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1 Chromosome structure due to phospho-mimetic H2A modulates DDR through increased
2 chromatin mobility

3

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12

13 **Abstract**

14 In budding yeast and mammals, double strand breaks trigger global chromatin mobility
15 together with the rapid phosphorylation of the histone H2A over an extensive region of the
16 chromatin. To assess the role of H2A phosphorylation in this response to DNA damage, we
17 have constructed strains where H2A has been mutated to the phospho-mimetic H2A-S129E.
18 We show that H2A-S129E mutant increases global motion of chromosomes even in the
19 absence of DNA damage. The intrinsic chromatin mobility of H2A-S129E is not due to
20 checkpoint activation, histone degradation or kinetochore anchoring. Rather, the increased
21 intra-chromosomal distances observed in H2A-S129E mutant are consistent with chromatin
22 structural changes. In this context, the Rad9^{53BP1}-dependent-checkpoint becomes
23 dispensable. The increase in chromatin dynamics is favorable to NHEJ of a single double-
24 strand break but is accompanied by a sharp decrease in inter-chromosomal translocation
25 rates. We propose that changes in chromosomal conformation due to H2A phosphorylation
26 are sufficient to modulate the DDR and maintain genome integrity.

27

28 **Introduction**

29 Eukaryotic cells have developed sophisticated machineries to respond to the multiple
30 stresses they are constantly confronted with. In the presence of DNA Double-Strand Breaks
31 (DSBs), the DNA damage response (DDR) protects the genome by detecting and repairing
32 the potentially lethal DSBs that could lead to genome instability or tumorigenesis. Inherited
33 or acquired defects in DDR can result in various diseases, such as immune deficiency,
34 neurological degeneration, premature aging, and severe cancer susceptibility (reviewed in
35 (Jackson and Bartek, 2009; Goldstein and Kastan, 2015)).

36
37 The DDR starts by the recruitment of surveillance proteins that activate cell cycle checkpoint,
38 promote chromatin remodeling allowing DNA accessibility to the repair machinery and trigger
39 DNA repair pathway choice (for reviews see (Mehta and Haber, 2014; Symington and
40 Gautier, 2011; Ceccaldi et al., 2016; Aylon and Kupiec, 2004)). Two of the most well-known
41 DSB repair mechanisms are homologous recombination (HR) and non-homologous end
42 joining (NHEJ). While HR repairs DNA breaks by copying the missing information across the
43 lesion from an undamaged template, as from the replicated sister chromatid, NHEJ does it
44 by ligation of the broken ends after their juxtaposition (reviewed in (Mehta and Haber, 2014;
45 Symington and Gautier, 2011; Ceccaldi et al., 2016; Aylon and Kupiec, 2004)). Long
46 considered as error-prone, the classical form of NHEJ is now regarded as a versatile,
47 adaptable and essential pathway for the maintenance of genomic stability, because joining
48 of the juxtaposed ends of the breaks does not necessarily involve nucleotide deletions.
49 However alternative forms of end-joining exist that can induce DNA aberrations, including
50 chromosomal translocations (reviewed in (Emerson and Bertuch, 2016; Bétermier et al.,
51 2014; McVey and Lee, 2008)) .

52
53 To repair the damage, the DNA damage checkpoint delays cell cycle progression. This can
54 trigger arrests at the G1/S transition, during the S-phase, or at the G2/M transition,
55 depending on the nature of the damage and the phase of the cell cycle in which the lesion
56 happens (for reviews see (Finn et al., 2011; Shaltiel et al., 2015; Waterman et al., 2020)).
57 The highly conserved MRX^{MRN} complex (Mre11-Rad50-Xrs2 in yeast; Mre11-Rad50-Nbs1 in
58 mammals) is among the earliest sensors of DSB by binding directly to DNA broken ends.
59 Sensing includes activation of the phosphatidylinositol3-kinase-related kinase (PIKK) family
60 such as mammalian ATM (Ataxia-Telangiectasia-Mutated) and ATR (ATM- and Rad3-
61 related), called Tel1 and Mec1, respectively, in budding yeast. These sensing kinases are
62 required independently after a DSB, at different points of the DDR. Tel1^{ATM} is rapidly
63 recruited and activated after the recognition of the DSB by the MRX^{MRN} complex, whereas

64 Mec1^{ATR} is recruited by the ATR-interacting protein Ddc2^{ATRIP} after 5' to 3' resection of the
65 DSB ends yields single-stranded DNA and binding of RPA (Kondo et al., 2001; Tibbetts et al.,
66 1999; Nakada et al., 2003; Shroff et al., 2004; Li et al., 2020; Zhou and Elledge, 2000). Both
67 kinases phosphorylate several other proteins involved in cell cycle checkpoint control and
68 DNA repair. An important landmark is the phosphorylation of histone H2A in yeast, or
69 histone variant H2AX in mammals. Phosphorylation of histone H2A occurs near its C
70 terminus (S129 in yeast H2A and S139 in mammalian H2AX, (Rossetto et al., 2014; Redon
71 et al., 2003; Burma et al., 2001; Celeste et al., 2003; Shroff et al., 2004). Phosphorylation of
72 H2A(X) in yeast and mammals, also known as γ -H2A(X), rapidly accumulates at the DSB,
73 spreads over long distances and contributes to further DNA signaling and repair (Downs et
74 al., 2000; Shroff et al., 2004; Burma et al., 2001; Lee et al., 2014; Renkawitz et al., 2013).

75
76 In particular, phosphorylation of H2A(X) at the site of DSB allows recruitment of chromatin
77 remodeling complexes (INO80, SWR1) and other proteins, including 53BP1 (the ortholog of
78 yeast Rad9) (Hammet et al., 2007; van Attikum et al., 2004; Morrison et al., 2004; Tsukuda
79 et al., 2005). Rad9^{53BP1} is a critical checkpoint adaptor protein that transmits the signal from
80 Mec1^{ATM} and Tel1^{ATR} to the downstream effector Rad53^{CHK2} and Chk1^{CHK1} (Blankley and
81 Lydall, 2004; Harrison and Haber, 2006; Sweeney et al., 2005). Rad9^{53BP1} was originally
82 identified in a pioneering study in budding yeast because it controls cell cycle progression by
83 arresting cells in the G2/M phase in case of unrepaired damage (Weinert and Hartwell,
84 1988). Rad9 and 53BP1 both contain BRCT and Tudor domains that recognize histone H3
85 methylation as well as γ -H2A(X) phosphorylation (Hammet et al., 2007; Lancelot et al.,
86 2007). Deletion of Rad9 or 53BP1 results in a more rapid degradation of DSB ends (Lazzaro
87 et al., 2008; Ferrari et al., 2015) Rad9^{53BP1}'s protective role in genome integrity is evidenced
88 by $\Delta rad9$'s poor survival upon genotoxic treatments including phleomycin, γ -rays and UV
89 (Menin et al., 2019; Mirman and de Lange, 2020)

90
91 Neither the functional relevance of chromatin modifications nor the mechanism by which the
92 DDR is activated by chromatin itself is fully understood. It has been shown that robust
93 targeting of repair factors or kinase sensors, in the absence of damage, can elicit the DDR
94 both in yeast or in mammals, indicating that local concentration of sensor protein and/or the
95 higher order of chromatin structure are key in the DDR cascade (Soutoglou and Misteli,
96 2008; Bonilla et al., 2008). In fact, in mammals, chromatin at the site of damage experiences
97 two successive waves of changes. A first local expansion dependent on γ -H2A(X) and
98 MRN(X) is followed by a dynamic compaction, the latter being enough to activate the DDR
99 (Ziv et al., 2006; Kruhlak et al., 2006; Khurana et al., 2014; Burgess et al., 2014). Chromatin

100 compaction, due to the tethering of methyltransferase SUV3-9 or heterochromatin protein
101 HP1, can trigger ATM signaling and activate upstream (γ -H2A(X)) but not downstream
102 (53BP1) components of the DDR cascade, showing that chromatin compaction is an integral
103 step of the DDR (Burgess et al., 2014). Strikingly, a global alteration of chromatin and
104 chromosome structure, induced by hypotonic condition or mechanical stress, is sufficient to
105 activate ATM or ATR in the absence of any DNA damage, respectively, (Bakkenist and
106 Kastan, 2003; Kumar et al., 2014). Yeast lacks homologs of SUV3-9 or HP1, but
107 interestingly, Mec1^{ATR} might respond to chromatin dynamics during S phase, when
108 replication stress occurs and causes mechanical stress on the nuclear membrane (Forey et
109 al., 2020; Bermejo et al., 2011).

110

111 Global modification of damaged genomes is also evidenced through an increase of
112 chromosome mobility, a process that may favor repair in yeast (Miné-Hattab and Rothstein,
113 2012; Seeber et al., 2013; Strecker et al., 2016; Hauer et al., 2017; Herbert et al., 2017;
114 Lawrimore et al., 2017; Smith et al., 2018). The molecular mechanisms at play in global
115 mobility are not fully deciphered, but regulatory networks are becoming clearer (reviewed in
116 (Zimmer and Fabre, 2018; Smith and Rothstein, 2017; Seeber et al., 2018; Haber, 2018)).
117 The DDR activation by Mec1^{ATM} and Tel1^{ATR} is a critical first step in the response (Seeber et
118 al., 2013; Smith et al., 2018; Dion et al., 2012; Miné-Hattab and Rothstein, 2012). Mec1^{ATM}
119 and Tel1^{ATR} activation have diverse consequences, such as centromeric relaxation
120 (Strecker:2016jj; Lawrimore et al., 2017) and chromatin modifications (Herbert et al., 2017;
121 Hauer et al., 2017; Miné-Hattab et al., 2017). It has been proposed that the repulsive forces
122 of the negative charges due to H2A phosphorylation could render the chromosome stiffer
123 (Herbert et al., 2017), or that histone depletion could be the cause of a more expanded
124 chromatin (Hauer et al., 2017; Cheblal et al., 2020), both chromatin alterations resulting in
125 the observed increase of chromosome mobility. It is remarkable that increased chromosome
126 mobility, as a response to genomic insults, is a conserved feature in metazoan genomes,
127 with regulations involving repair proteins 53BP1 or γ -H2AX, like in yeast (Ryu et al., 2015;
128 Clouaire et al., 2018; Lottersberger et al., 2015; Dimitrova et al., 2008; Schrank et al., 2018).

129

130 To better understand how γ -H2A(X) affects different aspects of chromosome structure and
131 movement, we have created yeast strains in which both copies of the H2A encoding genes
132 have been mutated to S129E, which appears to be phosphomimetic (Eapen et al., 2012).
133 We find that H2A-S129E fully recapitulates the increased apparent stiffness of chromatin
134 observed after damage, resulting in increased intra-chromosomal distances and
135 chromosome dynamics. An increased repair by NHEJ and a decreased rate of translocation

136 correlate with this change in chromatin. Surprisingly, we find that H2A-S129E rescues $\Delta rad9$
137 survival deficiency after DNA damage, further supporting the notion that changes in
138 chromatin structure are a key contributor to DNA damage response.

139

140 **Results**

141 *In the absence of DNA damage, mimicking H2A phosphorylation increases chromosome*
142 *mobility.*

143 To study the consequences of mimicking H2A-S129 phosphorylation on chromosome
144 mobility, we created isogenic yeast strains in which both *HTA1* and *HTA2* were mutated to
145 H2A-S129E by CRISPR/Cas9-mediated gene editing (see Materials and Methods). We used
146 four strains (P1-P4) that carry fluorescently labeled green loci (LacI-GFP bound to an array
147 of LacO sites) at different regions of the right arm of chromosome IV (**Figure 1A**). As control,
148 we replaced S129 by an alanine to impede phosphorylation, using the same strategy (H2A-
149 S129A). We first compared the growth of the H2A-S129 mutants to their wild-type
150 counterpart, in the presence or in the absence of DNA damage generated by the radio-
151 mimetic drug Zeocin (Dion et al., 2012; Seeber et al., 2013; Herbert et al., 2017). In the
152 absence of Zeocin, growth rates of P1-P4 WT, H2A-S129E and H2A-S129A strains were
153 indistinguishable, suggesting no particular endogenous damage caused by these mutations.
154 WT strains exhibited a sensitivity to Zeocin (**Figure 1A, Supplementary Figure 1A**), as
155 previously shown (Seeber et al., 2013; Strecker et al., 2016; Krol et al., 2015). In the
156 presence of Zeocin, growth of H2A-S129E was similar to that of WT, whereas H2A-S129A
157 mutants showed increased sensitivity (**Figure 1A, Supplementary Figure 1A**). To precisely
158 measure the sensitivity to Zeocin of the mutant strains, we performed colony forming unit
159 (CFU) assays by calculating the ratio between colonies grown on media with and without
160 Zeocin. CFU ratios were close to 70% in P1-P4 WT cells (**Figure 1B**). Likewise, CFU ratios
161 in H2A-S129E mutant strains ranged between $81\% \pm 3.1$ and $96 \pm 3.2\%$, with no significant
162 difference from WT, except for the P4 strain, which showed a slightly higher survival (Downs
163 et al., 2000; Moore et al., 2007; Redon et al., 2003). In H2A-S129A strains, CFU ranged
164 from $47.5\% \pm 4.0$ to $64.5\% \pm 7.2$ confirming the enhanced sensitivity of these mutants to
165 Zeocin compared to WT, as previously shown (Downs et al., 2000; Moore et al., 2007;
166 Redon et al., 2003). The simplest interpretation for a similar survival to DNA damage
167 between H2A-S129E mutant and WT is that glutamic acid can replace the function normally
168 provided by S129 phosphorylation, a conclusion further supported by the analysis of the
169 H2A-S129A mutants.

170 We then explored chromosome mobility in H2A-S129E mutant cells by tracking the
171 fluorescently labeled green loci (Herbert et al., 2017). Green labels are located at 180 kb and
172 522 kb from the centromere IV or at 476kb and 180kb from the right telomere IV (Gr1, Gr3,
173 Gr2 and Gr4 respectively, **Figure 1A**). We analyzed Mean Square Displacements (MSDs) of
174 the four Gr1-Gr4 loci by tracking hundreds of cells in each P1-P4 strain, by high-speed
175 100ms time-lapse microscopy, for a time period of 5 minutes (Spichal et al., 2016; Herbert et
176 al., 2017). To induce DNA damage, we used a final concentration of Zeocin of 250 $\mu\text{g/ml}$ for
177 6h. Under these conditions, in which $\sim 80\%$ of the cell population showed damage, as seen
178 by Rad52-GFP foci formation (**Supplementary Figure 1**) (Herbert et al., 2017), WT cells
179 exhibited a global increase in chromosome mobility (**Figure 1C**). Strikingly, in the absence of
180 Zeocin treatment, the MSDs in the H2A-S129E mutant strains, were as high as those
181 observed in wild type strains after induction of damage (**Figure 1C** and **Supplementary**
182 **Figure 2A**). In these mutant cells, addition of Zeocin for 6h did not significantly increase
183 MSDs, suggesting that H2A-S129E mutation maximizes mobility that Zeocin cannot increase
184 any further (**Figure 1C** and **Supplementary Figure 2A**). After Zeocin exposure, the lower
185 increase in mobility of the four tagged Gr1-Gr4 loci in H2A-S129A mutated cells
186 (**Supplementary Figure 2B** and **2C**), confirmed that $\gamma\text{-H2A(X)}$ is required for full global
187 mobility upon DNA damage (Herbert et al., 2017). Together, these results suggest that the
188 function normally performed by H2A phosphorylation is mirrored by H2A-S129E and that this
189 mutation is sufficient to induce an increase in chromosomal dynamics.

190
191 *H2A-S129E mutant does not trigger cell cycle arrest, histone loss or centromere*
192 *detachment.*

193 To understand the mechanism underlying the enhanced dynamics observed in the H2A-
194 S129E mutant, we explored different hypotheses. First, it is well documented that checkpoint
195 activation induces global mobility upon DNA damage (Seeber et al., 2013; Hauer et al.,
196 2017; Smith et al., 2018). To test whether H2A-S129E may induce mobility due to the
197 physiological DDR, we explored the effect of this mutant on cell cycle progression. FACS
198 results on asynchronous populations indicated an equally low number (19-23%) of cells in
199 the G2/M cell cycle phase in the WT and H2A-S129E mutant strains, indicating that the DDR
200 checkpoint leading to cell cycle arrest is not activated in H2A-S129E (**Figure 2A**). In
201 addition, phosphorylation of Rad53, the signal for effective checkpoint (Pelliccioli et al., 2001),
202 was not observed in both strains in the absence of damage (**Figure 2A**). However, both WT
203 and H2A-S129E mutant strains exhibited a similar DNA damage response after 6h Zeocin
204 treatment, since the percentage of G2/M arrested cells was enriched (71-77%) in both

205 strains, with an accompanying hyper-phosphorylation of Rad53 (**Figure 2A, Supplementary**
206 **Figure 3**). These results reject the hypothesis that enhanced chromatin dynamics observed
207 in H2A-S129E mutants in the absence of damage is triggered by constitutive activation of
208 the cell cycle checkpoint. Second, histone loss was shown to elicit enhanced chromatin
209 mobility (Hauer et al., 2017). We therefore examined the global stability of histones. By
210 immuno-blotting, we found comparable levels of total H4 in WT and H2A-S129E mutant cells
211 (**Figure 2B**). Total H4 abundance strongly decreased upon 6h of Zeocin treatment, as
212 previously reported (**Figure 2B**, (Hauer et al., 2017)). Finally a previous study had shown
213 that induction of a single DSB in the genome led to kinetochore protein Cep3
214 phosphorylation by Mec1 and also correlated with an increase in global mobility associated
215 with an increase in Spindle Pole Body (SPB) - Centromere (CEN) 2D distances (Strecker et
216 al., 2016). We therefore measured the distances between SPC42, a protein of the SPB
217 fused to mCherry and CenIV labeled by an array of TetO repeats bound by tetR-GFP (He et
218 al., 2000). In the absence of Zeocin, the SPB-CenIV distances between WT or H2A-S129E
219 strains were identical and in both cases the distances increased after cells were treated by
220 Zeocin (**Figure 2C**), indicating that a general loosening of the anchoring of the centromeres
221 to the SPB is probably not the cause of the increase in chromosomal mobility observed in
222 H2A-S129E mutants. Thus, the high intrinsic mobility of H2A-S129E mutated chromatin must
223 be due to factors other than checkpoint activation, histone degradation or loosening of
224 centromere tethering to the spindle pole body.

225

226 *Mimicking H2A-S129 phosphorylation increases intra-chromosomal distances without DNA*
227 *damage.*

228 It has been established that an increase in genomic mobility could be explained by a change
229 in chromatin structure (Hauer et al., 2017; Herbert et al., 2017; Miné-Hattab et al., 2017).
230 Based on polymer modeling and multi-scale tracking of chromatin after damage, a global
231 stiffening of the chromatin fiber is consistent with a simultaneous increase in chromosomal
232 mobility and spatial distances between loci on the same chromosome (Herbert et al., 2017;
233 Miné-Hattab et al., 2017). H2A phosphorylation was proposed as a potential molecular
234 mechanism, since the negative repulsive charges due to H2A phosphorylation could
235 increase the stiffness of the chromatin fiber, as seen *in vitro* and by modeling (Cui and
236 Bustamante, 2000; Qian et al., 2013; Herbert et al., 2017). In addition, based on similar
237 experiments, but using different modeling assumptions, chromatin decompaction has also
238 been proposed to play a role (Hauer et al., 2017; Amitai et al., 2017). We therefore
239 hypothesized that the increase in H2A-S129E chromosome motion could be linked to a

240 change in chromosomal structure. To address this question, we measured red-green
241 pairwise distances in P1-P4 WT and H2A-S129E mutated strains (**Figure 2D**). In these
242 strains, TetO arrays bound by tetR-mRFP are inserted at about 200 kb from the green labels
243 (see **Figure 1A**). As expected, intra-chromosomal distances increased in WT strains after
244 prolonged treatment to Zeocin (3h), ranging from (~ 350 - 414nm to ~ 384 - 628nm in the
245 absence and presence of Zeocin respectively, (**Figure 2D**, (Herbert et al., 2017))). In the
246 absence of Zeocin treatment, large increases of intra-chromosomal distances (from ~ 396 -
247 496 nm) were observed all along the chromosome arm in H2A-S129E mutants, similar to
248 those observed in WT upon Zeocin treatment (**Figure 2D**). In contrast, intra-chromosomal
249 distances do not increase in H2A-S129A mutants (Herbert et al., 2017), showing that the
250 effect on distances was specific to H2A-S129E mutation. A structural change in chromatin is
251 accordingly the most probable cause for the increased mobility observed in the H2A-S129E
252 mutant, in the absence of any DNA damage.

253

254 *Rad9 checkpoint control on cell survival and global mobility upon Zeocin treatment is*
255 *suppressed by mimicking H2A phosphorylation.*

256 The observation that H2A-S129E causes an increase in chromosome dynamics independent
257 of cell cycle arrest suggests that structural changes in the chromatin of this mutant may
258 make checkpoint factors dispensable for damaged-induced chromatin mobility (Seeber et al.,
259 2013; Smith et al., 2018; Dion et al., 2012; Miné-Hattab and Rothstein, 2012; Bonilla et al.,
260 2008). To test this hypothesis, we mutated the key checkpoint adaptor Rad9^{53BP1} protein in
261 the DDR that mediates the interaction between modified histones such as γ -H2A(X) and
262 several effector proteins in the DDR (Finn et al., 2011). We deleted *RAD9* in the WT and
263 H2A-S129E P2 strains and analyzed single ($\Delta rad9$) and double ($\Delta rad9$ H2A-S129E) mutant
264 phenotypes on cell survival, cell cycle profile and chromosome mobility. In the absence of
265 Zeocin treatment, both mutants grew similarly to their wild type *RAD9* counterpart in spot
266 assays (**Figure 3A**). As expected, the growth of the $\Delta rad9$ mutant was affected in the
267 presence of Zeocin (Seeber et al., 2013). Interestingly, spot assay and CFU ratio
268 quantification of treated versus non-treated cells, indicated that the $\Delta rad9$ H2A-S129E
269 double mutant was indistinguishable from WT or H2A-S129E single mutant strains (**Figure**
270 **3A, 3B**). Moreover, while cell cycle arrest due to Zeocin was deficient in $\Delta rad9$ mutant, as
271 predicted, it was restored in $\Delta rad9$ H2A-S129E double mutant (after Zeocin treatment,
272 $p=0.49$ between WT and $\Delta rad9$ H2A-S129E double mutant and $p=4.4 \cdot 10^{-14}$ between WT and
273 $\Delta rad9$, as determined by a contingency chi-square test (**Figures 2A, 3C**). Surprisingly,
274 Rad53 phosphorylation was not restored in the $\Delta rad9$, H2A-S129E double mutant (**Figures**

275 **3C**). Thus, the failure of the $\Delta rad9$ mutant to survive and activate cell cycle arrest in
276 response to Zeocin, is rescued by H2A-S129E mutation independently of Rad53
277 phosphorylation. These results suggest that an early DDR response – the phosphorylation
278 of histone H2A – enables cells to delay cell division in the absence of Rad9 and thus provide
279 more time for DNA repair (see Discussion).

280 We then measured global chromatin mobility in these mutants in the absence and the
281 presence of Zeocin (**Figure 3D**). Whereas the $\Delta rad9$ mutant behaved similarly to WT in
282 undamaged conditions, the absence of *RAD9* partially impeded the enhancement of global
283 chromatin mobility observed in WT strains upon DNA damage, in agreement with (Seeber et
284 al., 2013). Interestingly, in both undamaged and damaged conditions, the $\Delta rad9$ H2A-S129E
285 double mutant induced a massive increase of global dynamics, comparable to the single
286 H2A-S129E mutant (**Figure 3D, Supplementary Figure 4**). To check whether changes in
287 chromosome dynamics also translate into chromatin structural changes in the $\Delta rad9$ H2A-
288 S129E double mutant, we also measured intra-chromosomal 2D distances (**Figure 3E**). We
289 confirmed that in the absence of Zeocin treatment, the distances in $\Delta rad9$ mutant were not
290 significantly different to those of the wild-type strain and that the distances in the $\Delta rad9$ H2A-
291 S129E double mutant were comparable to the single H2A-S129E mutant. As expected from
292 the dynamic behavior, in the presence of Zeocin, $\Delta rad9$ showed a modest increase in intra-
293 chromosomal distances as compared to the WT but increase as much as H2A-S129E in the
294 double $\Delta rad9$ H2A-S129E mutant (**Figure 3E**). Thus, the structural changes induced by the
295 H2A-S129E mutant in the $\Delta rad9$ background corroborate the observations on the
296 chromosomal dynamics. Our results highlight the capacity of H2A-S129E mutant to suppress
297 the specific contribution of Rad9 to cell survival, DNA damage checkpoint and global
298 chromosome dynamics when genome is damaged, possibly through chromatin structure
299 modification.

300

301 *Mimicking H2A-S129 phosphorylation increases NHEJ and reduces translocation rates of*
302 *$\Delta rad9$.*

303 Rad9 and its mammalian ortholog 53BP1, are known to facilitate NHEJ (Ferrari et al., 2015;
304 Zimmermann et al., 2013). Since the Zeocin sensitivity of $\Delta rad9$ is suppressed by the H2A-
305 S129E mutant that increases chromosome mobility, and since chromosome mobility could be
306 a means for promoting the joining of double-stranded extremities by NHEJ (Lottersberger et
307 al., 2015; Dimitrova et al., 2008), we investigated the effect of both $\Delta rad9$ and H2AS129E
308 mutants on the NHEJ repair pathway. We used a plasmid repair assay, in which repair of a
309 linear plasmid can only be mediated by NHEJ. We generated linear plasmids with cohesive

310 ends, by cutting a *HIS3*-containing centromeric plasmid by *EcoRI* enzyme. In this assay,
311 only circularized plasmids confer histidine prototrophy to the transformed cells. A $\Delta yku70$
312 mutant, which is impaired in NHEJ, was used as control for plasmid linearization. After
313 transformation by the digested plasmids, His⁺ colonies were obtained in the $\Delta yku70$ mutant
314 10-fold less than in WT cells. These data indicate that most transforming molecules were
315 efficiently re-circularized in WT (**Figure 4A**). The number of His⁺ colonies recovered in the
316 H2A-S129A mutant was almost as low as in $\Delta yku70$ mutant, revealing that plasmid
317 circularization by NHEJ is compromised in the absence of γ -H2A(X). This result is consistent
318 with previous studies showing that truncated forms of H2A lacking S129 are deficient for
319 NHEJ and is consistent with an increased resection rate observed in H2A-S129A mutant
320 (Downs et al., 2000; Moore et al., 2007; Eapen et al., 2012). In contrast, the number of His⁺
321 colonies was ~ 1.3 times higher in the H2A-S129E mutant than in WT, indicating that
322 mimicking H2A phosphorylation allowed more effective repair by NHEJ than in WT (**Figure**
323 **4B**). In the $\Delta rad9$ mutant, the number of His⁺ cells was 4 to 8 times lower than in the WT
324 (**Figure 4B**), an expected result given the role of Rad9 in limiting 5' DNA end resection
325 (Ferrari et al., 2015). Remarkably, the number of His⁺ colonies was similar to WT in the
326 $\Delta rad9$, H2A-S129E double mutant. Thus the restoration of cell survival to DNA damage
327 observed in $\Delta rad9$ H2A-S129E double mutant could be linked to an increase in NHEJ
328 efficiency. In turn, increased chromosome motion observed in H2A-S129E could promote
329 NHEJ, as it was observed for dysfunctional telomeres in mammalian cells (Lottersberger et
330 al., 2015; Dimitrova et al., 2008).

331 We next explored whether large-scale chromatin mobility could also promote the joining of
332 DSBs that are at long range distance, leading to translocation mis-repair, as observed in
333 mammals (Lottersberger et al., 2015). To test for translocation rates, we used a genetic
334 system in which recovery of Ura⁺ *MAT α* colonies is mediated by a reciprocal translocation
335 between two DSBs generated in truncated *URA3* on two distinct chromosomes,
336 chromosomes III and V (Lee et al., 2008) (**Figure 4C**). DSBs were induced by expressing
337 the HO endonuclease from the galactose inducible *GAL1-10* promoter. We first checked by
338 qPCR that the cutting efficiency was similar after 1h of galactose induction at the two HO
339 cutting sites in WT and H2A-S129E mutant cells (**Supplementary Figure 5**). Then,
340 translocation rates were measured after permanent induction of HO on galactose-containing
341 plates. The number of Ura⁺ colonies growing on GAL-URA plates was divided by the total
342 number of cells grown in non-selective YPGAL plates for each strain. While phospho-
343 deficient H2A-S129A mutant increased translocation rates ~ 3 -fold compared to WT (13.5%
344 ± 2.6 and 42.4% ± 4.8 for WT and H2A-S129A, respectively), as previously documented

345 (Figure 4D and (Lee et al., 2008)), H2A-S129E decreased translocation rates by
346 approximately 13-fold ($1.1\% \pm 0.6\%$) (Figure 4D). As expected, the efficiency of
347 translocations in the mutant $\Delta rad9$ is comparable to that of WT (Figure 4D and (Lee et al.,
348 2008)) and significantly reduced in the double mutant $\Delta rad9$ H2A-S129E (Figure 4D).

349 Altogether, our results indicate that H2A phosphorylation mimicry induces an increase in
350 chromosomal dynamics favorable to a local NHEJ repair concomitant with a sharp decrease
351 in the inter-chromosomal translocation rate.

352

353 Discussion

354 Here we show that in the absence of DNA damage induction, the H2A-S129E mutation fully
355 recapitulates global chromosome/chromatin dynamics previously observed upon Zeocin
356 treatment (Hauer et al., 2017; Herbert et al., 2017). The specificity of this H2A-S129E mutant
357 is to mimic H2A phosphorylation by the criterion of being recognized by a γ -H2AX-specific
358 antibody (Eapen et al., 2012). One intriguing consequence of H2A-S129E mutation is to
359 allow bypassing an essential function of the DNA damage checkpoint protein, $RAD9^{53BP1}$,
360 when Zeocin is present. Locus tracking in living cells reveals that H2A-S129E mutant
361 restores the high chromatin mobility induced by DNA damage, which is defective in $\Delta rad9$.
362 Enhanced global dynamics was not related to tethering of the centromeres, histone depletion
363 or checkpoint activation, these mechanisms being proposed to explain the increase in
364 overall chromosome mobility observed when the genome is damaged (Strecker et al., 2016;
365 Smith et al., 2018; Lawrimore et al., 2017; Seeber et al., 2013). Rather, we found that the
366 negative charges of glutamic acid (but not the uncharged alanine) enhanced global
367 dynamics together with increased intra-chromosomal distances. These two characteristics
368 indicate a structural modification of the chromatin compatible with its stiffening (Herbert et al.,
369 2017). If transcription of some key regulators involved in the DDR would be modified in H2A-
370 S129E mutated cells, a similar phenotype could be expected. However, no such changes in
371 transcription could be detected by RNAseq analyses of H2A-S129E mutant compared to WT
372 (S. Bohn, B. Lemos, J.E. Haber and N. Krogan, unpublished result). The fact that $\Delta rad9$
373 DNA damage sensitivity and checkpoint defects are rescued by H2A-S129E suggests that
374 H2A-S129E may have a positive effect on repair. We tested this proposal by examining the
375 repair by NHEJ of a linearized plasmid and found that it was favored in H2A-S129E cells.
376 These observations are consistent with the idea that chromatin mobility of DNA extremities
377 may favor their rejoining as proposed in mammalian cells (Lottersberger et al., 2015).
378 However, the direct measurement of DSB translocations revealed that the level of joining in

379 *trans* was severely reduced in H2A-S129E. We propose that changes in chromosomal
380 conformation due to H2A phosphorylation are a means to efficiently modulate the DDR.
381

382 **A model for the regulation of DDR by changes in chromatin structure**

383 Chromatin structure is one of the key factors in the DDR since it is the first in being altered
384 both by the damage itself that generates DSBs and by the accumulation of histone
385 modifications and repair proteins at the damaged site. DSB might alter topological
386 constraints on the chromatin fiber, although little is known about these constraints
387 associated with higher order chromosome organization (Canela et al., 2017). Histone post-
388 translational modifications on the other hand, such as phosphorylation of H2A, which can
389 extend from the DSB over areas as large as 50-kb on either side of the DSB (Shroff et al.,
390 2004; Lee et al., 2014), also change the chromatin structure, the repulsive negative charges
391 due to phosphorylation being compatible with a stiffer chromatin (Reina-San-Martin et al.,
392 2003; Celeste et al., 2003; Lee et al., 2008; Miné-Hattab et al., 2017; Herbert et al., 2017).
393 Normally, γ -H2AX modifications occur around the site of a DSB, which exhibits enhanced
394 local motion, while inducing global mobility changes in undamaged regions. Here we see
395 that H2A-S129E-containing chromatin in undamaged regions display enhanced mobility.
396 Whether this represents global motion or is a manifestation of a local motion, we yet cannot
397 determine.

398
399 In mammals, regulation of ATM response by chromatin structure was also proposed to
400 contribute to the extremely rapid and sensitive response to damage, across the nucleus, as
401 shown by ATM activation in the absence of detectable damage, during hypotonic shock or
402 during histone deacetylation (Bakkenist and Kastan, 2003; Kumar et al., 2014). The changes
403 in chromatin mobility that we report here, where no damage was induced, were
404 systematically accompanied by an increase in distances between the arrays along the
405 chromosome, a parameter which can be explained by a change in chromatin structure
406 (Hauer et al., 2017; Amitai et al., 2017; Herbert et al., 2017). The higher order chromatin
407 structure modification due to H2A-S19E was previously proposed to correspond to a
408 decreased compaction (Downs et al., 2000), in agreement with a more relaxed profile of 2 μ
409 plasmid from H2A-S12E containing cells and a faster digestion of the H2A-S129E chromatin
410 by MNase (Downs et al., 2000). The fact that in the H2A-S129E mutant, the protection
411 pattern or the length of nucleosomal repeats was conserved (Downs et al., 2000), is also
412 compatible with a stiffer chromatin, but the number of nucleosomes per length unit (i.e.
413 compaction) remains to be determined. Similarly, treatment with Zeocin has been shown to
414 cause nucleosome loss, as documented by histones H3 and H4 degradation (Hauer et al.,

415 2017). Histone degradation could explain the additional modest effect on mobility and intra-
416 chromosomal distances observed in H2A-S129E mutant when treated with Zeocin since the
417 global level of H4 was not affected in undamaged H2A-S19E cells, but substantial
418 degradation was observed in both, WT and H2A-S19E cells in the presence of Zeocin. Thus
419 chromatin stiffening due to H2A phosphorylation and chromatin de-compaction due to
420 histone loss could exist together in Zeocin treated cells. Only close examination of the
421 chromatin structure by super resolution will help to determine the nature of these chromatin
422 changes.

423 Our live-cell chromosome dynamics observations combined with the genetic evidence
424 presented here suggest a novel mode of DDR regulation via an extremely efficient
425 modification of the chromosome structure. The activation of DDR is evident after DNA
426 damages, which restores cell cycle arrest in the double mutant $\Delta rad9$, H2A-S129E. Which
427 effectors could be responsible for the reactivation of the checkpoint? It was tempting to test
428 whether the phosphorylation of Rad53 was restored under these conditions. The lack of
429 detectable phosphorylated form of Rad53 indicates that downstream checkpoint activation
430 occurs through other effectors that have yet to be identified. Another hypothesis is linked to
431 the Mad2-mediated Spindle Assembly Checkpoint (SAC) that we previously show to sustain
432 arrest (Dotiwala et al., 2010). Activation of this extended G2/M arrest is dependent on
433 formation of γ -H2AX (Dotiwala et al., 2010). A possible explanation to the bypass of $\Delta rad9$
434 by H2A-S129E could be an extended arrest through the Mad2 SAC. Regardless the nature
435 of the effectors activated by H2A-S129E, our results indicate that H2A post-translational
436 modification of the chromatin that can be achieved in the absence of any protein synthesis,
437 results in a very proficient DDR, probably faster than any transcriptional regulation.

438

439 **What is the function of H2A-S129E?**

440 In the presence of H2A-S129E, Rad9 is no longer essential for arresting cells in G2/M after
441 DNA damage or for facilitating damage-associated chromosome mobility. Based on the
442 evidence presented here, we propose that chromosome mobility induced by chromatin
443 structural changes, is a manner to efficiently signal damage and help repair. The chromatin
444 changes and the enhanced dynamics may favor accessibility of the NHEJ repair machinery
445 to the extremities of the break. In agreement with this, Tel1^{ATM} that phosphorylates H2A-
446 S129, facilitates NHEJ repair protein recruitment and prevents dissociation of broken DNA
447 ends (Lee et al., 2008). Consequently, inappropriate rejoining of chromatin fragments that
448 can result in genetic translocations is decreased, as we and others have observed both in
449 yeast and mammals (Reina-San-Martin et al., 2003; Celeste et al., 2003; Lee et al., 2008).
450 Besides, DSB ends resection is thought to regulate the γ -H2A(X)-mediated recruitment of

451 remodeling complexes, SWR1, Fun30 and INO80, to promote repair by respectively NHEJ
452 and HR (van Attikum et al., 2007; Horigome et al., 2014; Eapen et al., 2012). Notably, the
453 interaction between Fun30 and H2A-S129E chromatin is impaired (Eapen et al., 2012),
454 raising the possibility that resection factors in H2A-S129E mutant would also be impaired,
455 thus favoring NHEJ. The role of chromosome mobility in repair by NHEJ is consistent with
456 the observation that increased roaming of deficient telomeres facilitates NHEJ in mammals
457 (Lottersberger et al., 2015; Dimitrova et al., 2008). Concerning translocations, i.e. the
458 junction between two DSBs on distinct chromosomes, two theories are proposed. On the
459 one hand, the limited motion of DSB ends would have a greater probability of forming a
460 translocation provided they are spatially close, on the other hand, mobile ends would favor
461 the connection between ends which have lost their proper interaction (Roukos et al., 2013;
462 Lottersberger et al., 2015). Thus a NHEJ made efficient thanks to the mobility of DSB
463 extremities would counterbalance an ectopic repair between two distant breaks. Our results
464 favor this last hypothesis. Mobilizing DSBs to promote faithful repair may be preserved by
465 evolution. The reason why such H2A-S129E mutation has not been positively selected may
466 be linked to the fact that it would be useful to keep such additional level of regulation,
467 through increased mobility, otherwise impossible to reach in the case of “permanent”
468 increased mobility.

469
470 Finally, the evolutionary conservation of chromosome mobility nurtures the possibility that
471 similar mechanisms through chromatin modification exist in other organisms. In both yeast
472 and mammals, the initial recognition of DSBs and activation of the checkpoint kinases is not
473 dependent on the formation of γ -H2A(X) (Celeste et al., 2003; Dotiwala et al.; Kruhlak et al.,
474 2006), but this modification is important in maintaining damage arrest and in the recruitment
475 of DSB repair factors. In mammalian cells, the response to DNA damage is complex and
476 includes modification due to damage include protein modifications that do not exist in yeast,
477 such as the poly ADP-ribosylation by poly (ADP-ribosyl) polymerases (PARPs). PARP also
478 induces chromatin mobility (Sellou et al., 2016). It would be interesting to test if chromatin
479 mobility induced by parylation in mammalian cell lines helps to preserve chromatin integrity.

480

481

482

483 **Legends to the figures**

484 **Figure 1. H2A-S129E increases global dynamics in absence of DNA damage.**

485 **A.** Left, schematics of chromosome IV, indicating the genomic positions of fluorescently
486 labeled loci investigated in this study. Numbers above the black line indicate genomic
487 distance from the centromere in kb. Blue dashed bars indicate the centromere (CEN IV) and
488 the right telomere (TEL IVR). Red and green bars indicate the four loci tagged in red (R1–
489 R4) or in green (Gr1-Gr4). Strains P1 to P4 correspond to individual red-green pairs,
490 separated by ~200kb (R1-Gr1, R2-Gr2, Gr3-R3, Gr4-R4, respectively). Right, drop assays
491 (tenfold dilutions) showing comparable growth of wild-type (WT) and H2A-S129E mutants in
492 the absence of damage and comparable sensitivity to long exposition to Zeocin (12, 5µg/ml).
493 As a control, effect of the Zeocin on cell survival is shown for H2A-S129A mutants. **B.**
494 Colony forming units (CFU) calculated as the ratio of cells growing in the presence Zeocin
495 over cells grown in the absence of Zeocin after spreading of ~ 200 colonies on each
496 medium, for the four strains tested (P1-P4) in wild-type (WT, black bars), H2A-S129E
497 mutants (grey bars) and H2A-S129A mutants (light grey bars). Error bars represent the
498 standard deviation of the mean CFU counted of at least three independent experiments; P
499 values are calculated after a non- parametric t test (n.s, not significant $P>0.05$, * $P\leq 0.05$, **
500 $P\leq 0.01$, *** $P\leq 0.0001$). **C.** Mean square displacements (MSDs) of Wild-Type cells and H2A-
501 S129E mutant as function of time interval of the four Gr1 to Gr4 green loci in P1 to P4
502 strains, as computed from 2D time-lapse microscopy data (cell population average). Blue
503 and red curves are for untreated and Zeocin-treated wild-type cells (WT) respectively; cyan
504 and magenta curves are for untreated and treated H2A-S129E mutant, respectively. The
505 numbers of cells used to compute each curve (n) are indicated.

506 **Figure 2. Increase in global dynamics in H2A-S129 mutated cells is not linked to**
507 **checkpoint activation, histone loss or defective centromeric tethering but to increased**
508 **intra-chromosomal distances.**

509 **A.** Flow cytometry analyses of asynchronous WT and H2A-S129E mutant cell populations
510 treated or not by Zeocin. H2A-S129E mutants show normal cell cycle in the absence of
511 damage and a G2/M arrest in the presence of damage, similarly to the WT. Statistical
512 analyses between WT and H2A-S129E mutated cells with a chi square-test shows not
513 significant values of 0.32 and 0.15 for untreated and treated conditions,
514 respectively. Representative results from immunoblotting showing the phosphorylation status
515 of Rad53–HA in WT and H2A-S129E cells in response or not to Zeocin treatment. Actin was
516 used as a loading control. **B.** Immunoblot analyses and quantification with H4 specific
517 antibodies on whole cell extracts in WT and H2A-S129E mutant before and after Zeocin
518 treatment. Dpm1 was used as a loading control. Immunoblot quantification with ImageJ
519 (mean and s.d.). **C.** Cumulative distributive function (CDF) from SPB-CenIV 2D distances of

520 WT and H2A-S129E mutant cell populations treated or not with Zeocin. **D.** Bar plots show
521 2D distances of untreated WT (blue) and H2A-S129E (cyan) and Zeocin-treated (red and
522 magenta, respectively), for the four pairs of green-red labeled loci in P1-P4 strains. The
523 horizontal line at the center of each box indicates the median value; the bottom and top limits
524 indicate the lower and upper quartiles, respectively. The whiskers indicate the full range of
525 measured values, except for outliers, which are shown as small red dots. Brackets indicate
526 the result of a Wilcoxon rank-sum test between distributions with “n.s.” for “not significant” for
527 $P > 0.05$, * for $P \leq 0.05$, ** for $P \leq 10^{-2}$ and *** for $P \leq 10^{-3}$. Number of analyzed (n) cells is
528 indicated.

529

530 **Figure 3. H2A-S129E mutant suppresses Rad9 checkpoint control on cell survival,**
531 **global dynamics and intra-chromosomal distances upon Zeocin treatment.**

532 **A.** Drop assay (tenfold dilutions) of Wild-Type (WT), $\Delta rad9$ and $\Delta rad9$ H2A-S129E double
533 mutant in the absence and the presence of 12,5 μ g/ml of Zeocin for the P3 strain. The single
534 H2A-S129E mutant is also shown. **B.** Colony forming units (CFU) calculated as the ratio of
535 cells growing in the presence Zeocin over cells grown in the absence of Zeocin after
536 spreading of ~ 200 colonies on each medium in wild-type (WT, black bar), H2A-S129E
537 mutant (dark grey bar), $\Delta rad9$ (light grey bar) and $\Delta rad9$ H2A-S129E double mutant (grey
538 bar). Error bars represent the standard deviation of the mean CFU counted of at least three
539 independent experiments; P values are calculated after a non- parametric t test (n.s, not-
540 significant $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.0001$). **C.** FACS analyses in the same
541 strains in the absence or the presence of 250 μ g/ml of Zeocin. Representative results from
542 immunoblotting showing the phosphorylation status of Rad53–HA in WT, $\Delta rad9$ and $\Delta rad9$,
543 H2A-S129E cells in response or not to Zeocin treatment. Actin was used as a loading
544 control. **D.** Mean square displacements (MSDs) calculated as in figure 1, WT compared to
545 $\Delta rad9$ on the top; H2A-S129E compared to $\Delta rad9$ H2A-S129E double mutant on the bottom.
546 Non-treated cells are blue, dotted blue, cyan and dotted cyan and treated cells are red,
547 dotted red, magenta and dotted magenta, respectively. **E** Box plots show 2D distances of
548 untreated and Zeocin-treated cells (same color code as in **D**) of WT compared to $\Delta rad9$ on
549 the left and H2A-S129E compared to the double mutant $\Delta rad9$ H2A-S129E on the right.
550 Analyzed cells range from (n) ~150 - ~1200. P values are calculated after a non- parametric
551 t test (n.s, not significant $P > 0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.0001$).

552

553 **Figure 4. Mimicking H2A S129 phosphorylation increases NHEJ and decreases**
554 **translocation rates.**

555 **A.** Schematic of the NHEJ assay principle. A replicative plasmid carrying HIS3 as an
556 auxotrophic marker (pRS413) is linearized by *EcoRI* enzyme and used for yeast
557 transformation. In strains efficient for NHEJ, plasmid extremities are joined and His⁺
558 colonies are recovered on plates lacking histidine (-HIS). **B.** Rejoining efficiency is calculated
559 as the ratio of His⁺ colonies recovered after transformation of a linearized plasmid relative to
560 introduction of a non-linearized plasmid. A $\Delta yku70$ mutant is impaired in NHEJ and is used
561 as a control for plasmid digestion efficiency. Bar graphs show mean \pm s.e.m. P values are
562 calculated after a non-parametric t test (n.s, not significant $P>0.05$, * $P\leq 0.05$, ** $P\leq 0.01$, ***
563 $P\leq 0.0001$). **C.** Schematic of the strain used to measure translocation. Two DSBs generated
564 by HO endonuclease (HOCs), an intronic sequence and truncated forms of *URA3* are
565 depicted on two distinct chromosomes. Reciprocal end-joining produces Ura⁺ cells. **D.**
566 Translocation efficiency is calculated as the ratio of Ura⁺ colonies recovered after DSB
567 persistent induction (-URA plates containing Galactose) relative to non-translocated
568 conditions (SC plates containing Galactose). P values are calculated after a non-parametric t
569 test (n.s, not significant $P>0.05$, * $P\leq 0.05$, ** $P\leq 0.01$, *** $P\leq 0.0001$).

570

571 **Supplementary Figure 1.**

572 **Absence of growth defects and intrinsic DNA damages in H2A-S129E mutant.**

573 **A.** Representative growth curves for the WT, H2A-S129A, H2AS129E strains in the absence
574 (left) or presence (right) of 250 μ g/ml Zeocin treatment. Mean values for two independent
575 experiments are plotted for each time point, with error bars showing standard error of the
576 mean (SE).

577 **B.** No intrinsic DNA damage in H2A-S129E in absence of Zeocin treatment but prolonged
578 exposure to Zeocin increases DNA damage. Rad52-GFP foci are shown (arrowheads) in
579 representative images of yeast cells that were either untreated or exposed to 250 μ g/ml of
580 the genotoxic drug Zeocin for 4 and 6 h in WT (black) and H2A-S129E strain (grey). Bar
581 graphs show mean \pm s.e.m. P values are calculated after a non-parametric t test (n.s, not
582 significant $P>0.05$).

583

584

585 **Supplementary Figure 2.**

586 **Values of MSDs at 10 sec. in WT, H2A-S129E and H2A-S19E mutated strains**

587 **A.** Values of MSD at 10 sec for WT, H2A-S129E and H2A-S129A. Boxplots show the
588 distribution of MSD at 10 s in absence of Zeocin (WT, blue; H2A-S129E, light blue) or after 3
589 h Zeocin exposure (WT, red; H2A-S129E, pink), for the four loci Gr1–Gr4. The horizontal line
590 at the center of each box indicates the median value, the bottom and top limits indicate the
591 lower and upper quartiles, respectively. The whiskers indicate the full range of measured
592 values, except for outliers, which are shown as small red dots. Brackets indicate the result of
593 a Wilcoxon rank-sum test between distributions, with “n.s.” for “not significant” ($P > 0.05$), *
594 for $P < 0.05$, ** for $P < 10^{-2}$ and *** for $P < 10^{-3}$.

595 **B.** MSDs for WT and H2A-S129A. Mean square displacement measured as in figure 1D as
596 function of time interval of the four Gr1 to Gr4 green loci in H2A-S129A background. Blue
597 and red curves are for untreated and treated Wild-Type cells (WT); grey and pink curves are
598 for untreated and treated H2A-S129A mutant, respectively. The numbers of cells used to
599 compute each curve (n) are indicated.

600 **C.** Values of MSD at 10 sec for H2A-S129A in the presence or absence of treatment.
601 Boxplots show the distribution of MSD at 10 s as in B, in absence of Zeocin (blue) or after 3
602 h Zeocin exposure (red), for the four loci Gr1–Gr4 in H2A-S129A mutated background.

603

604 **Supplementary Figure 3.**

605 **Percent of G2/M arrested cells in the absence or the presence of Zeocin for WT, H2A-**
606 **S129E, $\Delta rad9$ and $\Delta rad9$ H2A-S129E mutated cells.**

607 Cells expressing fluorescent LacI-GFP protein to lacO-array were inspected after image
608 acquisition. Unbound LacI-GFP was used as a nuclear staining. G2/M arrested cells show
609 nuclear masses separated between mother and daughter cells, but no septum formed.
610 Examples are shown on the right.

611

612 **Supplementary Figure 4.**

613 **Values of MSDs at 10 sec for WT, H2A-S129E, $\Delta rad9$ and $\Delta rad9$ H2A-S129E mutated**
614 **cells.**

615 Boxplots show the distribution of MSD at 10 s of WT compared to $\Delta rad9$ strains (left) and
616 H2A-S129E compared to $\Delta rad9$ H2A-S129E double mutant (right) in absence of Zeocin
617 (bleu and cyan, respectively) or after 6 h Zeocin exposure (red and magenta, respectively).
618 The horizontal line at the center of each box indicates the median value, the bottom and top
619 limits indicate the lower and upper quartiles, respectively. The whiskers indicate the full
620 range of measured values, except for outliers, which are shown as small red dots. Brackets

621 indicate the result of a Wilcoxon rank-sum test between distributions, with “n.s.” for “not
622 significant” ($P > 0.05$), * for $P < 0.05$, ** for $P < 10^{-2}$ and *** for $P < 10^{-3}$. Analyzed cells range
623 from (n) ~100 - ~1000.

624

625 **Supplementary Figure 5**

626 **Efficiency of the two HO-cleavages in translocation assay strains by Q-PCR.**

627 Efficiency of HO cleavage in WT, H2A-S129A, H2A-S129E, $\Delta rad9$ and $\Delta rad9$, H2A-S129E
628 strains carrying the two HO cleavage sites at the $MAT\alpha$ and $URA3$ locus was determined by
629 quantitative PCR using primers flanking HO recognition sites before (t0h) and after (t1h)
630 galactose induction, normalized by the amount of $ACT1$ sequence.

631 **Supplementary Table 1. Strains used in this study.**

632

633 **Materials and Methods**

634 *Strains construction*

635 All strains, plasmids and primers used in this study are listed in Table 1. Fluorescent labeled
636 strains used in this study were constructed by insertion of Tet-Operator and Lac-Operator
637 arrays inserted at specific locations on the chromosome IV (Robinett et al., 1996). These
638 arrays are bound by Tet-Repressor and Lac-Inducer, which are fused to the fluorescent
639 proteins mRFP and eGFP, respectively.

640 yH2A129A/ yH2AS129E mutants were constructed using CRISPR/Cas9 technology as
641 described by (Anand et al., 2017). Briefly, a 20 nucleotide sequence, corresponding to the
642 sgRNA, flanked by a NGG sequence (Cas9 PAM) was selected from the sequence of both
643 H2A genes ($HTA1/HTA2$) containing the serine 129. A complementary sequence is designed
644 to form a duplex with *BpI* overhangs and cloned into *BpI*-digested (pEF562) Yeast Cas9
645 plasmid (1:1). To introduce the modifications into the yeast genome, a single-stranded 80 nt
646 donor sequence was designed, consisting of 40 nucleotides upstream and 40 nucleotides
647 downstream of the targeted sequence and containing an alanine 129 (H2AS129A) or
648 glutamic acid 129 (H2AS129E) codon instead of the serine 129. Yeast cells were
649 transformed with 1 μ g yeast Cas9 plasmid containing the sgRNA against either $HTA1$ or
650 $HTA2$ (pEF567 and pEF568 respectively) and 2 μ g donor sequence. After transformation,
651 yCas9 plasmids were lost, and sequence modification of $HTA1$ and $HTA2$ genes were
652 verified by sequencing.

653 *Yeast cell cultures for in vivo microscopy fluorescence observations*

654 Yeast cells were grown in a selective medium overnight at 30°C, diluted 1:50 in the morning
655 and grown for two generations. When relevant, Zeocin at a concentration of 250 µg/ml was
656 added to the culture 6 h before imaging. Cells reaching exponential phase (1 O.D. at 600
657 nm) were then centrifuged, concentrated to 1.5×10^7 cells/ml and 3µl were spread on agarose
658 patches (made of synthetic complete medium containing 2% agarose). Patches were sealed
659 using VaLaP (1/3 Vaseline, 1/3 Lanoline, and 1/3 Paraffin).

660 *Wide-field microscopy*

661 Live cell imaging was done using a wide field microscopy system featuring a Nikon Ti-E
662 body equipped with the Perfect Focus System and a 60x oil immersion objective with a
663 numerical aperture of 1.4 (Nikon, Plan APO). We used an Andor Neo sCMOS camera, which
664 features a large field of view of 276×233 µm at a pixel size of 108 nm. We acquired 3D z-
665 stacks consisting of 35 frames with z-steps of 300 nm using a dual band filter set (eGFP,
666 mRFP). For each z position, two color channels are consecutively acquired with an exposure
667 time of 100 ms. The complete imaging system including camera, piezo, LEDs (SpectraX) is
668 controlled by the NIS-elements software.

669 *Wide-field image analysis and statistics*

670 Image analyses were performed using Fiji plugins (Herbert et al., 2017). Briefly, images were
671 corrected for chromatic aberrations using the plugin “Descriptor-based registration (2d/3d)”.
672 Once correction applied to all acquired images, the “Detect ROI” script allowed automatically
673 selecting non-dividing cells and computing the (x, y) coordinates of the red and green loci
674 using a custom-made Fiji plugin implementing Gaussian fitting. For tracking in time-lapse
675 microscopy, we used the same custom-written Fiji plugin, which allowed extracting locus
676 positions over the entire time course for each nucleus. A custom-made MATLAB script that
677 corrected global displacements and computed MSD curves for each trajectory using non-
678 overlapping time further analyzed these trajectories. Finally, other MATLAB scripts were
679 used to fit power laws to individual MSD curves or population-averaged MSDs over time
680 intervals 0.1 - 10 s.

681 *Colony Forming Unit assay*

682 Each strain was grown overnight in 3 ml of YPD. The day after each culture was
683 appropriately diluted and ~ 200 colonies were plated onto YPD plates and YPD plates
684 supplemented with Zeocin at a concentration of 12.5 µg/ml in order to induce random DSBs
685 (as described in Seeber et al., 2013). Colonies were counted after 2–3 days of incubation at

686 30°C. For each strain, three independent experiments were performed with the
687 corresponding controls.

688 *Plasmid repair assay*

689 The plasmid pRS413 (*HIS3*) was linearized with either *EcoRI*, which generates cohesive
690 ends. Equal numbers of competent cells are transformed with 100ng of either linear or
691 circular plasmids. Following transformation with linear plasmid, the cell must repair the *HIS3*-
692 containing plasmid to survive subsequent plating on Dropouts plates lacking histidine. Each
693 strain's repair efficiency was quantified, relative to the isogenic wild type, by assessing the
694 number of colonies obtained with the cut plasmid relative to the circular plasmid. Each
695 mutant was assayed a minimum of three times in triplicate, the averaged results, standard
696 errors and unpaired t tests are reported.

697 *Translocation assay*

698 Strains were grown overnight in 20mL of synthetic minimum medium SC (2% raffinose) at
699 30°C, diluted to a density corresponding to an OD of 0.2 at 600nm, and grown for 3 h in the
700 same synthetic minimum medium in order to reach exponential growth phase (OD = 1).
701 Cultures were diluted appropriately to have $\sim 10^4$ and $\sim 10^3$ colonies/plate after counting with
702 Malassez chamber and plated onto Galactose containing SC dishes lacking or not Uracile.
703 Colonies were counted after 2-3 days of incubation at 30°C. Translocation efficiency (%) is
704 done by calculating the ratio between the number of colonies in -URA SC plates containing
705 Galactose and the number of colonies in non selective SC plates containing Galactose. For
706 each strain, three independent experiments were performed.

707 *FACS analyses*

708 Cells were grown to mid-log-phase in liquid cultures, and treated or not with Zeocin at 250
709 mg/ μ l during 6h at 30°C. After incubation, samples were fixed in 70% ethanol and kept at
710 4°C for 48 h. Cells were then resuspended in 50 mM Sodium Citrate (pH 7) containing
711 RnaseA at 0.2 mg/mL final concentration. After incubation at 37°C for 1h, Sytox Green was
712 added to a final concentration of 1mM. A total of 10^6 cells were analyzed with a CANTO II
713 flow cytometer (BD Biosciences). Aggregates and dead cells were gated out, and
714 percentages of cells with 1C and 2C DNA content were calculated using FLOWJO software.

715 *Western Blotting*

716 Cells were grown to mid-log-phase in liquid cultures, and then treated with Zeocin at 250
717 mg/ μ l during 3h at 30°C. After incubation, samples were washed once with water and

718 resuspended in 20% TCA. Cells were lysed by sonication three times for 30 sec. and the
719 protein lysates were pelleted by centrifugation at 14000 rpm for 5 min at 4°C. The pellets
720 were dissolved in 1x SDS sample loading buffer by boiling for 5 min. Samples were
721 centrifuged for 30 sec. at 18000 g in a microcentrifuge and the supernatant was retained as
722 the protein extract. Protein samples were resolved on a Bolt™ 10% Bis-Tris Plus gel and
723 transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore). Membranes
724 were probed with anti-H4 (ab10158) and anti-HA-HRP (26183-HRP) antibodies. As a loading
725 reference, we use anti-Dpm1 (5C5A7) anti-Actin antibody (MA1-744). Anti-mouse and anti-
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746

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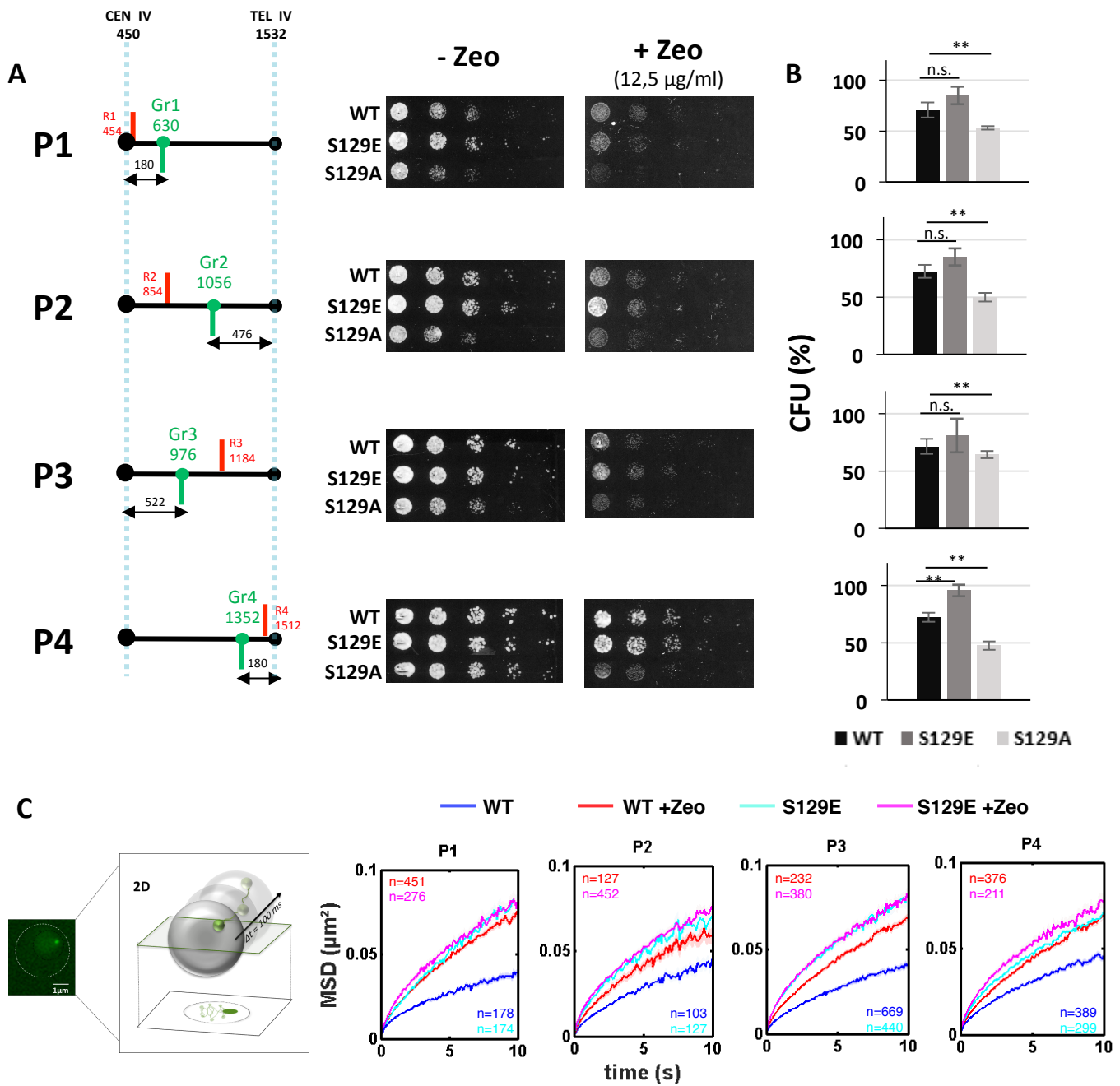
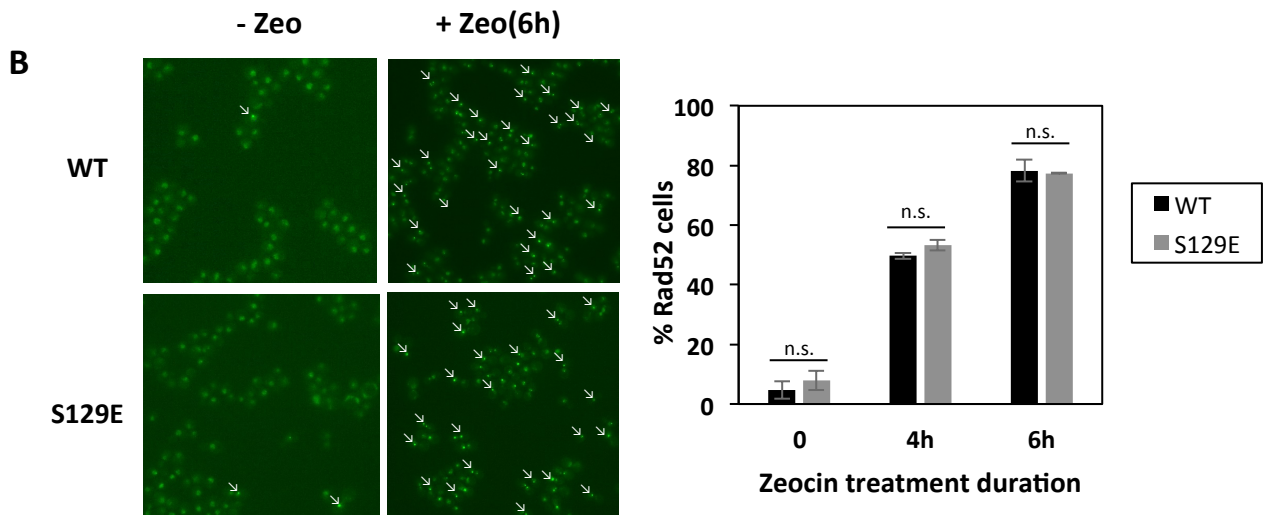
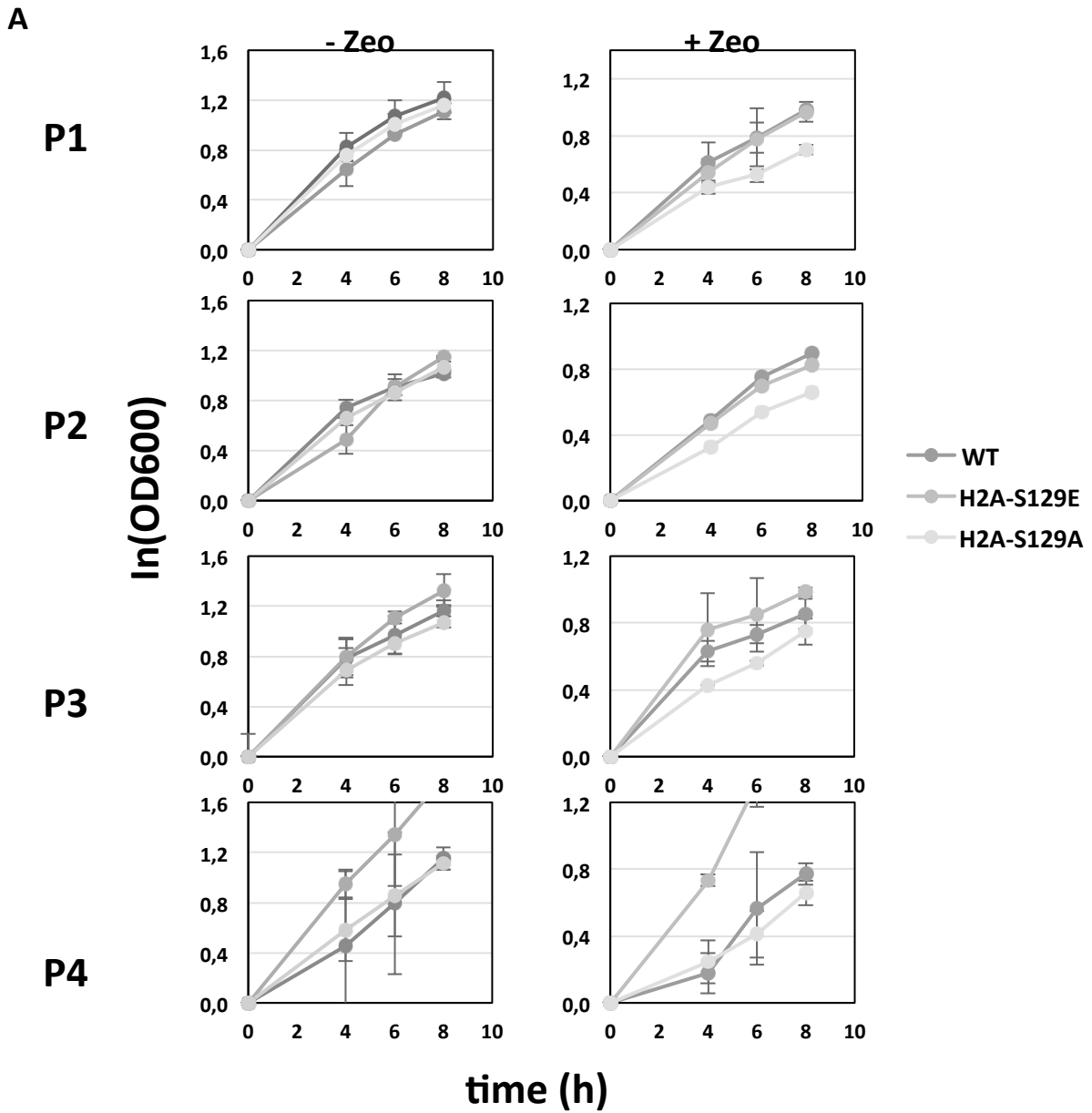
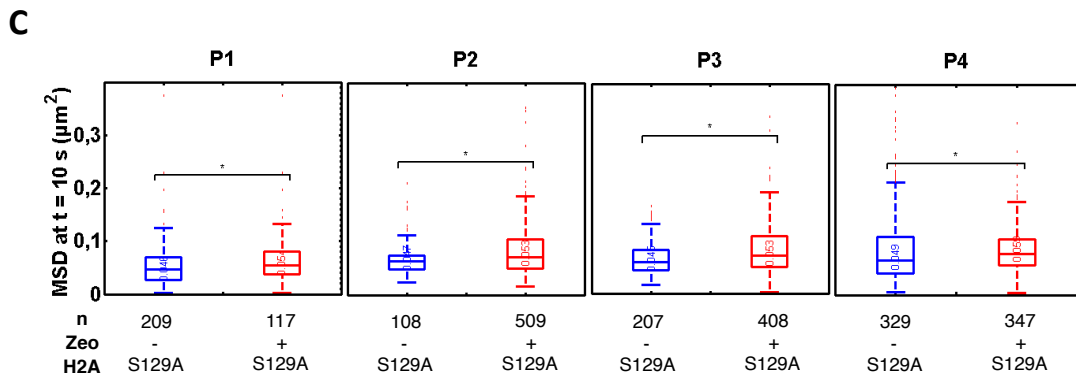
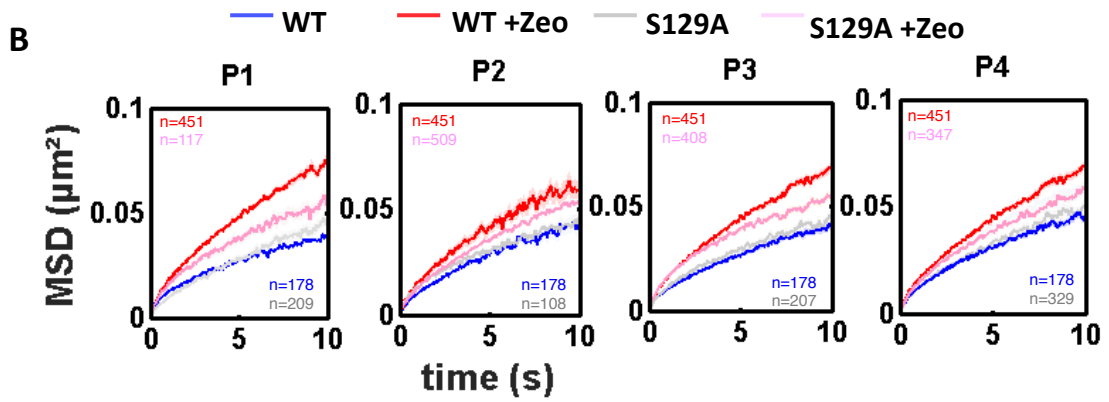
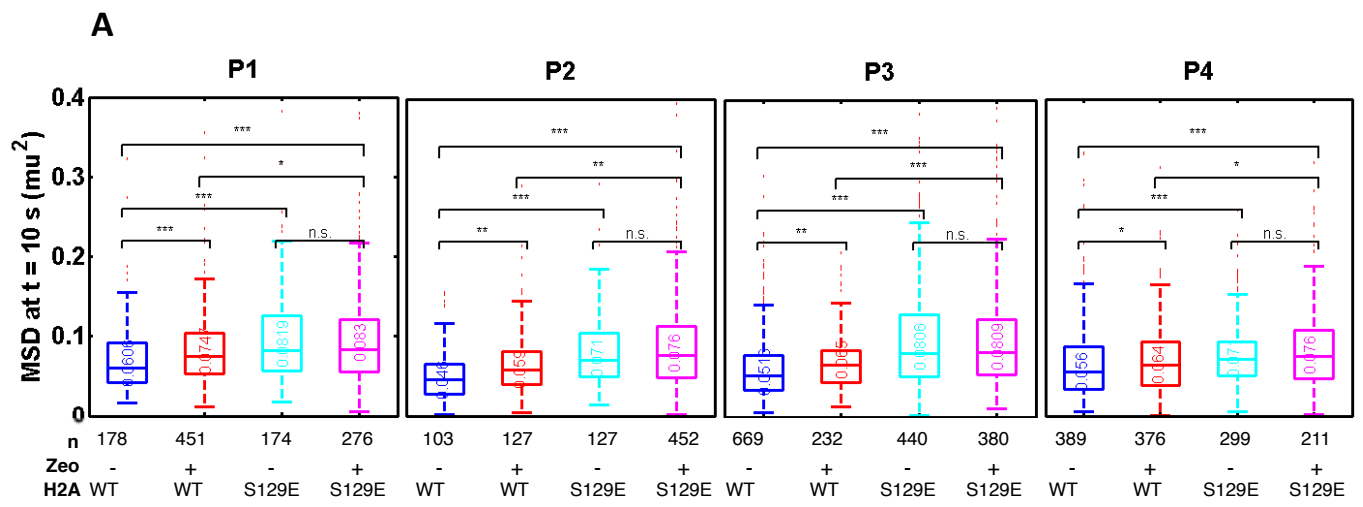


Figure 1





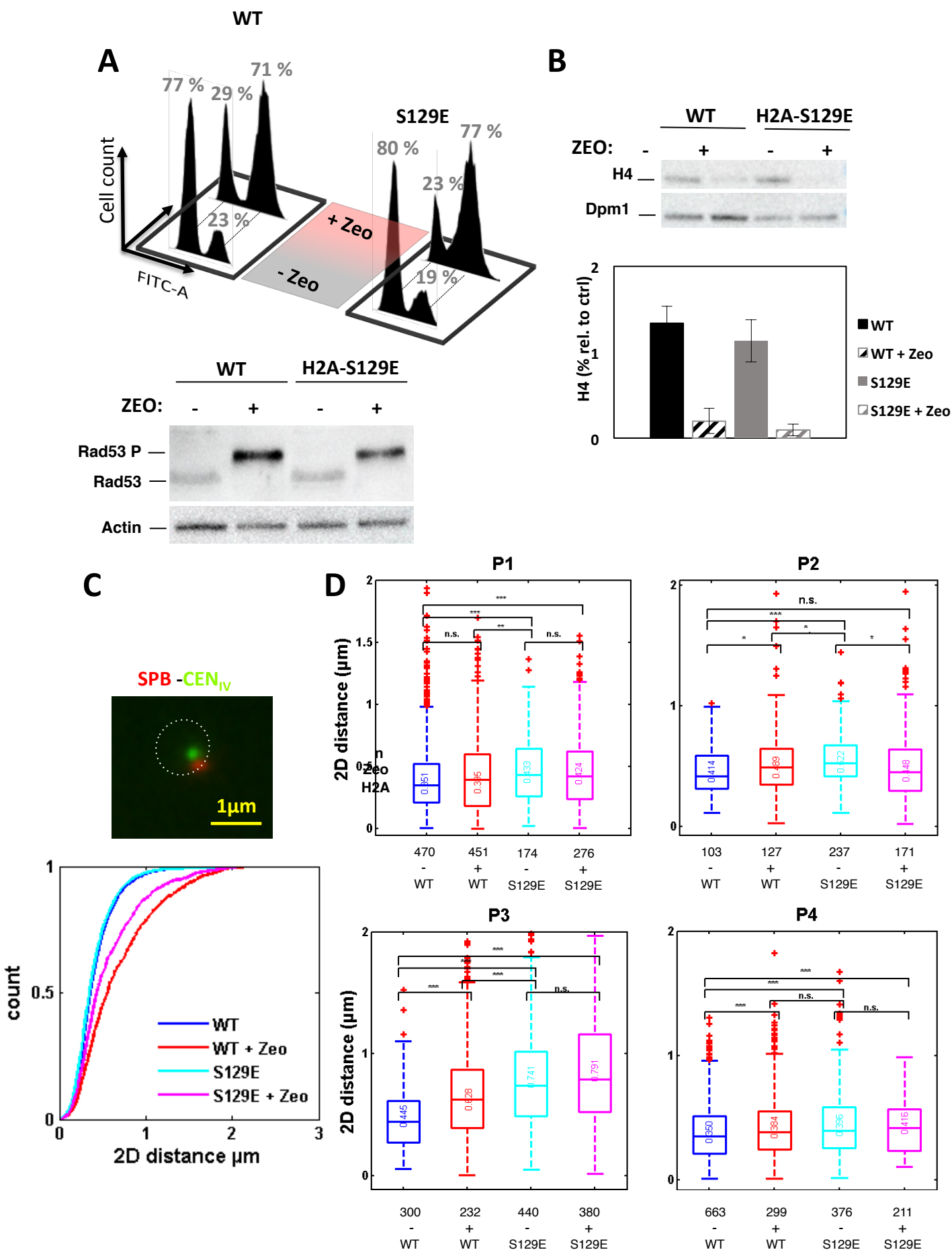
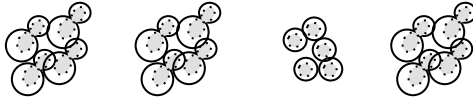


Figure 2

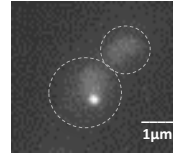
% of G2/M arrested cells

ZEO	WT		H2A-S129E		$\Delta rad9$		$\Delta rad9$ H2A-S129E	
	-	+	-	+	-	+	-	+
%	10	92	9	89	3	4	7	94

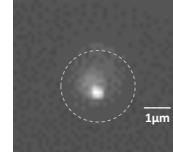


+ Zeo

$\Delta rad9$
S129E



$\Delta rad9$



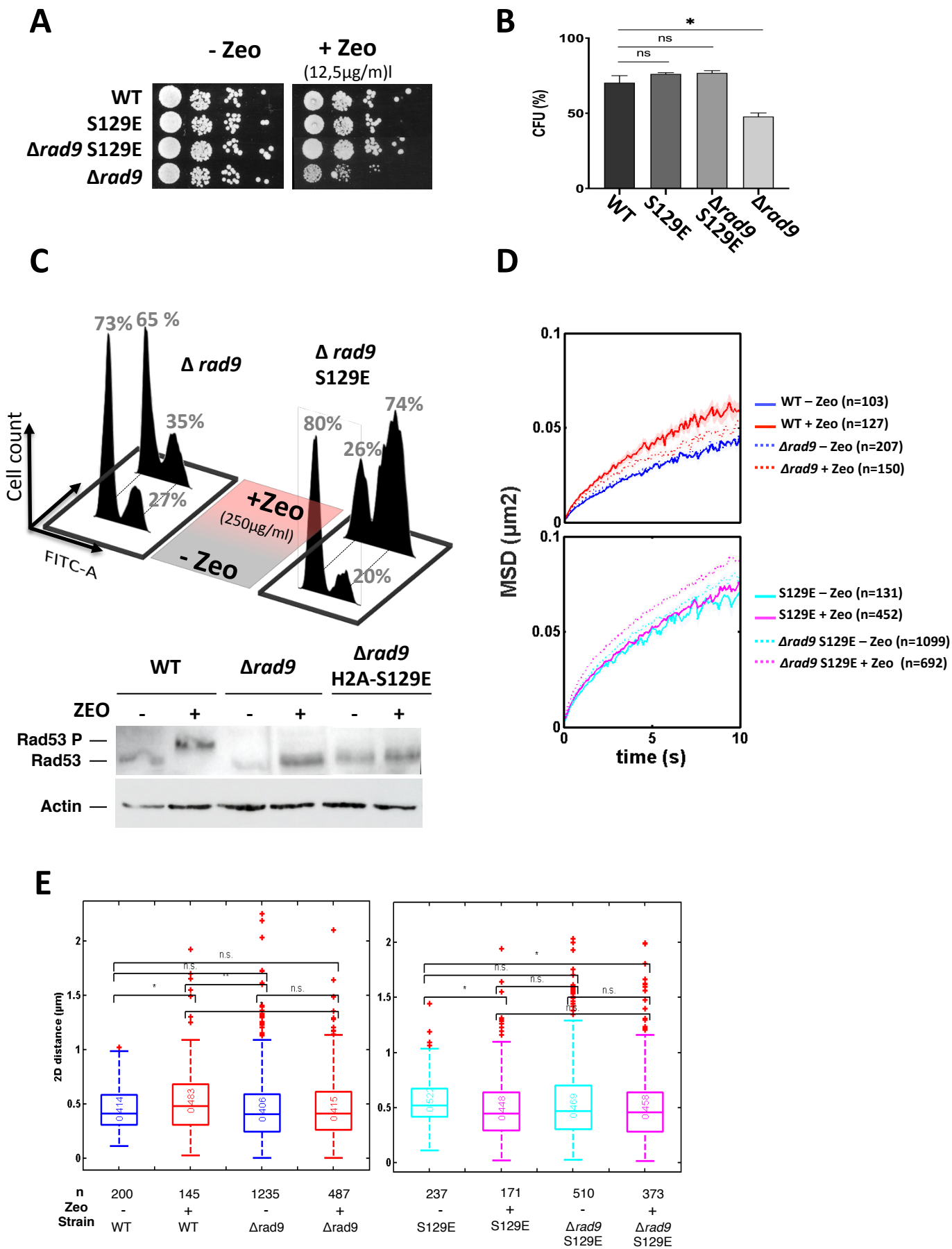
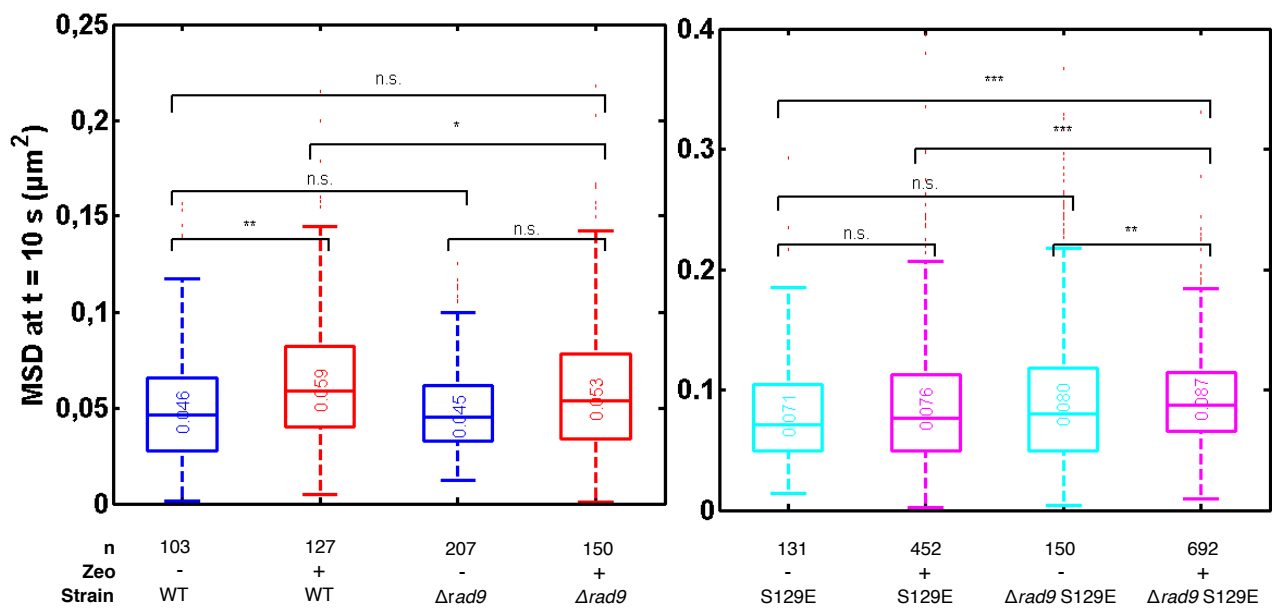
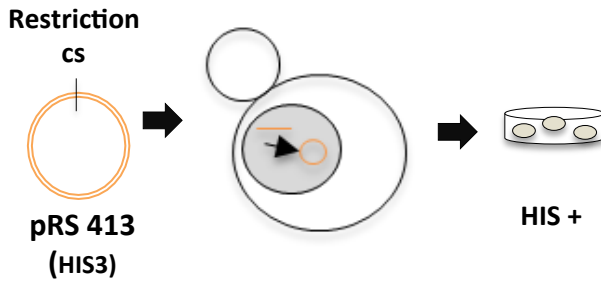
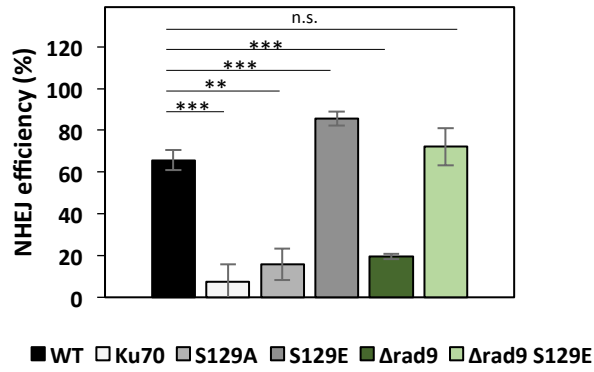
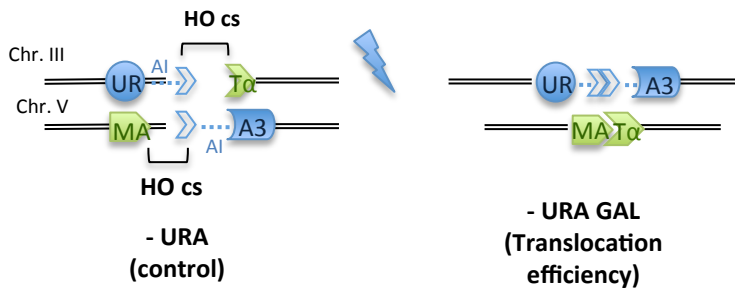


Figure 3



A**B****C****D**