes compared to those observed in G1-arrested cells that
157 contain little, if any, Scc1 (Figures S1A). Therefore, DNA loops and local contact enrichment
158 observed along mitotic chromosomes are cohesin-dependent.

159 Because of the population average nature of Hi-C, the systematic presence of all these
160 loops in each cell cannot be directly addressed here. However, to further characterize loops
161 and to analyze whether they correspond to contacts between Scc1 enrichment sites

162 Verzijlbergen et al., 2014), we generated pile-up plots. Pile-up plots aim at depicting the
163 average contact signal between pairs of Scc1 enrichment sites (Verzijlbergen et al., 2014).
164 Briefly, for each condition, contacts between all pairs of 80 kb windows, centered on Scc1,
165 and separated by 0 to 20 kb, were aggregated. A randomized set of windows separated by
166 similar distances was also computed. The pile-up plot resulted from the ratio between the
167 Scc1-centered aggregated maps and the randomized set. The same process was applied for
168 pairs of cohesins enrichment sites separated by increasing distances, from 0 kb to 140 kb,
169 with 20 kb steps (Figure 1E, Methods, (Gassler et al., 2017; Muller et al., 2018)). The color
170 scale of the plots reflects the differences in contacts between the two aggregated maps: the
171 redder, the more contacts are enriched on the maps centered on Scc1 enrichment sites. In
172 metaphase, a strong dot signal appeared at the center of the pile-up, *i.e.* between pairs of
173 cohesin-binding sites (Figure 1F, Methods), that vanishes over distances larger than 40 kb.
174 These dots point as enriched contacts between pairs of discrete cohesin binding sites, e.g.
175 loop-like structures. The strength of the dot signal can be assessed by dividing the median
176 value of the central 3x3 square (*i.e.* the signal value), by the median of the top-right corner
177 square (*i.e.* the background value). Pile-up plots confirm that the yeast mitotic chromosomes
178 are organized into chromatin loops anchored at cohesin binding sites.

179 Peaks of cohesin enrichment sites overlap, on average, with convergent transcription
180 sites (Busslinger et al., 2017; Glynn et al., 2004; Hu et al., 2015; Lengronne et al., 2004; Paldi
181 et al., 2019). The basis of DNA loops, which involve such cohesin enriched regions, were
182 indeed and unsurprisingly also found to be preferentially positioned within convergent genes
183 (as illustrated in Figure S1C).

184

185 ***Cohesin dependent loops are independent of sister chromatid cohesion***

186 Mitotic loops could arise from the bridging of cohesins involved in sister chromatid
187 cohesion. To test this hypothesis, we generated Hi-C maps of mitotic chromosomes from cells
188 depleted for Cdc45, which reach mitosis without replication (Tercero et al., 2000) (Figures 2A
189 and S2A). Contact maps and their associated DNA loops (Figures 2B and S2B), $P_c(s)$ (Figures
190 2C and S2D) and pile-up plots of mitotic unreplicated and replicated cells showed little, if
191 any, differences (Figure 2D). In addition, DNA loops of unreplicated mitotic chromosomes
192 disappear in the absence of Scc1 (Figures 2B, 2C, 2D and S2C). Altogether, these results
193 show that sister chromatid cohesion is not necessary for generating Scc1-dependent
194 structures.

195 To further determine whether cohesin-mediated loops along mitotic chromosomes
196 depend on a mitosis specific activity, we induced cohesin loading on unreplicated
197 chromosomes in G1. An engineered version of Scc1 that cannot be cleaved by separase was
198 induced in G1 arrested cells (Uhlmann et al., 2000) (Figures 3A and S3A) and contact maps
199 were generated (Figure 3B). Loading of cohesin on G1 chromosomes led to an accumulation
200 of loops between pairs of Scc1 binding sites (Figures 3B and 3C) and a significant increase in
201 intra-chromosomal contacts (Figures 3D, S3B, S3C and S3D). In this condition, loops
202 appeared slightly larger than in mitosis, suggesting that their expansion is constrained in
203 metaphase-arrested cells (Figure 3C). Altogether those results demonstrate that the
204 establishment of cohesin-mediated DNA loops along mitotic chromosomes is independent of
205 sister-chromatid cohesion and of a mitosis specific activity.

206

207 ***Wpl1-mediated releasing activity counteracts loop expansion***

208 In the absence of Wpl1, cohesins cannot be released from DNA. In mammals, Hi-C
209 studies of WAPL-depleted cells revealed an enlargement of cohesin loops along interphase
210 chromosomes which was interpreted as a hint of a loop extrusion process (Wutz et al., 2017).

211 To gain insights into the mechanism generating cohesin loops in yeast metaphase
212 chromosomes, we therefore tested whether intra chromosomal contacts, $P_c(s)$ and DNA loops
213 spread to greater distances in Wpl1-depleted cells. Wild-type and *wpl1Δ* cells were
214 synchronized in metaphase by depleting Cdc20 (Figure S4A) and genome-wide chromatin
215 interactions were quantified by Hi-C (Figure 4A). In Wpl1-depleted cells, the contact map
216 displayed a very different pattern as compared to wild-type, with less short-range contacts
217 overall (but a crispier constriction at the centromere level, clearly separating the two
218 chromosome arms), as well as chromatin loops punctuating the map resulting in a grid-like
219 dotted pattern (Figures 4A and S4B). The former positions of wild-type loops often displayed a
220 weaker signal in the absence of Wpl1 as compared to wild-type cells. On the other hand,
221 contacts between discrete, cohesin enriched sites now appeared enriched over longer
222 distances, as shown by pile-up plots (up to ~130 kb in the absence of Wpl1 compared to ~<40
223 kb in wild type) (Figures 4B and S4D). The $P_c(s)$ also confirmed the decrease in short-range
224 contacts and the enrichment over longer distances (Figures 4C and S4C).

225 The resulting grid-like loop pattern was often, but not always, stronger in
226 chromosomal regions proximal to the centromeres. This may be due to high levels of cohesin
227 loading at centromeric regions or due to the fact that the centromeric region represents a
228 roadblock to cohesin progression. Similarly to wt metaphase cells, the basis of DNA loops in
229 Wpl1 depleted cells involve enriched cohesion sites preferentially positioned within
230 convergent genes (Figure S4E). The effects of Wpl1 depletion were further supported by
231 performing the experiment in cells harboring *pds5-S81R*, an allele abolishing cohesin release
232 even in presence of Wpl1 (Figure S4).

233 Expansion of DNA loops induced by Wpl1 depletion was not specific to mitosis, as it
234 was also observed in G1-arrested cells expressing the non-cleavable version of Scc1 (Figure
235 S5).

236 Our data therefore show that similarly to human cells, the size of yeast cohesin-
237 dependent loops becomes deregulated in the absence of Wpl1, extending over much longer
238 distances, though still involving hotspots of cohesin depositions. It suggests that yeast cohesin
239 organizes mitotic chromosome presumably by loop extrusion, similarly to the mechanism
240 proposed by others to explain establishment of DNA loops by cohesin during mammalian
241 interphase.

242

243 ***Dual roles for Pds5 in DNA looping***

244 As Wpl1 works with Pds5 to mediate “releasing activity” we tested how Pds5
245 contributes to DNA loop regulation. G1-arrested cells were depleted for Pds5 using the
246 degron system, released into S-phase and arrested in metaphase (Figure S6A). Loss of both
247 Pds5/Eco1-mediated Smc3-K113 acetylation (Figure 5A) and sister chromatid cohesion
248 (Figure 5B) confirmed efficient Pds5 degradation. The contact map of Pds5-depleted cells
249 was generated (Figure 5C) and compared to control (Figures 5C and S6B) and *wpl1Δ* cells
250 (Figures 5C and S6C). Comparison of maps (Figure 5C) revealed that the absence of Pds5
251 increased the amount of long-range intra-chromosomal DNA contacts to a much higher extent
252 than in *wpl1Δ* cells, as confirmed by log ratio between maps (Figures S6B and S6C) and $P_c(s)$
253 curves (Figures 5E and S6D).

254 A close inspection of Hi-C map also revealed that, as in metazoan (Wutz et al., 2017),
255 Pds5 depletion induced a decrease in the number of discrete spots/DNA loops compared to
256 wild-type and *wpl1Δ* cells (Figure 5C). While in wild-type and *wpl1Δ* cells, loops bridge
257 discrete pairs of Scc1-enriched sequences, in Pds5-depleted cells loop bases were more
258 loosely defined, with no clear-cut dots in the normalized Hi-C maps (Figure 5C). This was
259 confirmed by pile-up plots showing that in Pds5 depleted cells, DNA loops did not

260 accumulate within those cohesin-rich loci as clearly as in wild-type cells (Figures 5D and
261 S6E).

262 In metaphase-arrested Pds5-depleted cells, the insulation of peri-centromeric regions
263 from the rest of chromosome arms was reduced. Whereas in wt and Wpl1-depleted cells the
264 immediate left and right centromere-flanking regions appear to form a discrete “domain”
265 (Figure S6F, see pink rectangle), this small structure disappears in the absence of Pds5.
266 Instead, both regions now became able to engage in long-range contacts with their entire
267 adjacent arm, up to the telomere. In addition, the ratio plot between Pds5-depleted and wild-
268 type mitotic maps also showed a significant loss of contacts between the two arms (Figure
269 S6B). As a result, each chromosome arm appears on the maps as a large self-interacting
270 domain (Figure 5C).

271 To analyze whether pericentromeric, long-range contacts affect centromere clustering
272 in the absence of Wpl1 or Pds5, we looked at the positioning of the fluorescently labelled
273 Ndc80 kinetochore protein in the nuclei of nocodazole-arrested cells. Whereas in wild-type
274 and Scc1-depleted cells all Ndc80 fluorescent signals appeared as two bright dots, both in
275 *wpl1Δ* and Pds5-depleted cells this structure was accompanied by multiple, small discrete
276 dots (Figure S6G). Given that the main difference between these different conditions
277 consisted in the size of the loops, we propose that the engagement of each centromeric region
278 in large loops induces a mechanical force promoting centromere de-clustering.

279

280 Chromosome contact patterns observed in Pds5-depleted cells suggest that Pds5
281 regulates loop formation not only *via* a Wpl1-mediated releasing activity, but also by an
282 unknown, Wpl1-independent mechanism. As Pds5, but not Wpl1, is essential to maintain
283 sister chromatid cohesion, we hypothesized that the increase in DNA loops sizes over longer
284 distances in Pds5-depleted cells could result from loss of cohesion. Indeed, cohesins involved

285 in sister chromatid cohesion may act as physical barriers, or roadblocks, halting the loop
286 expansion process and defining loop basis positions along chromosomes. Inactivation of Pds5
287 would alleviate those discrete roadblocks, allowing loop expansion to proceed over longer
288 distances, and resulting in an increase in long-range contacts. To test this, we measured the
289 effects of Pds5 or Wpl1 loss on 3D folding of un-replicated (*cdc45*) chromosomes (Figures
290 6A, B and C). The depletion of either Wpl1 or Pds5 had the same effect on chromosome
291 structure than that observed in metaphase, with Pds5 inactivation leading to intra-
292 chromosomal contacts bridging loci over longer distances than in *wpl1Δ* cells, as illustrated
293 by log ratio between Hi-C maps (Figure 5F). Moreover, inactivation of Pds5 in Cdc45
294 depleted cells also induced a decrease in the number of discrete spots/DNA loops compared to
295 wild-type and *wpl1Δ* cells (Figures 6C and D).

296 Impact of Pds5 and Wpl1 depletion was also quantified in living cells: fluorescently-
297 labelled centromeres and a locus positioned 400 kb away on chromosome XV got closer
298 together compared to control cells (Figure 6E), backing the Hi-C results showing increased
299 loop sizes.

300 Those results therefore demonstrate that Pds5 regulates DNA contacts and suppresses
301 loop expansion through two mechanisms, the recruitment of Wpl1, as well as a Wpl1-
302 independent pathway.

303

304 ***Eco1 inhibits loop expansion***

305 Since increase in loop length in Pds5 depleted cells does not only result from the
306 absence of releasing activity, we envisioned that Pds5 recruitment of Eco1 (Chan et al., 2013;
307 Minamino et al., 2015; Noble et al., 2006; Vaur et al., 2012) might regulate a second
308 mechanism required to inhibit DNA loop expansion. We tested the effects of Eco1 depletion
309 using an inducible degron (Figures S7A and S7B; Methods) and showed that loss of Eco1 is

310 sufficient to result in spreading of contacts (Figures 7A, S7C and S7D) and loops (Figures
311 7A, C and S7F) to longer distances. As the absence of Eco1 promotes dissociation of cohesin
312 from DNA by Wpl1 (Chan et al., 2012), these longer loops cannot result from an increase in
313 cohesin residence time on DNA. Therefore, this result shows that in addition to promoting
314 sister chromatid cohesion, Eco1 also inhibits the translocation process extending DNA loops.
315 Remarkably, loop length (Figure 7A, C and S7F), contact probability ($P_c(s)$) (Figures 7B and
316 S7B) and centromere clustering defects in Eco1 depleted cells (Figure S7G) were comparable
317 to those observed in Wpl1 depleted cells, despite Eco1 and Wpl1 having opposing effects on
318 releasing.

319 Since Pds5 recruits both Eco1 and Wpl1 on cohesin, we tested whether their co-
320 inactivation would mimic the effect of Pds5 depletion. Indeed, depletion of Eco1 in *wpl1Δ*
321 cells promoted long-range intra-chromosomal contacts and DNA loops nearly identical to
322 those observed in Pds5 depleted cells (Figures 7, S7B, D and E). This suggests that the
323 positioning of loop basis (hence the size of the loops) is regulated through two independent
324 pathways, Eco1 and Wpl1.

325

326 **DISCUSSION**

327 In the present study, we show that yeast mitotic chromosomes organize into loops
328 along individual chromatids, independently of replication. These loops are enriched in
329 cohesin subunits at their basis. The cohesin binding factors Pds5, Wpl1 and Eco1 affect the
330 sizes and distribution of these loops, with Pds5 regulating loop size *via* two pathways: the
331 Wpl1-mediated releasing activity and an Eco1-dependent mechanism (Figure 7).

332

333 *Yeast mitotic chromosomes are organized into cohesin-dependent loops*

334 We show that DNA loops are present along yeast mitotic chromosomes. The bases of
335 these loops are enriched in cohesin. Whether all cohesin deposition sites identified by
336 chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) are involved in loop
337 formation in all cells, or whether a loop heterogeneity exists within the population remains to
338 be characterized. Cohesin enrichment sites, and the bases of DNA loops in *wt* or Wpl1
339 depleted cells, correspond mostly to site of convergent genes. It has been proposed that
340 accumulation of cohesin at these genomic positions may be driven by transcription
341 (Lengronne et al., 2004). RNA polymerases along convergent genes may indeed represent
342 boundaries or roadblocks to cohesin-dependent loop expansion, resulting in an accumulation
343 of cohesin-dependent loop basis at sites of convergent transcription. We also noticed that
344 Pds5 is crucial to maintain accumulation of cohesin-dependent loop at those sites. This
345 implies that in absence of Pds5 the loop formation mechanism somehow manages to bypass
346 the roadblocks.

347

348 ***Cohesin binding factors regulate loops expansion***

349 It has been proposed that cohesin could promote chromatin loops formation *via* a loop
350 extrusion mechanism (Fudenberg et al., 2016; Goloborodko et al., 2016; Nasmyth, 2001).
351 This model predicts that the length of DNA loops should be dependent on cohesin residence
352 time on DNA. Since Wpl1 inactivation increases cohesin's residence time on DNA, cohesin
353 would extrude longer DNA in Wpl1-depleted cells. Indeed, Hi-C studies in mammals
354 revealed an enlargement of cohesin-mediated loops along chromosomes in WAPL1-depleted
355 cells. Our present study shows that the inactivation of Wpl1-mediated releasing also induces
356 enlargement of DNA loops in *S. cerevisiae*. By analogy with mammals, we propose that this
357 enlargement of DNA loops is the consequence of an increase of cohesin's residence time on
358 DNA and that cohesin structure yeast mitotic chromosomes through a loop extrusion process.

359 Alternatively, one can envision another hypothesis whereby the longer range contacts
360 observed in Wpl1-depleted cells result from cohesin loading defects due to Wpl1 depletion
361 (Rowland et al., 2009). Indeed, the inactivation of Wpl1 could reduce the amount of cohesin
362 being loaded on the chromatin, hence decreasing the number of complexes expanding DNA
363 loops present along a chromosome arm. The lower density of cohesin may in turn lead to
364 longer DNA tracks being transformed into loops by the same complex before colliding into
365 another complex, and thus resulting in the formation of longer loops on average. Future works
366 will determine whether loop enlargement in Wpl1-depleted cells is the consequence of
367 cohesin loading defects, of an increase in cohesin's residence time on DNA, or both.

368 We also analyzed the role of other cohesin binding factors on loop structures and
369 found that Pds5 regulates loop expansion *via* at least two pathways: the previously described
370 Wpl1-mediated releasing activity (Wutz et al., 2017) and a novel Eco1-dependent mechanism
371 (Figure 7). Our results point at a role of Eco1 in negatively regulating loop expansion through
372 a mechanism independent of cohesion establishment. In agreement with our result a recent
373 study reported that ESCO1 (human ortholog of Eco1) also inhibits the ability of cohesin to
374 form long DNA loops (Wutz et al., 2019).

375

376 ***Competition between Scc2 and Pds5 may regulate DNA loop expansion***

377 One may envision that Eco1-mediated acetylation inhibits translocase activity that
378 expands cohesin-dependent loops. It has been proposed that ATP hydrolysis stimulated by
379 Scc2 could be the driving force for loop expansion. As Pds5 competes with Scc2 for binding
380 the kleisin subunit (Petela et al., 2018), a possibility is that Pds5 inhibits the translocation
381 process required to expand DNA loops. Eco1-mediated acetylation may therefore improve
382 Pds5 binding on cohesin and consequently abolish the Scc2-mediated translocation process.
383 This idea is reinforced by *in vivo* FRAP measurements revealing that Eco1 inactivation

384 increases Pds5 turnover on chromosomes (Chan et al., 2013). Moreover, recent ChIP-seq data
385 show that Eco1 inactivation reduces Pds5 association with DNA while it has no effect on
386 cohesin ring association with DNA (Chapard et al., 2019).

387 Competition between Pds5 and Scc2 may also control loop expansion and cohesin
388 positioning in human cells. This hypothesis is supported by the fact that Scc2 does not co-
389 localize with CTCF which halts loop expansion. Wutz et al. (2017) showed that the ability of
390 CTCF to block loop extrusion is dependent on PDS5. As discussed by the authors,
391 replacement of Pds5 by Scc2 may be somehow inhibited at CTCF sites, which may
392 consequently abolish or decrease the rate of ATPase hydrolysis required to expand DNA
393 loops (Wutz et al., 2017).

394 How does Eco1 regulate competition between Scc2 and Pds5 during establishment and
395 maintenance of cohesin-dependent loops? It may involve Eco1-mediated Smc3 acetylation as
396 this reaction affects how Scc2 promotes loading of cohesin onto DNA (Hu et al 2015).
397 However, additional investigations are necessary to determine whether Smc3 acetylation is
398 involved in regulation of DNA loops. Deciphering how Eco1 influences DNA loop expansion
399 may provide new perspectives to understand how human Eco1 homologues (ESCO1 and
400 ESCO2) regulate gene transcription or how they deficiency induces the developmental
401 disorder called Roberts syndrome.

402

403 ***DNA looping is blocked at centromeres***

404 Our study points out that the yeast centromere is a strong roadblock to cohesin-
405 dependent loop expansion. The nature of this boundary raises interests beyond the yeast
406 community, as related mechanisms could apply in other species as well. In yeast, kinetochore
407 proteins bound to centromeres are connected through microtubules to the MTOC (spindle
408 pole body or SPB) which itself is embedded in the nuclear envelope. Such a physical bridge

409 could block the progression of incoming cohesin along the chromosome. It is also possible
410 that the huge kinetochore complex bound to centromeres is sufficient to stop DNA loop
411 expansion.

412 In wild type cells, Wpl1 mediates cohesin release and Eco1 restricts DNA loop
413 progression from chromosome arms to centromeric regions. When those activities are
414 impaired, loops emanating from chromosome arms can extend to centromeres, but no further,
415 resulting in sharp, discrete boundaries as observed on the contact maps. In addition, it is likely
416 that loops also originate from centromeres and spread towards chromosome arms.

417 Finally, we showed that centromere clustering is impaired in Wpl1, Pds5 or Eco1
418 depleted cells (Figure S6G and S7G). This suggests that centromere clustering is exposed to
419 opposite forces emanating from microtubules and, presumably, a loop extrusion-like
420 mechanism that promote loop expansion. Inhibiting such loop extruding mechanism in wild-
421 type cells may favor the insulation of clustered centromeres away from chromosome arms,
422 and consequently improve or stabilize microtubule attachment and chromosome segregation.
423 It remains to be investigated whether similar mechanisms occur in species with larger and
424 epigenetically defined centromeres compared to the budding yeast point centromere, and
425 whether the attachment of microtubules is mandatory to block DNA looping.

426

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434

435 **Author contributions**

436 LD, RK and FB designed research. LD and AT performed the experiments, with help
437 from LLS who initiated the study. RM analyzed the data, with contributions from AC. All
438 authors interpreted the data. LD, RK, FB wrote the manuscript, with contributions from RM.

439

440 **Declaration of interest**

441 The authors declare no competing interests.

442

443 **Data Availability**

444 Sample description and raw sequences are accessible on SRA database through the following
445 accession number: PRJNA528616.

446 **Figure Legends**

447 **Figure 1.** Mitotic yeast chromosomes are organized as cohesin-dependent loops and domains.

448 A) Left panel: schematic representation of the experimental protocol used to process cells
449 from G1 to metaphase in absence or presence of Scc1 (strains FB133-57B and yLD127-20b).
450 Right panel: cell-cycle arrest monitored by flow cytometry. Percentage of bi-nucleated
451 (anaphase) cells was measured to evaluate the efficiency of the metaphase arrest. B) Contact
452 maps of a part of chromosome IV (1 kb bin) for control and Scc1-depleted, metaphase-
453 arrested strains (strains FB133-57B and yLD127-20b) and G1 arrested cells (strain FB09-9C),
454 Scc1 ChIp seq profile and chromosomal contact along 100kb region of chromosome IV are
455 shown in the black square. C) Derivative of the curve plotting contact probability as a
456 function of genomic distance (log scale). D) Percentage of cells with paired or unpaired
457 fluorescently labelled URA3 loci (*ura3::3xURA3 tetO112; tetR-GFP*), carrying or not a Scc1-
458 AID degron (strains yLD126-36c and FB124), in presence of auxin. Scale bar, 2 μ m. E)
459 Schematic representation of the analysis pipeline used to generate pile-up ratio plots. F) Pile-
460 up ratio plots of 80 kb windows (2 kb bin) centered on pairs of Scc1-enriched or randomly
461 chosen positions (Methods). Ratios are ordered according to the distance between Scc1
462 enriched positions. Blue color: more contacts between the random genomic regions. Red
463 signal: more contacts between Scc1-enriched regions. The italic numbers atop each pile-up
464 reflect the strength of the central signal compared to background (Methods). See also Figure
465 S1.

466

467 **Figure 2.** Cohesin-dependent loops and domains on mitotic yeast chromosomes are
468 independent of sister chromatid cohesion. A) Schematic representation of the experimental
469 protocol used to generate unreplicated mitotic cells in absence or presence of Scc1 (strains
470 FB154 and FB149- 11B). B) Contact maps of a part of chromosome IV (1 kb bin) for

471 unreplicated mitotic cells (*cdc45*) with or without Scc1 (strains FB154 and FB149-11B) and
472 for metaphase-arrested cells (*cdc20*, strain FB133-57B). C) Derivative of the curve plotting
473 contact probability as a function of genomic distance (log scale). D) Pile-up ratio plots of 80
474 kb windows (2 kb bin) centered on pairs of Scc1-enriched or randomly chosen positions (see
475 Figure 1F). See also Figure S2.

476

477 **Figure 3.** Expression of Scc1 in G1-arrested cells induces DNA looping. A) Left panel:
478 Schematic representation of the experimental protocol followed to overexpress a HA-tagged,
479 non-cleavable Scc1 in G1. Right panel: western blot assessing expression of non-cleavable
480 Scc1 (*OE scc1*) with anti-HA antibody and expression of endogenous Scc1 (Scc1-PK) with
481 anti-V5 antibody. B) Contact maps of a part of chromosome IV (1 kb bin) for G1-arrested
482 cells with or without Scc1 (strains FB09-9C and FB09-4A) and for metaphase-arrested strain
483 (*cdc20*, FB133-57B). C) Pile-up ratio plots of 80 kb windows (2 kb bin) centered on pairs of
484 Scc1-enriched or randomly chosen positions (see Figure 1F). D) Derivative of the curve
485 plotting contact probability as a function of genomic distance. Black digits reflect the strength
486 of the central signal compared to background. See also Figure S3.

487

488 **Figure 4.** Wpl1 restricts DNA loop expansion. A) Contact maps of a part of chromosome IV
489 (1 kb bin) of wild type and *wpl1Δ* strains arrested in metaphase (strains FB133-57B and
490 FB133-49B). B) Pile-up ratio plots of 80 kb windows (2 kb bin) centered on pairs of Scc1-
491 enriched or randomly chosen positions (see Figure 1F). C) Derivative of the curve plotting
492 contact probability as a function of genomic distance. See also Figure S4 and S5.

493

494 **Figure 5.** A Wpl1-independent pathway regulates cohesin-dependent loops during mitosis. A)
495 Pds5 depletion was monitored by western blot through the Pds5-dependent acetylation of

496 Smc3-K113. Pgk1, loading control. B) Sister-chromatid cohesion was monitored by detecting
497 paired/unpaired fluorescently labelled URA3 loci in presence or absence of Pds5 (strains
498 yLD126-36c and yLD126-38b). Scale bar, 2 μ m. C) Hi-C contact maps of metaphase-arrested
499 cells in presence or absence of Pds5 or Wpl1 (1 kb bin). Chromosomes X and XI are
500 represented with pink and green lines, respectively. D) Pile-up ratio plots of 80 kb windows
501 (2 kb bin) centered on contacts between pairs of Scc1-enriched or randomly chosen positions
502 (see Figure 1F). E) Derivative of the curve plotting contact probability as a function of
503 genomic distance (log scale) for metaphase arrested strains. F) Log2 ratio between Hi-C maps
504 from metaphase-arrested strains or Cdc45-depleted cells expressing or not Wpl1 or Pds5 (1 kb
505 bin). Blue to red color scale reflects the enrichment in contacts in one population with respect
506 to the other. See also Figure S6.

507

508 **Figure 6.** Effect of Pds5 or Wpl1 loss on the 3D folding of un-replicated mitotic
509 chromosomes. A) Unreplicated mitotic cells without Pds5 or Wpl1 were generated with
510 strains FB154 (control), FB148-3C (*wpl1 Δ*) and FB156-5a (*pds5-AID*). Cell cycle was
511 monitored by flow cytometry. Percentage of bi-nucleated (anaphase) cells and budding
512 indexes were measured. B) Derivative of the curve plotting contact probability as a function
513 of genomic distance (log scale). C) Hi-C contact maps of unreplicated mitotic cells in absence
514 of Pds5 or Wpl1 (1 kb bin). D) Pile-up ratio plots of 80 kb windows (2 kb bin) centered on
515 contacts between pairs of Scc1-enriched or randomly chosen positions (see Figure 1F). E)
516 Fluorescent imaging of strains yLD162-13a (control), yLD162-2b (*wpl1 Δ*) and yLD163-22a
517 (*pds5-AID*) strains harboring fluorescently labelled kinetochores (Ndc80-GFP) and HIS3 gene
518 (TetO/TetR-mRFP, 400 kb away from the chromosome XV centromere). Cells were arrested
519 in early S phase by expressing a non-degradable version of Sic1 protein. Scale bar, 2 μ m.

520 Distances between kinetochores and the HIS3 locus were measured in each condition and
521 plotted as cumulative distributive functions. See also Figure S7.

522

523 **Figure 7.** Eco1 counteracts loop expansion. A) Hi-C maps (1 kb bin) for strains FB133- 57B
524 (wild-type), FB133-20C (*eco1-AID*), FB133-49B (*wpl1Δ*) and FB133-1D (*wpl1Δ eco1-AID*)
525 and yLD121-1a (*pds5-AID*) arrested in metaphase. The chromosomes are indicated for each
526 map. B) Derivative of the curve plotting contact probability as a function of genomic distance
527 (log scale) for metaphase arrested strains. C) Pile-up ratio plots of 80 kb windows (2 kb bin)
528 centered on pairs of Scc1-enriched or randomly chosen positions (see Figure 1F). D) Model
529 showing the two Pds5-regulated pathways inhibiting DNA loop expansion through a loop
530 extrusion mechanism. Left: Eco1 inhibits cohesin translocase activity; right: Wpl1 opens the
531 Smc3-Scc1 gate and dissociates cohesin from DNA.

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STAR METHODS

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Romain Koszul (romain.koszul@pasteur.fr)

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Yeast Strains and Plasmids

All strains are derivatives of W303. Strains and plasmids are listed in Key Resources Table and in Supplementary Table S2

Media and growth conditions

Strain yLD118-1a (MET3-CDC20) was grown overnight at 30°C in 150ml of synthetic complete medium deprived of methionine (SC-Met) (SC: 0.67% yeast nitrogen base without amino acids (Difco)), supplemented with a mix of amino-acids, uracil and adenine, 2% glucose) to reach $4,2 \times 10^8$ cells. To induce metaphase arrest, cells were arrested in G1 for 2h30 by addition of alpha-factor (Antibody-online, ABIN399114) every 30min (1µg/ml final), washed 3 times and released in rich medium (YPD: 1% bacto peptone (Difco), 1% bacto yeast extract (Difco) and 2% glucose) supplemented with methionine (2mM final). 2h latter cells were fixed for Hi-C. Strains yLD127-20b, yLD121-1a, FB133-57B, FB133-20C, FB133-49B, FB133-1D were processed as described above except auxin addition (Sigma-Aldrich, I3750) (1mM final) to the media 1h after starting alpha-factor treatment. Cells were released from G1 in YPD supplemented with methionine and auxin (1mM final). Strains FB08-5C, FB08-6A, FB09-4A, FB09-9C were grown overnight in 300ml of YP medium

558 supplemented with raffinose 2% (Sigma-Aldrich, R0250) to reach $8,4 \times 10^8$ cells. Expression
559 of Scc1(R180D,268D)-HA was induced 1h after starting alpha-factor treatment by addition of
560 newly-made galactose (Sigma-Aldrich, G0750) (2% final) to the cultures. Cells were fixed for
561 Hi-C after 2h30 in alpha-factor. Strains FB154, FB149-11B, FB148-3C, FB156-5a (CDC45-
562 AID) were grown overnight in 150ml YPD to reach $4,2 \times 10^8$ cells. G1 arrest and auxin
563 addition were conducted as previously, (except auxin concentration, 2mM final), cells were
564 released in YPD media supplemented with auxin (2mM final) for 80min and fixed for Hi-C.

565

566

567 **METHOD DETAILS**

568

569 **Flow cytometry**

570 About $2,8 \times 10^6$ were fixed in ethanol 70% and stored at -20°C . Cells were the pelleted,
571 washed and incubated overnight in Tris-HCl 50mM pH 7,5 complemented with RNase A (10
572 mg/ml; Sigma-Aldrich) at 37°C . Cells were pelleted, resuspended 400 μl of 1,0mg/ml
573 propidium iodide (Fisher, P3566) in 50mM Tris pH 7,4, NaCl, MgCl₂ and incubated for 1 h
574 at room temperature. Flow cytometry was performed on a CyFlow® ML Analyzer (Partec)
575 and data were analyzed using FloMax software.

576

577 **Microscopy**

578 Strains FB124, yLD126-38b, yLD126-36c were grown overnight in SC-Met at 30°C . The
579 next day cells were diluted in fresh media. Exponentially growing cells were arrested in G1
580 with alpha-factor treatment, induced with auxin (1mM final), washed and arrested in
581 metaphase as described above. Strains yLD162-13a, yLD162-2b, yLD163-22a were grown
582 overnight in YP-raffinose at 30°C . The next day, after dilution in fresh media, exponentially
583 growing cells were arrested in G1 with alpha-factor treatment and while being induced with

584 auxin (1mM final). Expression of non-degradable Sic1 was induced 30min before release by
585 addition of galactose (2% final) to the media. Cells were washed and released in YP
586 supplemented with raffinose and galactose for 120min. Cells were placed on 2% agarose pads
587 made of synthetic complete medium plus glucose. Live cell imaging was performed under a
588 spinning disk confocal system (Nipkow Revolution, Andor Technology) with an EM charge-
589 coupled device (CCD) camera (DU 888; Andor Technology) mounted on an inverted
590 microscope (IX-81; Olympus) featuring a CSU22 confocal spinning disk unit (Yokogawa
591 Corporation of America). Image acquisition was done at 30°C. 41 Z-stacking images with
592 0.25µm intervals were acquired by using IQ2 software with 200ms exposure time. Were used:
593 100× objective lens (Plan-Apochromat, 1.4 NA, oil immersion; Olympus) and single laser
594 lines for excitation, diode pumped solid state lasers (DPSSL). GFP fluorescence was excited
595 at 488 nm (50 mW; Coherent) and mCherry fluorescence at 561 nm (50 mW; Cobolt jive).
596 Green and red fluorescence were collected using a bi-bandpass emission filter (Em01-
597 R488/568-15; Semrock). Pixel were 65nm in size.

598

599 **Acetylation assays**

600 A pellet from 10^7 cells was frozen in liquid nitrogen and stored at -20°C overnight. The cell
601 pellet was resuspended in 100µl H₂O, 20µl trichloroacetic acid (Sigma-Aldrich, T8657) and
602 broken with glass beads at 4°C. Precipitated proteins were resuspended in Laemmly buffer/
603 Tris HCl pH 8,0 and extracted by cycles of 5min heating at 80°C- 5min vortexing at 4°C.
604 Eluates were analyzed by SDS-PAGE followed by western blotting with antibodies anti-V5
605 tag (VWR, MEDMMM-0168-P), anti-Pgk1 (Invitrogen, 459250) and anti-Smc3-K113Ac
606 (Beckouët et al., 2010).

607

608 **Hi-C libraries**

609 Hi-C was performed as described (Lazar-Stefanita et al., 2017), except cells were disrupted
610 using a Precellys apparatus (Bertin Instruments) instead of processed through zymolyase
611 treatment. Aliquots of $1-3 \times 10^9$ cells in 150 ml YPD/synthetic medium were fixed in 3%
612 formaldehyde (Sigma, F8775) for 20 min at room temperature and quenched with 25 ml
613 glycine 2.5 M for 20 min at 4°C. Cross-linked cells were recovered through centrifugation,
614 washed with YPD and a 150 mg pellet was stored at -80°C. Hi-C DNA libraries were 500 bp
615 sheared using CovarisS220 apparatus, and the biotin-labeled fragments were selectively
616 captured by Dynabeads Myone Streptavidin C1 (Invitrogen). The resulting libraries were used
617 as template for the Illumina amplification by PE-PCR primers and paired-end sequenced on a
618 NextSeq500 Illumina platform. All Hi-C libraries are listed in Supplementary Table 1.

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620

621 **QUANTIFICATION AND STATISTICAL ANALYSIS**

622 **Processing of the reads and generation of contact maps**

623 Pairs of reads were aligned independently using Bowtie2 in its most sensitive mode against
624 the latest *S. cerevisiae* W303 reference genome (GCA_002163515.1), corrected for a
625 chromosomal inversion on chromosome 16 revealed by the Hi-C data. Alignment was done
626 using an iterative procedure and each uniquely mapped read was assigned to a restriction
627 fragment. Uncuts, loops and religation events were filtered as described (Cournac et al.,
628 2012). Contact matrices were built with resolutions of 2 or 20kb (bin sizes) and normalized
629 using the sequential component procedure (Cournac et al., 2012). Log-ratios were generated
630 by dividing 2 normalized contact maps, with the same resolution, by one another and then
631 computing the \log_2 of the resulting matrix.

632

633 **Computation of the contact probability as a function of genomic distance**

634 Contact probability as a function of genomic distance $P_c(s)$ was determined as described
635 (Muller et al., 2018). Intra-chromosomal pairs of reads were selected and partitioned by
636 chromosome arms. Pairs oriented towards different directions or separated by less than 1.5 kb
637 were discarded. For each chromosome, the remaining pairs were log-binned as a function of
638 their genomic distance s using the formula: $\text{bin} = \lceil \log_{1.1}(s) \rceil$. The number of read pairs in
639 each bin was counted and weighed by the bin size $1.1^{(\text{bin}-1)}$, as well as the difference
640 between the length of the chromosome and the genomic distance.

641

642 **Identification of cohesin binding-sites and generation of pile-up plots**

643 Data from (Verzijlbergen et al., 2014) were used to generate Scc1 ChIP-Seq profiles with a
644 2kb resolution. Bins with a signal over 1.5 were labelled as cohesin binding sites (CBS). CBS
645 were determined for wild type and Pds5-AID (Petela et al., 2018) strains. All possible pairs of
646 CBS within chromosomal arms were determined and partitioned according to their genomic
647 distance. In 2kb resolution contact maps, windows surrounding these positions were extracted
648 and averaged. The resulting observed signal was divided by the expected signal, generated by
649 averaging the windows around random positions having the same genomic distance as the
650 pairs of CBS. For each window, uncovered bins were defined as bins with a total number
651 of reads under $\text{median}(\text{number of reads} / \text{bin}) - SD$ and excluded of the averaging operations
652 to reduce noise. The strength of the central (loop) signal was assessed by dividing the median
653 value of the central 3x3 square (i.e. the signal value), by the median of the top-right corner
654 square (i.e. the background value). As a consequence, the more “loop signal”, the higher the
655 score (> 1). Since loops will deplete longer range contacts, a strong loop signal will be
656 accompanied at longer distances by a weak one (< 1). No loops will result in a signal ~ 1 . See
657 Flyamer et al., 2019, for a similar approach.

658

659 **DATA AND CODE AVAILABILITY**

660 Sample description and raw sequences are accessible on SRA database through the following
661 accession number: PRJNA528616.

662 Codes and functions used to generate the figures from the raw data are available online
663 (<https://github.com/koszullab/hicstuff>).

664 Imaging data are available on Mendeley (<https://data.mendeley.com/datasets>) under the DOI:
665 10.17632/pgwsf28v2d.

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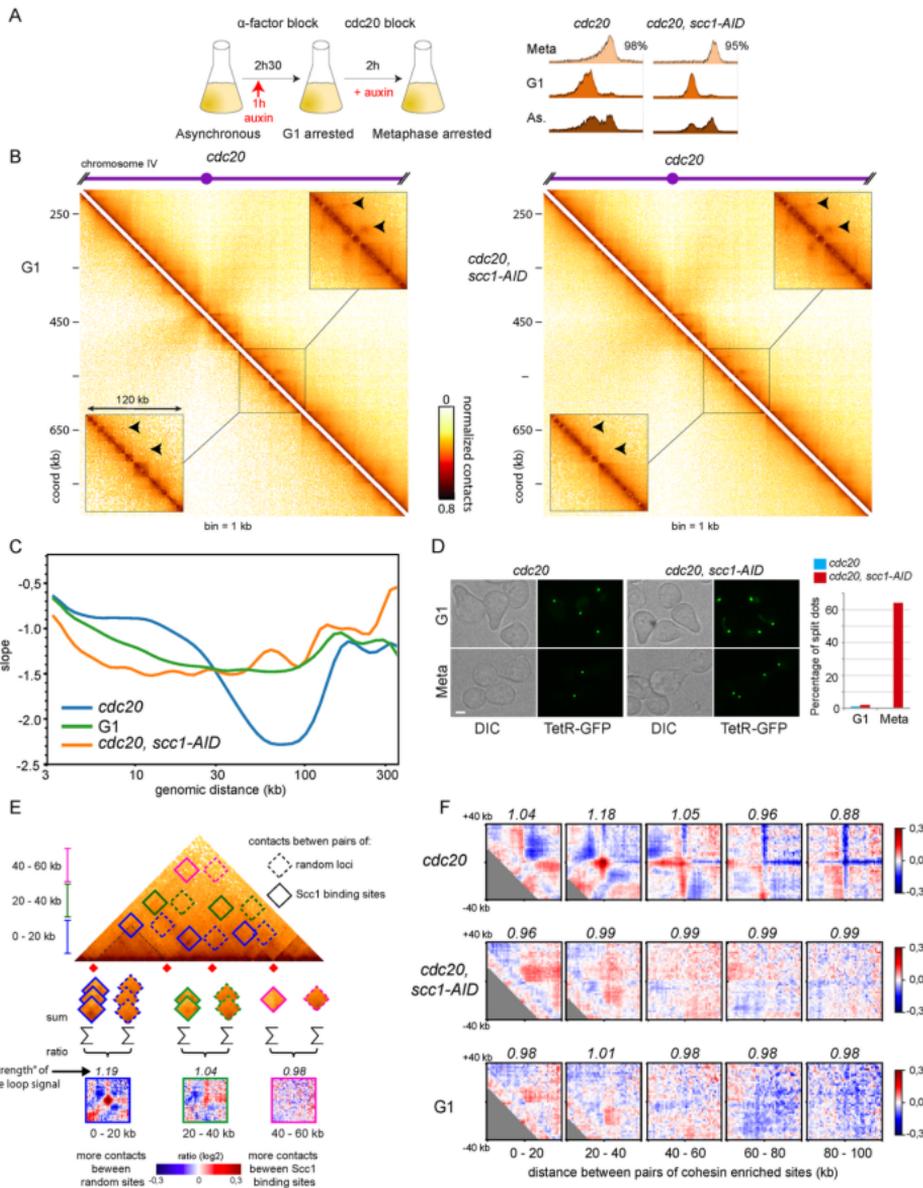
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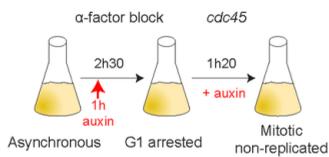
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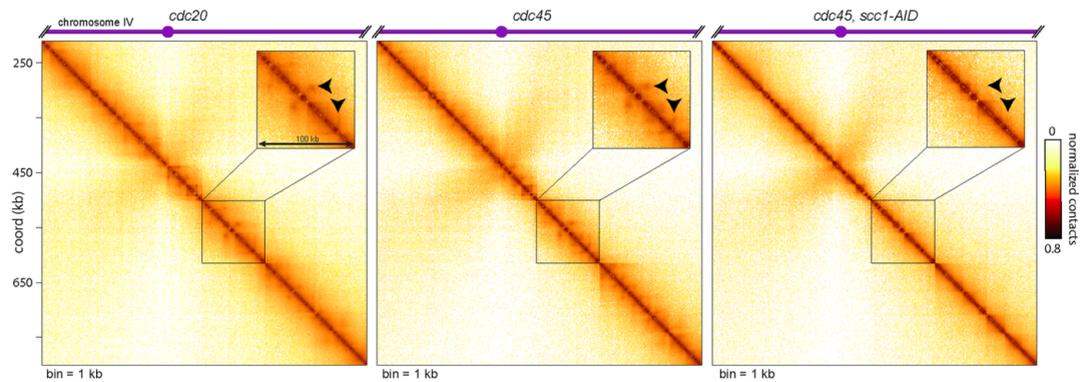
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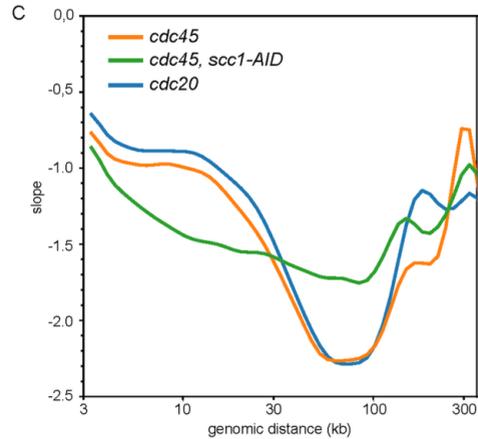
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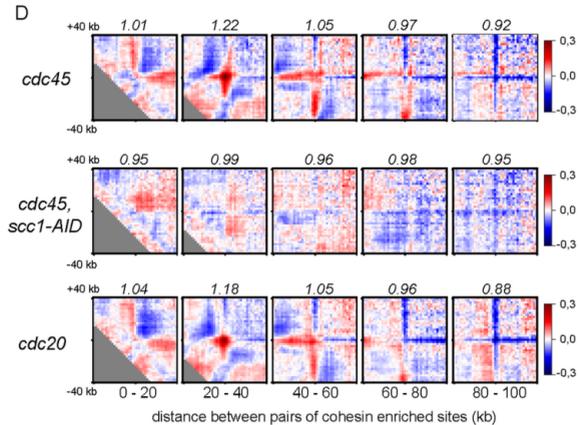
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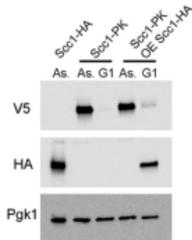
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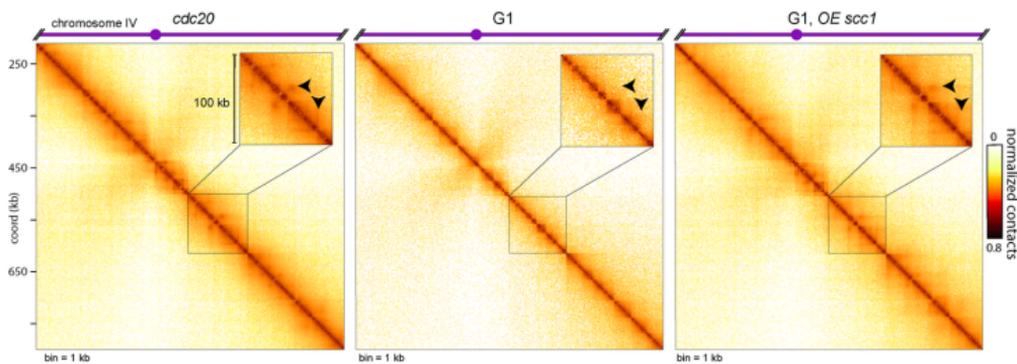
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