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The activation loop residue serine 173 of *S.pombe* Chk1 kinase is critical for the response to DNA replication stress

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13 0044-(0)-1248382526

16 Classification: BIOLOGICAL SCIENCES/Cell Biology

18 ORCID iD numbers: orcid.org/0000-0002-1450-4734

21 Key words: Chk1, kinase, cell cycle, DNA replication, checkpoint

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24 **Summary statement**

25

26 Mutation of serine-173 in the activation loop of Chk1 kinase may promote cancer as it
27 abolishes the response to genetic alterations that arise while chromosomes are being
28 copied.

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31

32 **Abstract**

33

34 Why the DNA damage checkpoint kinase Chk1 protects the genome of lower and higher
35 eukaryotic cells differentially is still unclear. Mammalian Chk1 regulates replication origins,
36 safeguards DNA replication forks and promotes fork progression. Conversely, yeast Chk1
37 acts only in G1 and G2. We report here that the mutation of serine 173 (S173A) in the
38 activation loop of fission yeast Chk1 abolishes the G1-M and S-M checkpoints without
39 affecting the G2-M arrest. Although Chk1-S173A is fully phosphorylated at serine 345 by
40 the DNA damage sensor Rad3 (ATR) when DNA replication forks break, cells fail to stop
41 the cell cycle. Mutant cells are uniquely sensitive to the DNA alkylation agent methyl-
42 methanesulfate (MMS). This MMS sensitivity is genetically linked with the lagging strand
43 DNA polymerase delta. Chk1-S173A is also unable to block mitosis when the G1
44 transcription factor Cdc10 is impaired. Serine 173 is equivalent to lysine 166 in human
45 Chk1, an amino acid important for substrate specificity. We conclude that the removal of
46 serine 173 impairs the phosphorylation of a Chk1 target that is important to protect cells
47 from DNA replication stress.

48 **Introduction**

49

50 *S.pombe* Chk1 is phosphorylated in its C-terminal domain at serine-345 by Rad3 (ATR)
51 after the kinase was recruited to a broken chromosome by Rad4 (TopBP1), Crb2 (53BP1)
52 and the Rad9-Rad1-Hus1 ring (Capasso et al., 2002) (Lopez-Girona et al., 2001) (Saka et
53 al., 1997) (Furuya et al., 2004) . Activated Chk1 delays cell cycle progression at the G2-M
54 boundary by stimulating Wee1 to phosphorylate the inhibitory tyrosine-15 residue of Cdc2
55 (CDK1) kinase and by simultaneously removing the activating tyrosine phosphatase
56 Cdc25 from the nucleus (Furnari et al., 1999) (O'Connell et al., 1997). It is generally
57 believed that the Chk1 response in yeast is limited to G2 even though DNA replication
58 forks collapse in S phase (Lindsay et al., 1998) (Francesconi et al., 1993) (Redon et al.,
59 2003). *S.pombe* Chk1 performs a second, more enigmatic role in G1 where it prevents
60 premature mitosis when the transcription factor Cdc10 is impaired (Carr et al., 1995). It
61 also phosphorylates Cdc10 in the presence of methyl-methanesulfonate that alkylates the
62 DNA template to delay G1-S transition (Ivanova et al., 2013).

63 Unlike in yeast, human Chk1 acts mainly during S phase. It is also phosphorylated at S345
64 by ATR when the kinase associates with stalled DNA replication forks via Claspin (Mrc1),
65 in a process aided by TopBP1 (Rad4) and the 9-1-1 ring. Modification of S345 depends on
66 the additional phosphorylation of S317 and is followed by the auto-phosphorylation of
67 Chk1 at S296 (reviewed in (González Besteiro and Gottifredi, 2015)). This auto-
68 phosphorylation event is important for the association of Chk1 with Cdc25A and the
69 subsequent degradation of the phosphatase (Kawahara et al., 2010). Modification of S280
70 by p90 RSK kinase ensures the nuclear localisation of Chk1 (Li et al., 2012). Human Chk1
71 associates also with DNA lesions independently of Claspin by binding to poly-ADP-ribosyl
72 modified PARP (Min et al., 2013). Activated Chk1 blocks late replication origins by
73 disrupting the TopBP1-Treslin complex, promotes translesion DNA polymerases, mediates

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74 homologous recombination at broken forks through Rad51 and BRCA2, regulates fork
75 elongation and arrests cell cycle progression by promoting the degradation of Cdc25A
76 (reviewed in (González Besteiro and Gottifredi, 2015)). While yeast Chk1 can be deleted
77 (Walworth and Bernards, 1996), mammalian cells depend on the kinase for viability.
78 Interestingly, only S345 phosphorylation is required for the essential roles of Chk1 (Wilsker
79 et al., 2008). Inhibition of human Chk1 in unperturbed cells interferes with S phase
80 (Petermann and Caldecott, 2006) and mitosis (Zachos and Gillespie, 2007). Cdc2 (CDK1)
81 phosphorylates human Chk1 at S286 and S301 during normal mitosis as well as in the
82 response to DNA damage (Shiromizu et al., 2006) (Ikegami et al., 2008) with as yet
83 unknown functional implications.

84 Another open question is how the catalytic activity of Chk1 is regulated. The generally
85 accepted model predicts an auto-inhibitory complex between the N-terminal kinase domain
86 and the C-terminal regulatory domain (Kosoy and O'Connell, 2008) (Palermo et al., 2008).
87 This complex is thought to open up when S345 is phosphorylated by ATR (Rad3) at sites
88 of DNA damage. Whether this model is correct is still unclear since only the N-terminal
89 kinase domain of human Chk1 has been crystallised (Chen et al., 2000). The activation
90 loop adopted an open conformation in this structure which implies that Chk1 does not
91 depend on the modification by an upstream activator as many other kinases do. How Chk1
92 is silenced at the end of the DNA damage response is also not fully understood. Human
93 Chk1 is degraded after its modification at S345 in a process that is independent of the
94 other phosphorylation sites (Zhang et al., 2005). A similar degradation does not occur in
95 yeast. Attenuation of Chk1 correlates with its dephosphorylation at S345 by Wip1 (PPM1D)
96 in human cells (Lu et al., 2005) and by Dis2 in *S.pombe* (den Elzen and O'Connell, 2004).
97 Interestingly, Wip1 is replaced by PPA2 in undamaged cells where it dephosphorylates
98 Chk1 at S317 and S345 (Leung-Pineda et al., 2006). Currently no information is available

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99 on the regulation of Chk1 in unperturbed yeast cells.
100 We report here a rare separation-of-function mutation in Chk1 kinase. Mutation of serine
101 173 (S173A) in the activation loop of *S.pombe* Chk1 abolishes the G1-M arrest, when cells
102 arrest at start in *cdc10* mutant cells, and the S-M arrest in the response to broken DNA
103 replication forks. The G2-M checkpoint responses are largely intact and the mutant
104 kinases is fully phosphorylated by Rad3. Chk1-S173 is also specifically sensitive to the
105 alkylation of the DNA template by methyl-methanesulfonate in a manner related to the
106 lagging strand DNA polymerase delta. We conclude that the S173A mutation impairs the
107 activation of a downstream target of Chk1 that is specifically involved in the response to
108 DNA replication stress. This conclusion is in line with the requirement of the equivalent
109 lysine 166 for substrate recognition in human Chk1 (Chen et al., 2000).

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110 **Results**

111

112 **Reduced S345 phosphorylation of Chk1-S173A in unperturbed cells**

113 Lysine 166 occupies a central position in the activation loop of human Chk1 opposite the
114 catalytic aspartate 130 (D155 in *S.pombe*, Fig. 1A) where it may determine substrate
115 specificity (Chen et al., 2000). The corresponding *S.pombe* residue is serine 173 (Fig. 1A)
116 and aspartate 189 in *S.cerevisiae*.

117 To find out whether S173 plays a role in Chk1 activity, we mutated this residue to alanine
118 and integrated the mutant gene with a C-terminal HA₃ tag (*chk1-S173A-HA*₃) at its
119 endogenous locus using the Cre-lox recombination system (Watson et al., 2008). The
120 integrated gene was amplified and the mutation was confirmed by DNA sequencing. We
121 also integrated the wild type gene (*chk1-HA*₃) (Walworth and Bernards, 1996) to exclude
122 any effects of the flanking lox DNA sequences on *chk1* expression (Fig. S1).

123 We first used the phos-tag electrophoresis assay (Caspari and Hilditch, 2015) to study the
124 phosphorylation pattern of wild type Chk1 to establish a base line for the analysis of Chk1-
125 S173A. Phos-tag acrylamide slows down the mobility of proteins relative to the extend of
126 their phosphorylation (Kinoshita et al., 2006). We activated wild type Chk1 with the
127 topoisomerase 1 inhibitor camptothecin (CPT) that breaks DNA replication forks in S
128 phase (Pommier et al., 2010). As previously reported (Wan et al., 1999), CPT induced the
129 mobility shift of Chk1-HA on normal SDS page which is triggered by the phosphorylation
130 of S345 (Capasso et al., 2002) (Fig. 1B). Analysis of the same samples on a phos-tag gel
131 revealed a larger number of phosphorylated Chk1 forms in untreated cells and a group of
132 additional bands when cells were treated with 10μM CPT for 3.5h (Fig. 1B,C). Since these
133 inducible bands were absent in the S345A mutant (*chk1-S345A-HA*₃) (Janes et al., 2012)
134 and in cells without Rad3 kinase (*chk1-HA*₃ Δrad3), they are related to the phosphorylation
135 of serine 345 (Fig. 1C). We also noticed that the hypo-phosphorylated material of Chk1 at

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136 the bottom of the phos-tag gel consists of at least two bands (A & B in Fig 1C). Mutation of
137 S173 to alanine (S173A) had no obvious impact on the normal band shift when cells were
138 treated with 12mM hydroxyurea (HU), which stalls DNA replication forks, with 10 μ M
139 camptothecin (CPT) or with the UV mimetic 4-nitroquinoline 1-oxide (4-NQO) at 10 μ M (Fig.
140 1D). Also the phosphorylation pattern of Chk1-S173A in untreated cells was not
141 significantly different from wild type (Fig. 1E).

142 To find out whether the unperturbed phosphorylation of Chk1 relates to cell physiology, we
143 grew cells from early logarithmic growth into stationary phase and withdrew samples at
144 different times (Fig. 1F). The band associated with S345 phosphorylation peaked during
145 the most active growth phase of wild type cells (time point 2 in Fig. 1G) and was later
146 replaced by a hypo-phosphorylated form once cells had exited the cell cycle (time point 4,
147 band C in Fig. 1G). The peak in S345 phosphorylation reflects most likely the occurrence
148 of endogenous DNA replication damage. It was however interesting to find that the S173A
149 mutation lowered the amount of S345 phosphorylation during the active growth phase
150 (time point 2 in Fig. 1G) suggesting an impaired response to replication stress.

151

152 **Chk1-S173A cells are sensitive to DNA alkylation**

153 Since Chk1 is crucial for the G2-M checkpoint (Walworth and Bernards, 1996), we
154 synchronised *chk1-HA*₃ wild type and *chk1-S173A-HA*₃ cells in G2 by lactose gradient
155 centrifugation (Luche and Forsburg, 2009) and released them into rich medium with or
156 without MMS (0.05%), 4NQO (10 μ M) or HU (12mM) at 30°C to measure the delay time.
157 The first telling observation came when we compared the untreated strains. While wild
158 type cells (*chk1-HA*₃) entered the second cycle at around 180 min, *chk1-S173A-HA*₃ cells
159 were delayed by 20 min (Fig. 2A). Such a second cycle delay is typical for agents like CPT
160 or HU which interfere with DNA replication (Mahyous Saeyd et al., 2014). It is therefore
161 possible that the *chk1-S173A-HA*₃ strain suffers from a DNA replication problem that

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162 triggers this short G2 delay. The UV mimetic 4-NQO and the DNA alkylation agent MMS
163 blocked both the passage through the first G2 since DNA is instantly damaged, whereas
164 HU caused the expected second cycle arrest as cells are only hit once they undergo DNA
165 replication (Lindsay et al., 1998). While the S173A mutation had no impact on the HU
166 arrest (Fig. 2B), it allowed cells to exit G2 earlier in the presence of 4-NQO and MMS (Fig.
167 2C, D). This partial G2-M checkpoint defect was more prominent for MMS as *chk1-S173A-*
168 *HA*₃ cells started to return to the cell cycle already after 80 min compared with wild type
169 cells which arrested throughout the experiment (Fig. 2D). This checkpoint defect correlated
170 with a high MMS sensitivity of the mutant strain (Fig. 2F, G). Interestingly, a similar loss of
171 viability was not observed when the *chk1-S173A-HA*₃ strain was treated with HU, CPT or
172 UV light (Fig. 2E). This is an important finding as it reveals S173A as a separation-of-
173 function mutation. MMS modifies both guanine (to 7-methylguanine) and adenine (to 3-
174 methyladenine) thereby inducing mismatches in the DNA that are repaired by base
175 excision repair. Inefficient BER results in single-stranded DNA breaks independently of the
176 cell cycle but causes DNA double-strand breaks when these gaps are encountered by a
177 replication fork (Lundin et al., 2005). The MMS sensitivity of the *chk1-S173A-HA*₃ mutant
178 was not related to a defect in S345 phosphorylation as the mutant kinase displayed the
179 characteristic band shift on phos-tag SDS page (Fig. 2H). Interestingly, the S345 shift was
180 strongest at the lowest MMS concentration of 0.01% and declined at the higher
181 concentrations.

182

183 **Chk1-S173A is defective in the G1-M checkpoint**

184 In addition to its key role in G2, Chk1 blocks mitosis when *S.pombe* cells arrest at start in a
185 *cdc10* mutant (Fig. 3A) (Carr et al., 1995). Cdc10 is a subunit of the MBF transcription
186 factor complex that activates S phase genes during the G1-S transition (Lowndes et al.,

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187 1992). We constructed *chk1-HA₃* and *chk1-S173A-HA₃* double mutants with the
188 temperature-sensitive *cdc10.V50* (H362Y) allele (Marks et al., 1992) and released G2-
189 synchronised cells into rich medium at 30°C and 37°C (Fig. 3B, C). As reported previously
190 (Carr et al., 1995), *chk1-HA₃* *cdc10.V50* cells progressed through the first cycle before
191 arresting in G2 at the restrictive temperature of 37°C (Fig. 3B). Entry into the first cycle
192 was delayed by 60 min due to the increase in the temperature (Janes et al., 2012). While
193 *chk1-HA₃* *cdc10.V50* cells leaked slowly out of this G2-M arrest with only a few cells
194 displaying the terminal cut phenotype where the new cell wall cuts through the nucleus,
195 *chk1-S173A-HA₃* *cdc10.V50* cells entered mitosis much faster with most cells showing the
196 cut phenotype (Fig. 3C, D). We concluded from this experiment that the activation loop
197 mutation impairs the G1-M checkpoint function of Chk1. Interestingly, this G1-M function of
198 Chk1 is independent of its S345 phosphorylation as the temperature up-shift from 30°C to
199 37°C did not trigger the band shift on normal SDS page (Fig. 3E).

200 Since Chk1 acts also upstream of Cdc10 to prevent entry into S phase when the DNA
201 template is alkylated by MMS (Fig. 3F) (Ivanova et al., 2013), we synchronised *chk1-HA₃*
202 and *chk1-S173A-HA₃* cells in metaphase using the cold sensitive *nda3.KM311* allele
203 (Hiraoka et al., 1984) and released cells into rich medium with or without 0.01% MMS by
204 raising the temperature from 20°C to 30°C. This experiment would allow us to measure the
205 delay in G1-S transition induced by MMS. Untreated wild type cells (*chk1-HA₃*
206 *nda3.KM311*) initiated DNA replication between 40 min and 60 min post-release which
207 increased the DNA content from 2C to 4C (Fig. 3G, H). The mutant strain (*chk1-S173A-*
208 *HA₃* *nda3.KM311*) showed a similar behaviour but displayed two interesting differences.
209 Not all cells were able to escape the mitotic arrest as they maintained a 2C DNA content,
210 and the proportion of cells that exited reached the 4C DNA content slightly earlier than wild
211 type cells (Fig. 3H). The delayed exit from the metaphase arrest could be linked with the

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ability of *S.pombe* Chk1 to sustain the activation of the spindle checkpoint that delays metaphase-to-anaphase transition (Collura et al., 2005). Hence, the S173A mutation may prolong this mitotic arrest. The faster progression of the mutant cells through S phase is consistent with the reduced S345 phosphorylation during the unperturbed cell cycle (Fig. 1G) as this indicates a lower checkpoint activation. Addition of MMS delayed the accumulation of the 4C DNA content in both strains, with the S173A mutant showing a more pronounced effect (Fig. 3I). This led us to conclude that the activation loop mutation affects only the down-stream function of Chk1 that restrains mitosis in the *cdc10* mutant, but not the up-stream function which delays G1-S transition in the presence of MMS.

221

222 **Chk1-S173A fails to respond to broken replication forks**

223 The next decisive observation came when we analysed the S-M checkpoint response to
224 broken DNA replication forks. As long as the structural integrity of a stalled fork is
225 protected by Cds1 kinase, Chk1 activity remains low (Xu et al., 2006). Cds1 (Chk2) kinase
226 shields stalled replication structures from nucleases and recombination enzymes (Kai et al.,
227 2005) (Boddy et al., 2003). Chk1 is however strongly activated when forks break in the
228 absence of Cds1, and cells without Chk1 and Cds1 are completely checkpoint defective
229 (Lindsay et al., 1998). To test whether the S173A mutation impairs this response, we
230 combined the *chk1-S173A-HA₃* allele with the deletion of *cds1* (Δ *cds1*). The double mutant
231 was as HU sensitive as the Δ *chk1* Δ *cds1* strain strongly implying that the activation loop
232 mutation blocks Chk1 activation when replication forks collapse in the absence of Cds1
233 (Fig. 3A). This conclusion was confirmed when we released G2-synchronised *chk1-*
234 *S173A-HA₃* Δ *cds1* cells into rich medium with 12mM HU. Like the checkpoint defective
235 Δ *chk1* Δ *cds1* strain, the *chk1-S173A-HA₃* Δ *cds1* mutant entered a fatal mitosis 140min
236 post-release (Fig. 3B). The majority of cell died while they re-entered the cell cycle
237 indicated by the cut phenotype where one daughter cells is anuclear or where the new wall

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238 cuts through the single nucleus (Fig. 3C). Collectively, these results demonstrate an
239 outright dependency of cells on serine 173 when replication forks break in the absence of
240 Cds1. As in the earlier experiments, Chk1-S173A was fully phosphorylated at S345 in
241 $\Delta cdc1$ cells (Fig. 3D). These results imply a defect of Chk1-S173A down-stream of
242 collapsed replication forks in the absence of Cds1.

243

244 **Chk1-S173A reduces the viability of DNA polymerase epsilon mutant cells**

245 Because deletion of *S.pombe chk1* compromises the viability of temperature-sensitive
246 mutants of DNA polymerase delta and epsilon (Francesconi et al., 1995), we combined
247 mutant alleles in the three replicative DNA polymerases alpha (*swi7-H4*), delta (*cdc6.23*)
248 and epsilon (*cdc20.M10*) with either *chk1-HA₃* or *chk1-S173A-HA₃*. While testing cell
249 growth at the semi-restrictive temperature of 33°C, we noticed that the S173A mutation
250 specifically reduced the viability of the pol epsilon (*cdc20.M10*) mutant as the *chk1-S173A-*
251 *HA₃* *cdc20.M10* double mutant grew only very poorly compared to the *chk1-HA₃*
252 *cdc20.M10* strain (Fig. 5A). DNA polymerase epsilon synthesises the leading strand
253 (Pursell et al., 2007), is involved in long-patch BER (Wang et al., 1993), associates with
254 the DNA replication checkpoint protein Mrc1 (Claspin) (Lou et al., 2008) and establishes
255 heterochromatin (Li et al., 2011). The reduced viability at 33°C could suggest two roles of
256 Chk1. Either the kinase responds to replication problems associated with the leading
257 strand or it promotes DNA pol delta that can remove mismatches left behind by pol epsilon
258 (Flood et al., 2015). Phos-tag analysis showed that some hypo-phosphorylated material
259 was absent from Chk1-S173A, but this was the case for both, pol delta and epsilon (Fig.
260 5B).

261 We next synchronised the strains in early S phase using the HU protocol (Luche and
262 Forsburg, 2009) and released them back into the cell cycle to follow their progression into

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263 G2. While the S173A mutation had no impact in the case of DNA polymerase delta (*chk1-*
264 *S173A-HA₃ cdc6.23*) (Fig. 5C), it did advance cell cycle progression in the DNA
265 polymerase epsilon strain (*chk1-S173A-HA₃ cdc20.M10*) (Fig. 5D). The mutation in the
266 activation loop allowed cells to acquire a G2 (2 copies, 2C) DNA content 90 min post-
267 release, approximately 30 min earlier than the wild type Chk1 kinase (*chk1-HA₃*
268 *cdc20.M10*). We did however find no evidence of S345 phosphorylation in any mutant
269 strain during this experiment (Fig. 5E). The faster progression of the *chk1-S173A-HA₃*
270 *cdc20.M10* mutant could explain why the pol epsilon strain loses viability at the semi-
271 permissive temperature. The activation loop mutation S173A might block the
272 phosphorylation of a down-stream target that is crucial for a reduction in leading strand
273 synthesis when DNA polymerase epsilon is impaired or when pol delta needs to remove
274 mismatched nucleotides.

275

276 **The MMS sensitivity of Chk1-S173A is linked with DNA polymerase delta**

277 Given the requirement of pol delta for the removal of alkylated bases by BER (Blank et al.,
278 1994), we tested the genetic relationship between *chk1-S173A-HA₃* and *cdc6.23*.
279 Intriguingly, the mutation in the catalytic subunit of pol delta affected survival on MMS
280 plates differentially depending on whether the *chk1-HA₃* wild type or *chk1-S173A-HA₃*
281 mutant allele was present. While *cdc6.23* cells containing the wild type kinase were MMS
282 sensitive, *cdc6.23* cells with the mutant kinase displayed some degree of resistance (Fig.
283 6A). We followed this observation up by conducting an acute survival test at 0.025% MMS
284 and noticed that the *chk1-HA₃ cdc6.23* double mutant was significantly more MMS
285 sensitive than the pol delta (*cdc6.23*) single mutant that contains the untagged *chk1* gene
286 (Fig. 6B). This implies that the tagged *chk1-HA₃* allele, which has been used in many
287 studies (Walworth and Bernards, 1996), differs from the untagged gene in a *cdc6.23*

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288 mutant background. Intriguingly, the mutation in the activation loop suppressed this hyper-
289 sensitivity to a level observed for the *chk1-S173A-HA₃* single allele (Fig. 6B). Collectively,
290 these data show that the MMS sensitivity of the *chk1-S173A* mutation is epistatic with the
291 *cdc6.23* mutation in the catalytic subunit of pol delta at 30°C and that the mutation also
292 suppresses the damaging activity of the tagged wild type Chk1 kinase. The nature of this
293 activity is as yet unknown. We suspect however that the C-terminal tag interferes with the
294 repair function of pol delta in BER (Blank et al., 1994). To test whether the polymerase
295 mutations interfere with S345 phosphorylation of Chk1 and Chk1-S173A, the
296 corresponding strains were treated with 0.01% MMS at 30°C and also exposed to the
297 semi-permissive temperature of 33°C without MMS. While both Chk1 proteins were
298 phosphorylated at S345 in the presence of MMS, the phosphorylation of the wild type
299 kinase was lower in the pol delta mutant coinciding with its high MMS sensitivity (Fig. 6C).
300 Chk1 was only weakly S345 modified at 33°C in both polymerase mutants indicating that
301 no or very little endogenous DNA damage occurs under these conditions.

302

303 **Discussion**

304 The only separation-of-function conditions known so far are the phosphorylation of S317 of
305 human Chk1, which is only required for the DNA damage response but not for its essential
306 functions (Wilsker et al., 2008), and the mutations E92D and I484T in *S.pombe* Chk1
307 which affect the S-M checkpoint but only at 37°C (Francesconi et al., 1997). We report
308 here a new separation-of-function mutation, S173A in the activation loop of *S.pombe* Chk1,
309 that abolishes the G1-M and S-M checkpoints independently of S345 phosphorylation
310 under normal growth conditions. When *chk1-S173HA₃* cells arrest at start during the G1-S
311 transition due to the *cdc10.V50* mutation, they cannot prevent mitosis (Fig. 3C,D). A
312 similar problem arises when DNA replication forks break in HU medium in the absence of
313 Cds1 (Fig. 4B, C). Since *cdc10.V50* cells arrest with unreplicated chromosomes at start
314 (Luche and Forsburg, 2009), both Chk1 requirements must reflect distinct G1-M and S-M
315 checkpoint activities of Chk1. What is however intriguing is that the *chk1-S173A* mutant is
316 not CPT sensitive (Fig. 2E), although camptothecin also breaks DNA replication forks
317 (Pommier et al., 2010). This implies that the activation loop mutation is only critical when
318 Cds1 is absent. Since Cds1 protects damaged forks from nucleases and recombinases
319 (Kai et al., 2005) (Boddy et al., 2003), it is possible that the activation loop mutation
320 activates a DNA repair factor that is redundant as long as Cds1 is active. The DNA
321 damage signal must reach Chk1-S173A as the mutant kinase is phosphorylated at S345 in
322 the presence of CPT (Fig. 1D), 4-NQO (Fig. 1D), MMS (Fig. 2H) and HU (Fig. 3D). It is
323 therefore unlikely that the S173A mutation interferes with Rad3 activation at damaged
324 chromosomes involving Crb2 (53BP1), Rad4 (TopBP1) and the 9-1-1 ring (Furuya et al.,
325 2004). Since the corresponding lysine-166 in human Chk1 is involved in substrate
326 specificity (Chen et al., 2000), it is more likely that the activation loop mutation blocks the
327 phosphorylation of a down-stream target that is required to restrain mitosis in *cdc10*

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328 mutant cells and when forks break in *cds1* deletion cells (Fig. 7A). This target appears to
329 be distinct from Wee1 and Cdc25 because the *chk1-S173A* strain is able to block mitosis
330 when *cds1+* cells are treated with HU or the UV mimetic 4-NQO (Fig. 2B, C). A clear

331 difference exists however when DNA is alkylated by MMS as *chk1-S173A* cells have a
332 partial G2-M checkpoint defect (Fig. 2D) and are highly sensitive (Fig. 2G; 6B).

333 The genetic link between DNA polymerase delta and Chk1-S173A may hint at this
334 unknown target. The observation that the activation loop mutation reduces the viability of

335 the pol epsilon (*cdc20.M10*) mutant at 33°C (Fig. 5A) could be explained by the faster
336 progression through S phase (Fig. 5D). This faster progression may however be linked

337 with DNA polymerase delta given that pol epsilon needs pol delta to repair any remaining
338 mismatches in the leading strand which are not removed by its own 3'-exonuclease

339 activity (Flood et al., 2015). Pol delta is also able to replicate the leading and the lagging
340 strand once a fork has collapsed (Miyabe et al., 2015). The requirement of S173 for

341 viability of the pol epsilon (*cdc20.M10*) mutant could therefore mean that Chk1 is involved
342 in the repair activities of pol delta either when mismatched bases remain in the leading
343 strand after MMS treatment or when the leading strand is elongated by pol delta during the
344 homologous recombination dependent re-start of collapsed replication forks in HU-treated

345 Δ *cds1* cells (Fig. 7A). This conclusion is strengthened by the epistatic relationship between
346 *chk1-S173A* and *cdc6.23*, the catalytic subunit of pol delta (Fig. 6B).

347 In summary, S173A is a rare separation-of-function mutation of Chk1 that may help to
348 dissect its role in S phase where it might link post-replication repair by DNA polymerase
349 delta with a block over mitosis. To uncover the identity of its proposed target will however

350 require further work. It is intriguing that one of the other known separation-of-function
351 mutations, E92D (Francesconi et al., 1997), sits at the beginning of a loop opposite the

352 activation loop where S173A is (Fig. 7B). The other interesting notion is that this intra-S

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353 activity of Chk1, which is not essential in yeast, may have become essential during the
354 evolution of higher eukaryotic cells (Petermann and Caldecott, 2006).

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355 **Materials and Methods**

356

357 **Yeast strains**

358 The genotype of the strains used in this study is *ade6-M210 leu1-32 ura4-D18*. The *rad3*
359 gene was deleted with the *ade6+* gene and the *cds1* gene was deleted with *ura4+*. The
360 *chk1* gene was deleted with *kanMX4* antibiotic resistance gene. *chk1-S345A-HA₃* (*h-*
361 *ade6-M210 chk1::loxP-chk1-S345A-HA3-loxM leu1-32 ura4-D18*); *chk1-S173A-HA₃* (*h-*
362 *ade6-M210 chk1::loxP-chk1-S173A-HA3-loxM leu1-32 ura4-D18*) (Fig. S1). See figure
363 legends for further details.

364

365 **Base strain construction and integration of the Chk1 point mutations**

366 The base strain was constructed as described in (Watson et al., 2008). The *loxP* and *loxM*
367 Cre-recombinase recognition sequences were integrated 84nt upstream of the start codon
368 and 84nt downstream of the stop codon (Fig. S1A) using the primers *Base-1* and *Base-2*
369 (Fig. S1C). The point mutations S173A and S345A were introduced using fusion PCR as
370 reported in (Janes et al., 2012). Genomic DNA from the *chk1-HA₃* strain (Walworth and
371 Bernards, 1996) was used as the PCR template to introduce the C-terminal HA affinity tag.
372 The two overlapping *chk1* gene segments were amplified using the primers *Base-3* and
373 the mutation reverse primer, and the primer *Base-4* and the mutation forward primer (Fig.
374 S1C). The full-length fusion fragments were cloned into the *lox-Cre* integration plasmid
375 using the restriction enzymes SphI and SacI. Integration of the mutated *chk1-HA₃* genes
376 resulted in the loss of 4nt upstream of the start codon and of 17nt downstream of the stop
377 codon (Fig. S1B).

378

379 **Cell synchronisation**

380 Cells were synchronised as described in (Luche and Forsburg, 2009). HU was used at a

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381 final concentration of 15mM for 3.5h at 30 °C in rich medium. Lactose gradients were
382 centrifuged for 8min at 800rpm. The *nda3.KM311* mitotic arrest was performed in rich
383 medium as reported in (Nakazawa et al., 2011). One volume of pre-warmed medium (40°C)
384 was added to the 20°C medium to quickly raise the temperature to 30°C at the up-shift to
385 re-start the cell cycle.

386

387 **Flow cytometry**

388 The DNA content was measured using a CUBE 8 (Sysmex) instrument as described in
389 (Luche and Forsburg, 2009). The histograms were produced using the free Flowing
390 Software (<http://flowingsoftware.btk.fi/>)

391

392 **Phos-tag SDS page**

393 Phostag gels (6%) were prepared and run as reported in (Caspari and Hilditch, 2015).

394

395 **Survival assays**

396 The drop tests and acute survivals assays are described in (Kai et al., 2006).

397

398 **Antibodies**

399 Anti-HA antibody (BioScource, Covance MMS-101P-200)

400

401 **Acknowledgements**

402 The authours would like to thank Dr Jacqueline Hayles (The Francis Crick Institute,
403 London, UK) for supplying the *cdc10.V50* strain.

404

405

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406 **Author Contributions**

407 NC performed some experiments. TC performed some experiments, designed the study
408 and analysed the results.

409

410 **Competing Interests**

411 No competing interest declared.

412

413 **Funding**

414 This work was not supported by a research grant. It was part of a research-informed
415 teaching program in which students (NC) engage with original research over a period of 3
416 years in the modules MSE-2021 Genomic Instability & Disease, MSE-3013 Research
417 Project and MSE-4073 Medical Master Research Project. The expenditure was funded
418 indirectly by tuition fee income to Bangor University.

419

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420 **Figure legends**

421

422 **Fig. 1. Reduced S345 phosphorylation of Chk1-S345 in unchallenged cells.** (A) Model

423 of the kinase domain of *S.pombe* Chk1. The Swiss model tool was used

424 (swissmodel.expasy.org). The underlying crystal structure is 4czt (34.5% identity)

425 (Chaves-Sanjuan et al., 2014). Serine-321 is the last C-terminal amino acid. (B) *chk1-HA₃*

426 wild type cells were treated in rich medium with 10μM camptothecin (CPT) for 3.5h at 30°C.

427 UT = untreated. Total protein extracts were separated on normal 10% SDS page or 6%

428 phos-tag SDS page [full image] (PT). The arrow indicates the group of shift bands related

429 to S345 phosphorylation. (C) PT-SDS page showing extracts from *chk1-HA₃*, *chk1-S345A-*

430 *HA₃* and *chk1-HA₃ rad3::ade6+* cells treated with 10μM CPT for 3.5h [full image]. A and B

431 indicated the hypo-phosphorylated double band. (D) Normal SDS page analysis of *chk1-*

432 *HA₃* and *chk1-S173A-HA₃* cells treated with 12mM hydroxyurea (HU) and 10μM CPT for

433 3.5h or with 10μM nitroquinoline 1-oxide (4NQO) for 1h at 30°C [full image] (Chk1 runs at

434 58kDa). (E) PT-SDS page analysing extracts from untreated *chk1-HA₃*, *chk1-HA₃*

435 *rad3::ade6+*, *chk1-S173A-HA₃* and *chk1-S173A-HA₃ rad3::ade6+* cells [full image]. (F, G)

436 Untreated *chk1-HA₃* and *chk1-S173A-HA₃* cells were grown in rich medium from a low cell

437 number into stationary phase. Samples were withdrawn at the indicated time points and

438 analysed on PT-SDS page [full image]. C indicates a phospho-band in stationary cells.

439

440 **Fig. 2. Chk1-S173A cells are MMS sensitive.** (A-D). *chk1-HA₃* and *chk1-S173A-HA₃*

441 cells were synchronised in G2 by lactose gradient centrifugation and released into rich

442 medium containing no drug (UT), 12mM hydroxyurea (HU), 10μM nitroquinoline 1-oxide

443 (4NQO) or 0.05% methyl-methanesulfonate (MMS). (E-F) Drop test of the indicated strains

444 on rich medium plates containing 4mM HU, 10μM CPT, 0.01% MMS or were treated with

445 50J/m² UV light (254nm). (G) Acute cell survival at 0.05% MMS. The *rad3::ade6+*

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446 *tel1::leu2+* double mutant is checkpoint defective (averages of 3 repeats). (H) PT-SDS
447 page analysis of total protein extracts from *chk1-HA₃* and *chk1-S173A-HA₃* cells treated
448 with the indicated MMS concentrations for 3.5h at 30°C in rich medium.

449

450 **Fig. 3. Chk1-S173A is defective in the G1-M checkpoint.** (A) Inactivation of Cdc10
451 induces a Chk1-dependent block of mitosis. (B-D) *chk1-HA₃ cdc10.V50* and *chk1-S173A-*
452 *HA₃ cdc10.V50* cells were grown in rich medium at 30°C, synchronised in G2 by lactose
453 gradient centrifugation and released into rich medium at 30°C (B) or 37°C (C). Note the 60
454 min delay of the first cycle due to the temperature up-shift. The majority of *chk1-S173A-*
455 *HA₃ cdc10.V50* cells enter a terminal mitosis (cut phenotype) (D). (E) *chk1-HA₃ cdc10.V50*
456 and *chk1-S173A-HA₃ cdc10.V50* cells were grown in rich medium at 30°C, at 37°C or at
457 30°C with 0.01% MMS for 3.5h. Total protein extracts were analysed on normal SDS page.
458 (F) MMS treatment delays G1-S transition by activating Rad3-Chk1 phosphorylation of
459 Cdc10. (G) The cold sensitive beta-tubulin gene *nda3.KM311* arrests cells with a 2x1C =
460 2C DNA content in metaphase. Cells reach a 4C DNA content after S phase. (H) Flow
461 cytometry histograms of untreated *chk1-HA₃ nda3.KM311* (blue) and *chk1-S173A-HA₃*
462 *nda3.KM311* cells (yellow) after release from the metaphase block in rich medium. Time is
463 post-release. (I) Flow cytometry histograms of MMS-treated (0.01%) cells after the release
464 from the metaphase block. Dark colours = plus MMS. Blue & yellow = untreated (identical
465 to Fig. 3H). The 2C and 4C DNA content is indicated by dotted lines.

466

467 **Fig. 4. Chk1-S173A fails to respond to broken replication forks.** (A) Drop test with the
468 indicated strains at 30°C on rich medium plates. (B, C) *chk1-S173A-HA₃*, *chk1-S173A-*
469 *HA₃ cds1::ura4+*, *cds1::ura4+* and *cds1::ura4+ chk1::kanMX4* strains were synchronised
470 by lactose gradient centrifugation and released into rich medium with 12mM HU at 30°C (B)

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471 = sepeated G1/S cells; C= cut cells). (D) *chk1-HA₃*, *chk1-HA₃ cds1::ura4+*, *chk1-S173A-HA₃*,
472 *chk1-S173A-HA₃ cds1::ura4+* strains were incubated for 3.5h at 30°C in rich medium
473 [U], in 12mM HU [H] or 10µM CPT [C]. Total protein extracts were analysed on a 10%
474 SDS page.

475

476 **Fig. 5. Chk1-S173A reduces the viability of DNA polymerase epsilon mutant cells.** (A)
477 Drop test with the indicated strains on rich medium plates. The *chk1-HA₃* (WT) and *chk1-*
478 *S173A-HA₃* (S173A) alleles were crossed into *swi7-H4* (pol alpha), *cdc6.23* (pol delta) and
479 *cdc20.M10* (pol epsilon). (B) PT-SDS and normal SDS (N) analysis of the strains used in
480 the drop test. Total protein was extracted after growth in rich medium for 3.5h at 30°C. The
481 arrow indicates the hypo-phosphorylated Chk1 protein. (C, D) *chk1-HA₃ cdc6.23*, *chk1-*
482 *HA₃ cdc20.M10*, *chk1-S173A-HA₃ cdc6.23* and *chk1-S173A-HA₃ cdc20.M10* cells were
483 synchronised in early S phase by incubating cells in rich medium with 15mM HU for 3.5h.
484 Flow cytometry histograms were recorded at the indicated times after HU was washed out.
485 The dotted lines indicate HU arrested and G2 (2C) cells. (E) Total protein samples were
486 prepared from samples taken from this experiment at the indicated times and analysed on
487 normal SDS page.

488

489 **Fig. 6. The MMS sensitivity of Chk1-S173A is linked with DNA polymerase delta.** (A)
490 Drop test with the indicated strains on rich medium plates at 30°C, 30°C with 0.01% MMS
491 or 37°C. (B) Acute MMS survival (0.025%) at 30°C (averages and s.d. errors of 3 repeats).
492 (C) *chk1-HA₃ cdc6.23*, *chk1-HA₃ cdc20.M10*, *chk1-S173A-HA₃ cdc6.23* and *chk1-S173A-*
493 *HA₃ cdc20.M10* cells were grown at 30°C, 33°C or 30°C plus 0.01% MMS for 3.5h in rich
494 medium. Total protein extracts were analysed on normal SDS page.

495

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496 **Figure 7. Model.** (A) Chk1-S173A may be defective in the phosphorylation of a protein
497 that allows DNA pol delta, which acts in front of the moving replication fork on the lagging
498 strand, to remove mismatches that remain in the leading strand in the presence of MMS.
499 Since pol delta also synthesizes both strands during the recombinogenic repair of collapse
500 forks, Chk1-S173A might also impair this function. CMG = Cdc45 + Mcm2–7 + GINS
501 replication complex. (B) Model of the kinase domain of *S.pombe* Chk1. The underlying
502 crystal structure is 4czt (34.5% identity).

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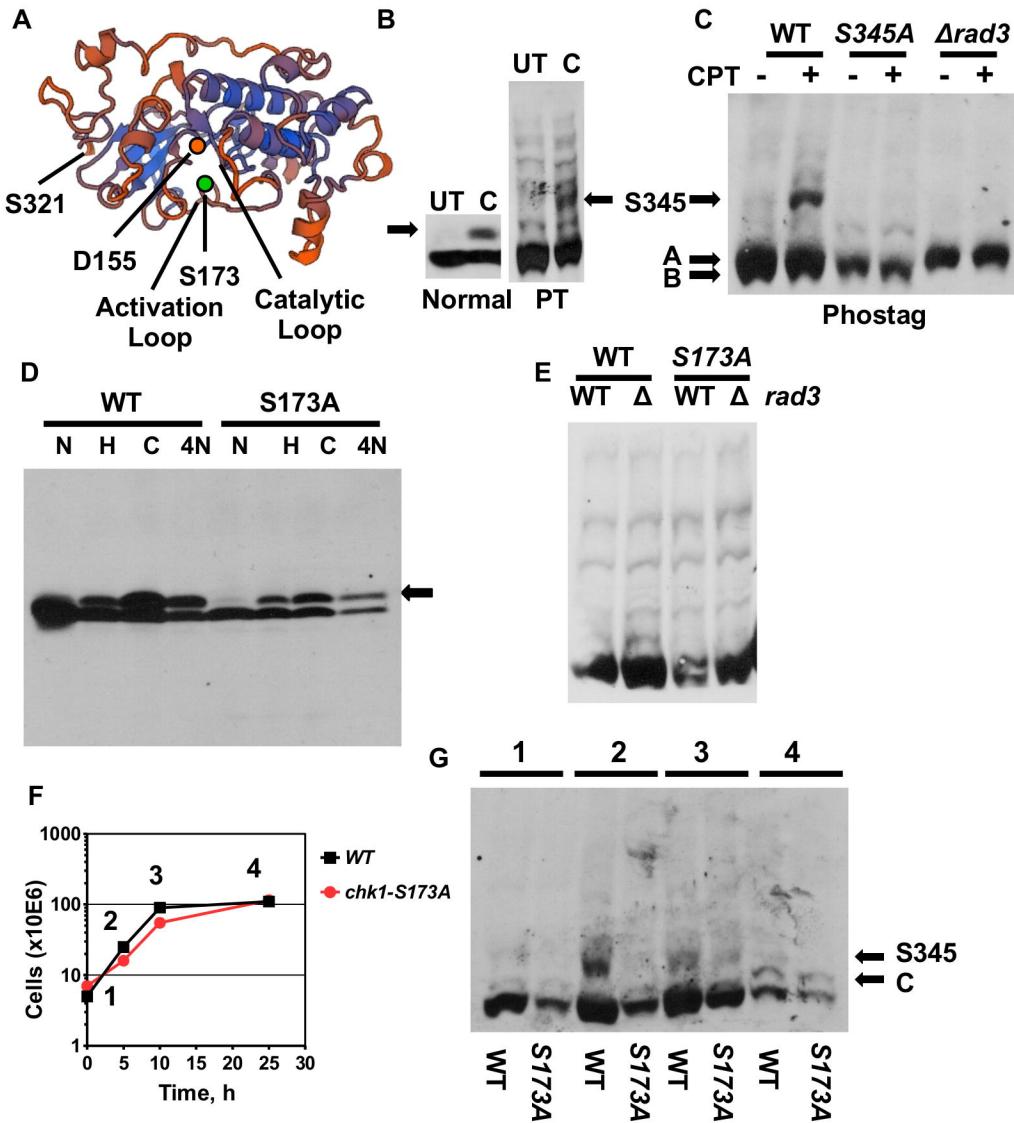
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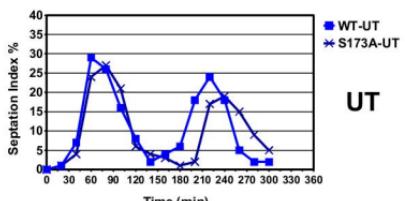
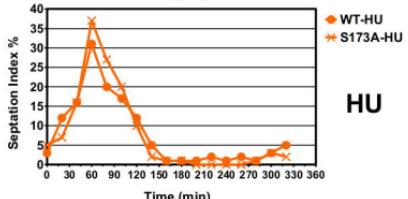
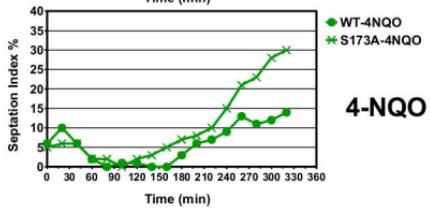
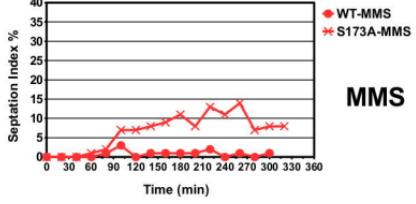
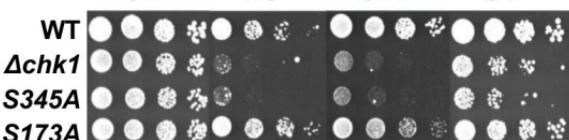
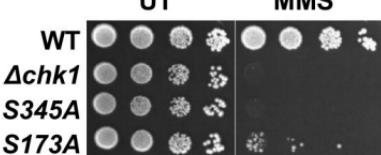
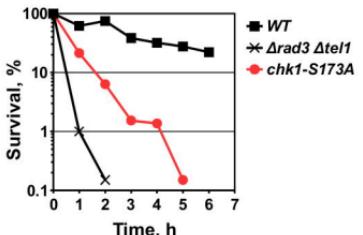
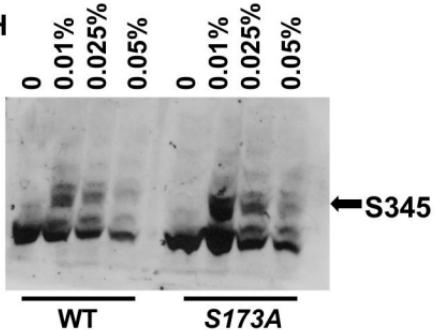
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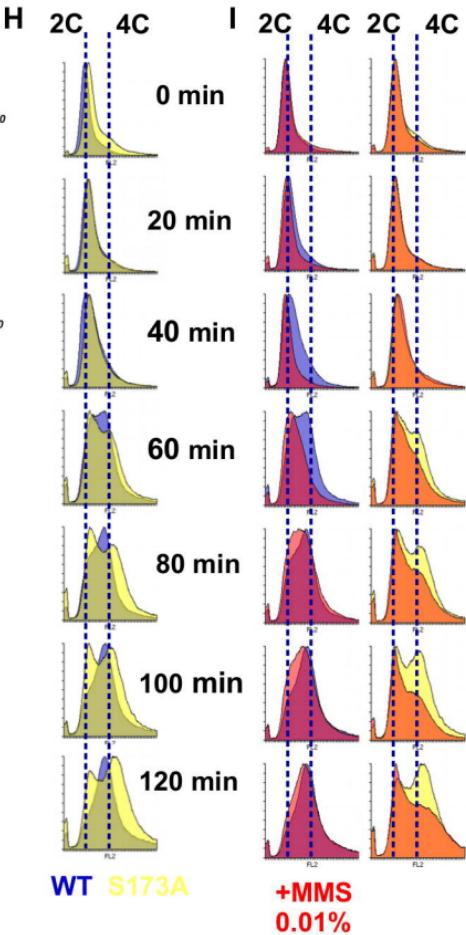
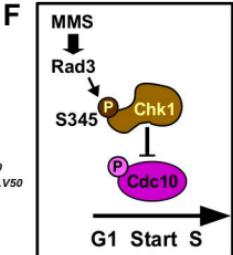
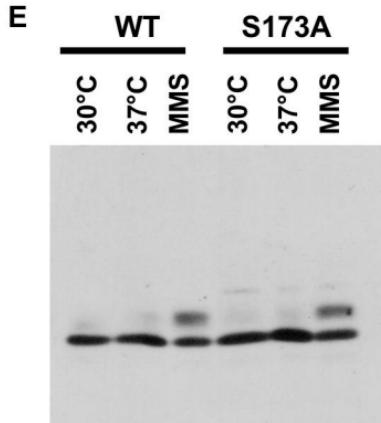
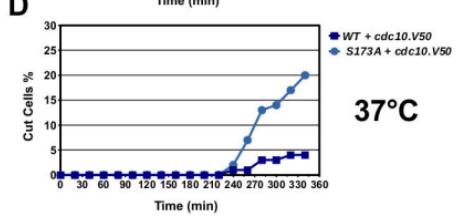
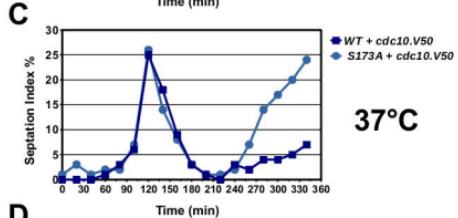
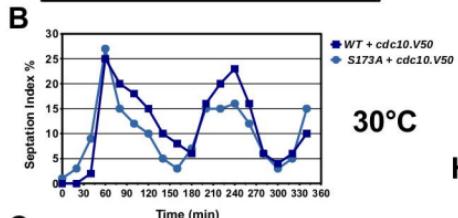
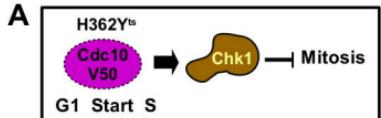
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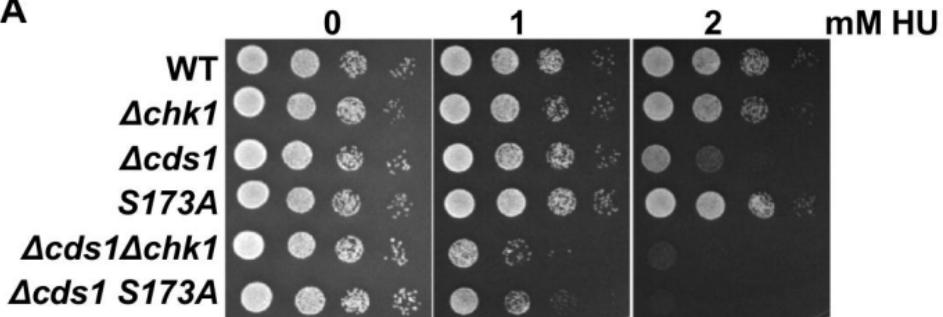
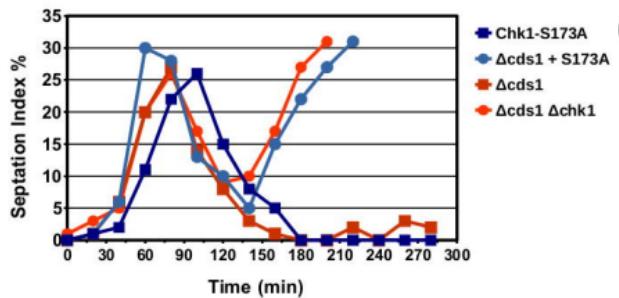
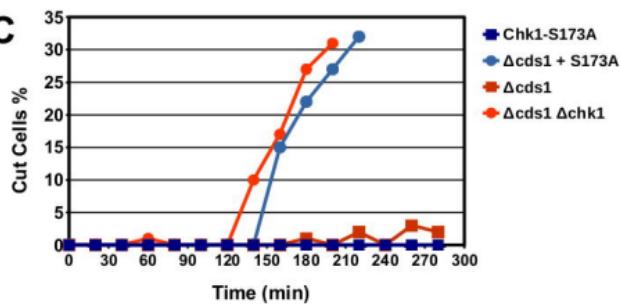
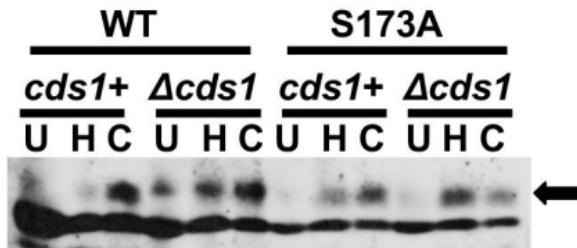
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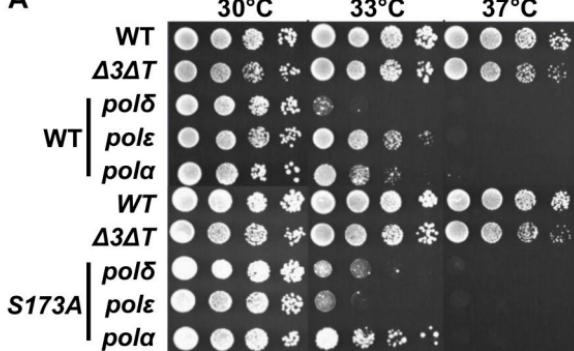
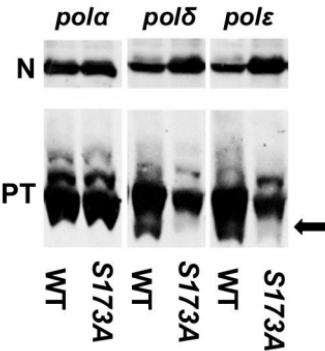
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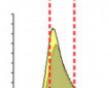
A**B****C****D****E****F****G****H**



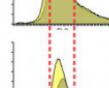
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A**B****C**

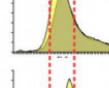
HU 2C



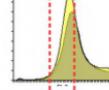
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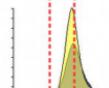
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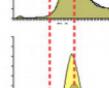
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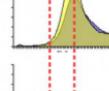
90 min



120 min

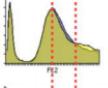
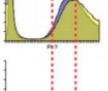
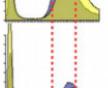
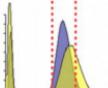
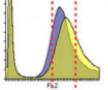
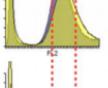
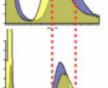
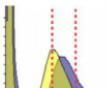
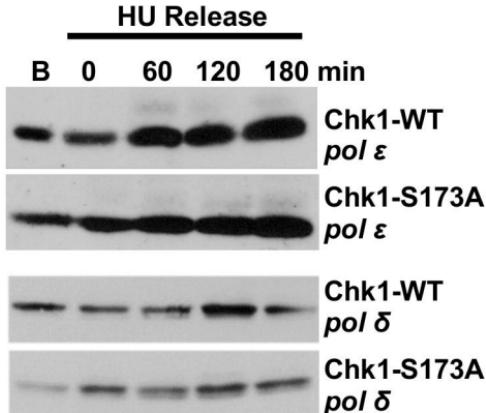


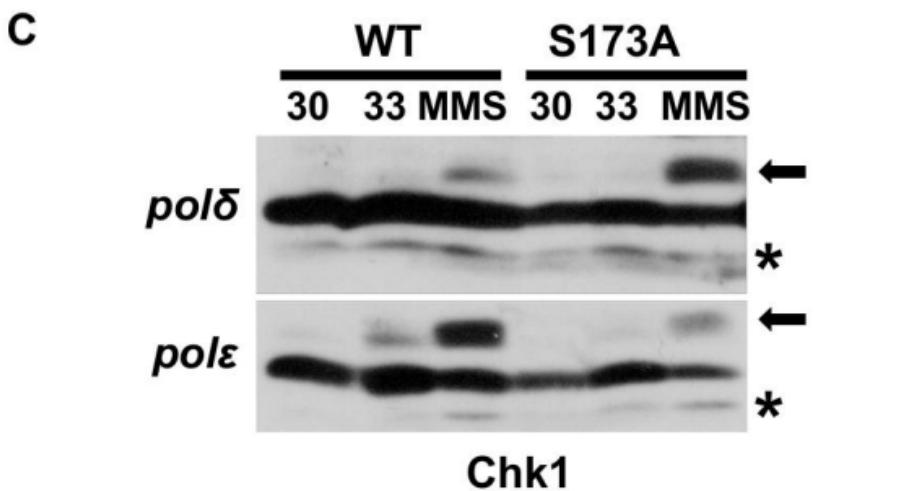
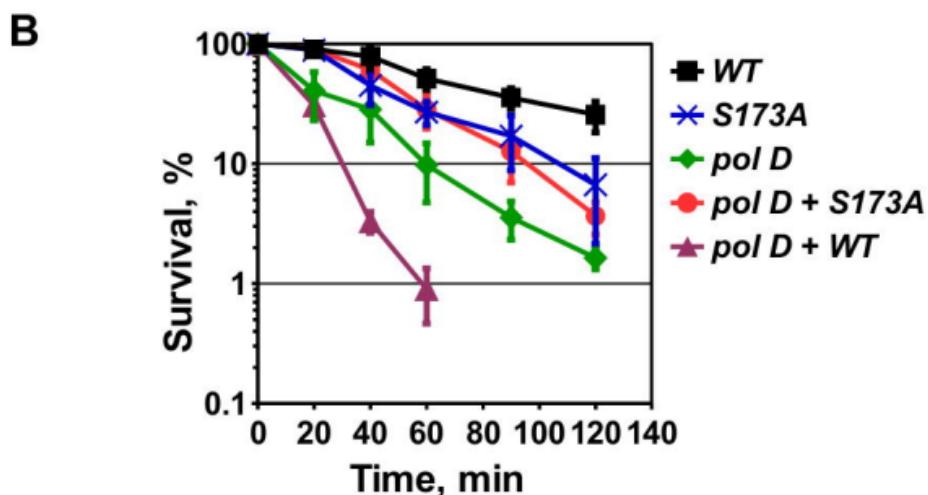
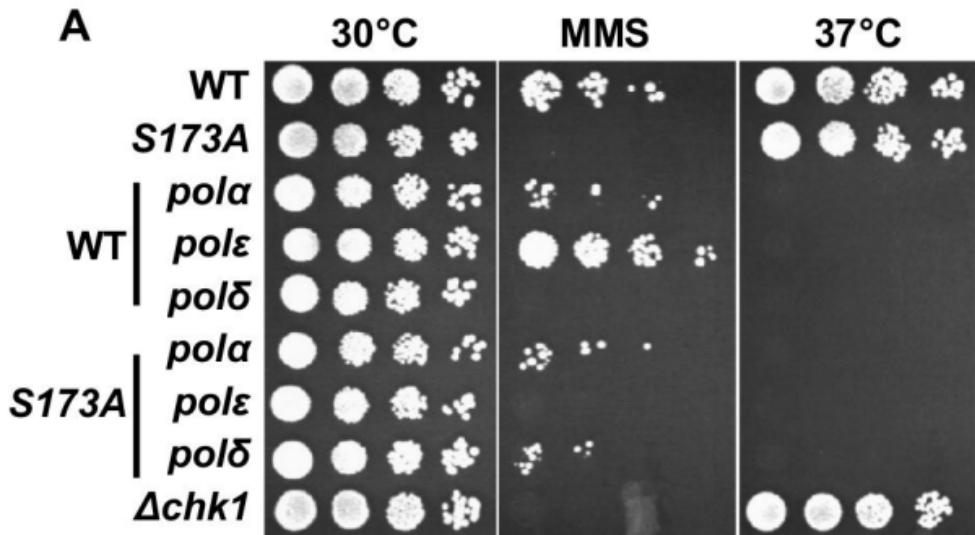
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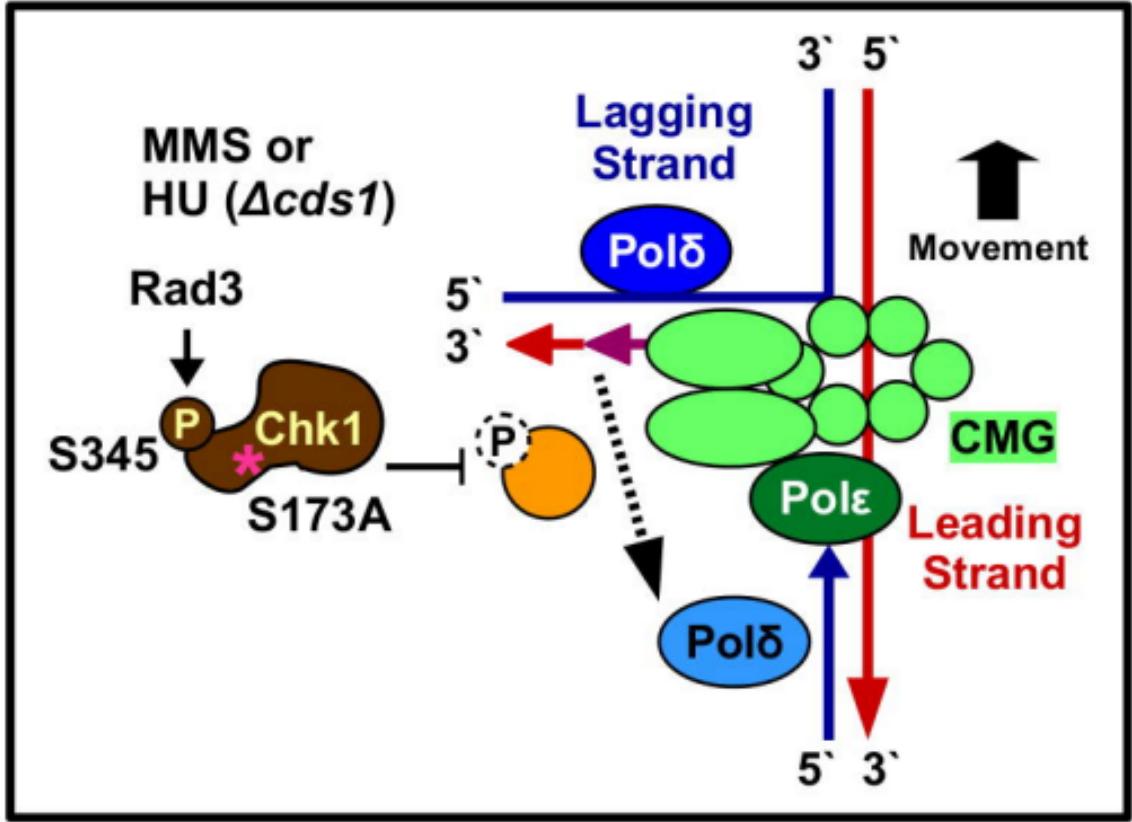
180 min

HU 2C

 $pol\delta$ chk1-WT $pol\delta$ chk1-S173A $pol\epsilon$ chk1-WT $pol\epsilon$ chk1-S173A**E**



A



B

