



The drinking water contaminant dibromoacetonitrile delays G1-S transition and suppresses Chk1 activation at broken replication forks

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Scientific Reports

DOI:

[10.1038/s41598-017-13033-8](https://doi.org/10.1038/s41598-017-13033-8)

Published: 01/01/2017

Peer reviewed version

[Cyswllt i'r cyhoeddiad / Link to publication](#)

Dyfyniad o'r fersiwn a gyhoeddwyd / Citation for published version (APA):

Caspari, T., Dyer, J., Fenner, N., Dunn, C., & Freeman, C. (2017). The drinking water contaminant dibromoacetonitrile delays G1-S transition and suppresses Chk1 activation at broken replication forks. *Scientific Reports*, 7, Article 12730. <https://doi.org/10.1038/s41598-017-13033-8>

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26 **Abstract**

27

28 Chlorination of drinking water protects humans from water-borne pathogens, but it also
29 produces low concentrations of dibromoacetonitrile (DBAN), a common disinfectant by-
30 product found in many water supply systems. DBAN is not mutagenic but causes DNA
31 breaks and elevates sister chromatid exchange in mammalian cells. The WHO issued
32 guidelines for DBAN after it was linked with cancer of the liver and stomach in rodents.
33 How this haloacetonitrile promotes malignant cell transformation is unknown. Using fission
34 yeast as a model, we report here that DBAN delays G1-S transition. DBAN does not
35 hinder ongoing DNA replication, but specifically blocks the serine 345 phosphorylation of
36 the DNA damage checkpoint kinase Chk1 by Rad3 (ATR) at broken replication forks.
37 DBAN is particularly damaging for cells with defects in the lagging-strand DNA polymerase
38 delta. This sensitivity can be explained by the dependency of pol delta mutants on Chk1
39 activation for survival. We conclude that DBAN targets a process or protein that acts at the
40 start of S phase and is required for Chk1 phosphorylation. Taken together, DBAN may
41 precipitate cancer by perturbing S phase and by blocking the Chk1-dependent response to
42 replication fork damage.

43 **Introduction**

44

45 Dibromoacetonitrile (DBAN) is generated at low nanomolar concentrations when bromide
46 reacts with nitrogenous organic matter during the chlorination of drinking water ¹. A survey
47 of 20 water supply systems in England and Wales revealed DBAN as the most abundant
48 haloacetonitrile (HAN) (≤ 40.2 nM, ≤ 8 μ g/L) ². Epidemiological studies in the USA linked the
49 consumption of chlorinated water with an increased risk in bladder, brain and rectal cancer
50 ^{3 4 5}. Very high concentrations of DBAN (> 250 μ M) induce cancer of the liver and stomach
51 in rodents ^{6 7}. The WHO issued guidelines for DBAN (0.35 μ M), dichloroacetonitrile
52 (DCAN; 0.18 μ M) and trichloroacetonitrile (TCAN; < 0.007 μ M) in the response to these
53 findings ⁸.

54 While DBAN is probably not mutagenic, it effectively alkylates DNA *in vitro* which may
55 explain how it breaks DNA and elevates sister chromatid exchange in yeast and
56 mammalian cells ^{9 10 11}. DBAN increases the levels of reactive oxygen species (ROS) in
57 rat cells as indicated by a rise in 8-hydroxy-2-deoxyguanosine (8OHdG) ^{12 13} and blocks
58 aldehyde dehydrogenase ¹⁴, dimethylnitrosamine-demethylase ¹⁵, glutathione-S-
59 transferase ¹⁶, superoxide dismutase and catalase ¹⁷ in the liver. Monobromoacetonitrile
60 (BAN) was shown to induce endoreplication in Chinese hamster ovary cells by blocking
61 mitosis ¹⁸. While these findings indicate a potential health risk, it is still unclear how DBAN
62 precipitates cancer.

63 Using the model organism fission yeast (*Schizosaccharomyces pombe*), we report here
64 that DBAN delays G1-S transition and specifically blocks the activation of the DNA
65 damage checkpoint kinase Chk1 at broken DNA replication forks.

66 The replication machinery assembles around the DNA helicase MCM₂₋₇ at dedicated
67 chromosomal sites (origins) in a stepwise process (reviewed in ¹⁹). The pre-replication

68 complex forms late in G1 when DDK, a heterodimer of Cdc7/Hsk1 kinase and its activating
69 subunit Dbf4/ASK, recruits Sld3 (Treslin) and Cdc45. The cell cycle regulator CDK (cyclin-
70 dependent kinase) activates this complex later at the start of S phase by loading the
71 BRCT-domain protein Rad4 (Dbp11, TopBP1), Sld2, DNA polymerase epsilon and the
72 GINS proteins²⁰. When DNA polymerase alpha-primase and MCM10 associate with this
73 structure, processive DNA replication begins. The lagging strand is displaced in front of
74 the moving replication fork where Cdc45 channels it into DNA polymerase delta. The
75 leading strand runs straight through the fork where it is copied by DNA polymerase epsilon
76^{21 22}. A drop in the nucleotide pool, which can be triggered by the RNR (ribonucleotide
77 reductase) inhibitor hydroxyurea (HU), stalls forks in early S phase by activating the intra-
78 S checkpoint kinase Cds1²³. Cds1 binds to Mrc1 (Claspin) at the stalled fork after both
79 proteins were phosphorylated by Rad3 (ATR)²⁴. Mrc1 associates also with early origins
80 during the G1-S transition, independently of the checkpoint, where it is modified by DDK
81²⁵. The collision of replication forks with immobilised topoisomerase 1, which can be
82 trapped on the DNA by camptothecin (CPT), triggers the phosphorylation of the DNA
83 damage checkpoint kinase Chk1 at serine 345 by Rad3²⁶. Activation of Cds1 and Chk1
84 block both the cell cycle activator Cdc2 (CDK1) thereby initiating a transient G2-M arrest
85²⁷. We report here that DBAN perturbs G1-S transition and blocks Chk1 phosphorylation at
86 a broken replication fork without affecting the Rad3-dependent modification of other
87 checkpoint proteins. DBAN may therefore act on a process or a protein that is required at
88 the start of S phase and later at damaged DNA replication forks. We conclude that DBAN
89 elicits DNA replication stress, a known driver of cancer development²⁸.

90

91 **Results**

92

93 **DBAN interferes with S phase**

94 Informed by the ability of bromoacetonitrile (12 μ M) to block mitosis in Chinese hamster
95 ovary (CHO) cells¹⁸, we first tested whether haloacetonitriles (HANs) impact on cell cycle
96 progression. Wild type cells were synchronised in G2 by lactose gradient centrifugation²⁹
97 and released into medium with 10 μ M bromo-, dibromo-, chloro-, dichloro- or
98 trichloroacetonitrile (BAN, DBAN, CAN, DCAN, TCAN). Samples were withdrawn every
99 20min to score septated G1/S cells. While the monohalogen compounds, BAN and CAN,
100 allowed cells to complete two cell cycle rounds, their dihalogen forms, DCAN and DBAN,
101 delayed entry into the second cycle by 40min and 60min, respectively (figure 1c, d). Unlike
102 CHO cells¹⁸, DBAN-treated yeast cells arrested in G2 before the onset of mitosis (figure
103 S1a, b). Since a second cycle G2 arrest is typical for drugs like hydroxyurea (HU) or
104 camptothecin (CPT) that interfere with DNA replication, we concluded that DBAN and
105 DCAN perturb S phase thereby triggering the G2 delay (figure 1g). HU stalls DNA
106 replication forks by depleting the dNTP pool, whereas CPT breaks forks by immobilising
107 topoisomerase 1 in front of the advancing replication complex. Both events delay onset of
108 mitosis through the Rad3-dependent activation of the checkpoint kinases Cds1 and Chk1,
109 respectively^{27 30}. To investigate whether the DBAN-induced S phase perturbations
110 activate the checkpoint, we synchronised a checkpoint-defective $\Delta rad3 \Delta tel1$ double
111 mutant (Tel1/ATR is the second checkpoint kinase besides Rad3/ATR) in G2 and
112 released cells into medium with or without 10 μ M DBAN. Very unexpectedly, the
113 checkpoint deficient strain arrested for longer compared to wild type cells (figure S1c)
114 showing that the delay is checkpoint-independent. The second unexpected observation
115 was made when we analysed the cell cycle impact of trichloroacetonitrile. Unlike DBAN or

116 DCAN, TCAN (10 μ M) blocked entry into the first cycle for around 280 min (figure 1e).
117 Since such a first cycle arrest is typical for agents that break DNA³⁰ and because HANs
118 were linked with DNA breaks in mammalian cells¹⁰, we tested whether they increase the
119 phosphorylation of histone 2AX at S129 by Rad3 and Tel1, an established marker of
120 chromosomal breaks³¹. Intriguingly, all HANs (BAN, CAN, DBAN, TCAN), with the
121 exception of DCAN, reduced the phosphorylation of H2AX showing that DNA breaks are
122 not the cause of the arrest (figure S1d). Since H2AX is also phosphorylated early during
123 unperturbed S phase³¹, this drop may be caused by depleting the pool of S phase cells
124 due to the G2 arrest. We can however not exclude the possibility that the HANs affect the
125 H2AX phosphorylation directly since BAN and CAN do not show a strong arrest (figure 1a,
126 b). The decline in H2AX phosphorylation was concentration dependent starting at 8 μ M
127 DBAN (figure S1e). We next tested whether the first cycle arrest is unique to TCAN and
128 found that a higher concentrations of DBAN (20 μ M) also delayed cells in the first G2
129 phase (figure 1f). This implies that TCAN is more effective than DBAN in eliciting this
130 response. As in the case of the second cycle arrest, the DNA damage checkpoint was not
131 required (figure S1f). A checkpoint-defective *Δ cds1 Δ chk1* double mutant delayed mitosis
132 for 220min, although this arrest was 40min shorter compared to wild type cells (figure S1f).
133 We did not further investigate this first cycle arrest as we wanted to learn more about how
134 DBAN perturbs S phase given the importance of DNA replication stress in malignant
135 transformation²⁸.

136

137 **DBAN delays G1-S progression**

138 To map the execution point of DBAN in S phase, wild type cells were enriched in G1 by
139 nitrogen starvation and released back into the cell cycle by replenishing the medium with a
140 nitrogen source²⁹. The DNA content was measured by flow cytometry over 8 hours to

141 monitor progression from G1 (1 copy of the chromosomes, 1C) into G2 (two copies, 2C)
142 (figure 2). While most untreated cells reached G2 4h post-release (figure 2a), DBAN and
143 TCAN (10 μ M) treated cells retained a 1C DNA content (figure 2b). We also blocked cells
144 in early S phase with 15mM HU to have an internal marker for unreplicated DNA (figure
145 2b, c). Eight hours after the release from G1, only HU and TCAN treated cells remained
146 arrested, while DBAN permitted the completion of DNA replication (figure 2c). This shows
147 that DBAN only delays G1-S transition, whereas TCAN blocks this step more effectively.
148 None of the other HANs (BAN, CAN, DCAN) had a similar effect, although cells had a
149 slightly larger DNA content at the 8h time point compared to untreated cells (figure 2c).
150 This difference indicates that untreated cells had re-entered the cell cycle as this would
151 average their DNA content at a slightly lower value, while CAN and DCAN treated cells
152 remained in G2 for longer with the full 2C DNA content.

153 To exclude the possibility that the G1 arrest protocol impacts on this interesting finding, we
154 synchronised *nda3-KM311* cells in mitotic prophase. This cold sensitive beta-tubulin
155 mutant stops with condensed chromosomes without a mitotic spindle at 20°C and returns
156 to the cell cycle within minutes upon a temperature up-shift to 30°C³². In line with the first
157 experiment, TCAN prevented the accumulation of G2 cells whereas DBAN only delayed it
158 (figure S1g).

159 We next arrested cells in early S phase by incubating a wild type strain in 15mM HU for
160 3.5h²⁹ to test whether DBAN or TCAN (10 μ M) would impact on DNA replication. While the
161 latter was not the case for DBAN, TCAN-treated cells delayed for 20min compared to
162 untreated cells (figure S2c). Since DNA replication was complete within 60min in the
163 absence of TCAN, a 20min difference (1/3 of S phase) may well be significant.

164 Taken together, these data show that DBAN delays G1-S transition, but allows cells to
165 complete DNA replication. In contrast, TCAN blocks cells effectively before the G1-S

166 transition and delays DNA replication. This conclusion is in line with the higher potency of
167 TCAN as a G2 blocker (figure 1e).

168

169 **DBAN affects DNA polymerase delta**

170 Since DBAN allows DNA replication to proceed after an initial G1-S delay (figure 2c, S1g),
171 we tested whether this affects the three replicative DNA polymerases, alpha (Pol1, *swi7*),
172 epsilon (Pol2, *cdc20*) or delta (Pol3, *cdc6*). Since these essential genes cannot be deleted,
173 we used temperature-sensitive mutants at the semi-restrictive temperature of 30°C. Serial
174 dilutions of the strains were applied to rich medium plates containing no HAN or 10µM
175 DBAN. We also incubated one plate at 37°C to confirm the temperature sensitivity. While
176 mutations in the three essential subunits of Pol delta (*cdc6.23* [catalytic], *cdc27.P11* [non-
177 catalytic], *cdc1.P13* [non-catalytic]) impaired cell viability, mutations in the catalytic
178 subunits of Pol epsilon (*cdc20.M10*) or Pol alpha (*swi7.H4*) did not (figure 3b).

179 Interestingly, deletion of the fourth, non-essential Pol delta subunit, *cdm1*, had also no
180 effect (figure 3c). We next tested mutations in the MCM₂₋₇ helicase that unwinds the DNA
181 template (MCM2 [*cdc19.P1*], MCM4 [*cdc21-M68*] or MCM5 [*nda4-108*]), but failed to
182 detect loss of viability (figure 3c). Also no impact on cell growth was found for mutations in
183 DDK (Cdc7/Hsk1) kinase (*hsk1-1312*) and in the replication factor Rad4 (TopBP1)
184 (*rad4.116*) (figure 3c). Whether a mutation in Ctf4 (*mcl1-1*), which binds Pol alpha to the
185 replication complex, impairs cell viability was difficult to judge since the strain grew very
186 poorly even in the absence of DBAN (figure 3b). Interestingly, none of the DNA pol delta
187 mutants showed a growth defect on TCAN plates even at 20µM (figure 3d). This was
188 unexpected given the high impact of TCAN on cell cycle progression. A possible
189 explanation is provided by the replication delay caused by TCAN (figure S2c) that may
190 prevent loss of viability. The pol delta mutants were also not sensitive to CAN, BAN or

191 DCAN at 10 μ M (figure 3e).

192

193 **DBAN overcomes the intra-S arrest of a pol delta mutant**

194 To find out why pol delta mutants are DBAN sensitive, we synchronised wild type, pol
195 alpha (*swi7.H4*), pol delta (*cdc27.P11*) and pol epsilon (*cdc20.M10*) strains in early S
196 phase using the HU arrest protocol ²⁹. After HU was washed out, cells were released into
197 medium with or without 10 μ M DBAN. Flow cytometry showed that untreated wild type cells
198 completed S phase within 60min (figure 4a). As previously reported ³³, the untreated
199 mutant strains delayed S phase progression at the semi-permissive temperature of 30°C
200 (figure 4b-d). While DBAN had no impact on S phase within the first 60min in the case of
201 the pol alpha and pol epsilon mutants, it did significantly advance DNA replication of the
202 pol delta (*cdc27.P11*) strain (figure 4c). This advancement was clearly detectable at the
203 60min and 90min time points (figure 4c). After 2h, the DBAN-treated *cdc27.P11* cells
204 initiated already the next cell cycle round compared to the untreated sample (figure 4c).
205 Cdc27 connects the catalytic (Cdc6) and non-catalytic (Cdc1) subunits, and binds Pol
206 delta to the DNA sliding clamp PCNA ³⁴. DBAN also advanced DNA replication of the
207 other two mutant strains but later and to a lesser extend (figure 4b, d).

208 These results imply that DBAN abolishes the intra-S phase arrest of the pol delta
209 (*cdc27.P11*) strain, which may be linked with its loss of viability on DBAN plates (figure
210 3b). Since the viability of pol delta mutants depends on Chk1 kinase ³³, we next tested
211 whether DBAN interferes with the activation of the checkpoint kinases Cds1 and Chk1.

212

213 **DBAN suppresses the activation of Chk1**

214 To find out whether DBAN perturbs activation of the intra-S checkpoint kinase Cds1 at
215 stalled forks, asynchronous *cds1-His₆HA₂* cells ³⁵ were incubated with 10 μ M DBAN, 12mM

216 HU or with both chemicals simultaneously for 4h. Total protein extracts were loaded onto a
217 6% phos-tag SDS gel to assay the phosphorylation status of Cds1. Phostag
218 electrophoresis reveals the phosphorylation pattern of proteins as their mobility is inversely
219 related to the extent of their modification³⁶. While DBAN did not promote the modification
220 of Cds1, the kinase was intensively phosphorylated when DNA replication forks stalled in
221 the presence of HU. Although DBAN did not impact on this hyperphosphorylation, it
222 induced a faster migrating band (figure 5a). The latter band could be a
223 hypophosphorylated form of Cds1.

224 We then repeated this experiment with a *chk1-HA₃* strain³⁷ but replaced HU with 12μM
225 camptothecin (CPT) to break DNA replication forks. In contrast to Cds1, DBAN effectively
226 suppressed Chk1 phosphorylation at serine 345 (figure 5b). On normal SDS page, S345
227 phosphorylation was detected as a band shift, as previously reported, which disappeared
228 upon DBAN exposure (figure 5b, lower panel)³⁷. DBAN also induced very slowly
229 migrating, hyperphosphorylated bands independently of CPT. The suppression of Chk1
230 phosphorylation at damaged replication forks could explain why DBAN impairs the viability
231 of DNA polymerase delta mutants which rely on this kinase for survival (figure 3b).

232 To test whether DBAN is an inhibitor of Rad3 kinase, we replaced CPT with MMS (methyl-
233 methanesulfonate) that damages DNA by alkylation³⁸. Since DBAN did not block the
234 MMS-induced phosphorylation of Chk1 (figure S1h), it is unlikely that the HAN impairs
235 Rad3 kinase directly. We then exposed wild type cells (*chk1-HA₃*) to CPT or to the
236 combination of DBAN and CPT on plates to gauge whether replication forks still break.
237 The latter seems to be the case as DBAN rendered wild type cells (*chk1-HA₃*) CPT
238 sensitive (figure 5d). This increase in sensitivity, which was similar to the sensitivity of a
239 kinase-dead *chk1* mutant (*chk1-D155E-HA₃*), indicates that forks still break while DBAN
240 prevents activation of Chk1.

241 To find out when DBAN acts on Chk1 in the cell cycle, we synchronised *chk1-HA₃* cells in
242 early S phase with HU and released them into medium with CPT (12µM) or with CPT and
243 DBAN or TCAN (10µM). In line with the idea that Chk1 is activated once bulk DNA
244 synthesis had been completed³⁹, the shift band of Chk1, indicative of serine 345
245 phosphorylation, appeared 60 min post-release after the levels of the DNA replication
246 marker Mrc1 had declined (figure 5e)²⁴. Since Chk1 is weakly phosphorylated during the
247 HU arrest, all samples displayed a weak shift band that increased strongly when forks
248 were damaged by CPT (figure 5e, bottom panel). The presence of DBAN or TCAN
249 effectively suppressed activation of Chk1 (figure 5f). Since Rad3 modifies also the Rad9
250 subunit of the 9-1-1 checkpoint ring at broken forks (figure 5c)⁴⁰, we repeated this
251 experiment with a HU-synchronised *rad9-HA₃* strain⁴¹. As in the case of Chk1, Rad9
252 phosphorylation is detectable as a band shift. This shift was not affected by DBAN strongly
253 suggesting that Chk1 is specifically targeted by the haloacetonitrile (figure 5g). We noticed
254 however that the Rad9 phosphorylation peaked 30 min earlier in the presence of DBAN
255 (figure 5g, panels 3+4). The phos-tag assay did not reveal any changes in the Rad9
256 phosphorylation pattern which were DBAN specific (figure S1i).

257 Collectively, these results suggest that DBAN kills pol delta mutants (figure 3b) and
258 renders wild type cells sensitive to camptothecin (figure 5d) by preventing the
259 phosphorylation of Chk1 at damaged DNA replication forks.

260

261 Discussion

262

263 The evidence presented here reveal novel activities of DBAN at two stages during the cell
264 cycle, at the G1-S transition and later at damaged replication forks (figure 6). DBAN
265 affects both processes in a negative way as it delays entry into S phase (figure 2) and
266 suppresses phosphorylation of Chk1 (figure 5b, f) without affecting the activation of Rad9
267 (figure 5g) or Cds1 (figure 5a). Replication fork damage can originate from the inhibition of
268 topoisomerase 1 by camptothecin or from mutations in the lagging strand DNA
269 polymerase delta ^{26 33}. Collectively, these findings imply that DBAN blocks an event or a
270 protein that is required for both, entry into S phase and activation of Chk1 at damaged
271 forks (figure 6). Early in S phase, human and yeast cells phosphorylate the histone H2AX
272 in a cell cycle specific manner independently of DNA breaks ^{31 42}. The same chromatin
273 modification is later required for the recruitment of Crb2 (53BP1) to a broken fork where
274 the scaffold protein associates with Chk1 ^{43 44}. DBAN and TCAN may therefore
275 compromise both processes by reducing H2AX phosphorylation (figure S1d,e). For
276 example, the HANs may up-regulate a phosphatase, like human PP2A-B56 ϵ , that
277 dephosphorylates H2AX at a damaged forks upon CPT treatment ⁴⁵. What argues against
278 this model is the survival of the DNA pol delta mutants in the presence of BAN, CAN or
279 TCAN (figure 3) which all reduce H2AX phosphorylation (figure S1d). An alternative
280 explanation is provided by the dual function of Rad4/Cut5 (TopBP1) during G1-S transition
281 and in the activation of Chk1 ^{46 47}. Rad4 associates with Sld3 and Sld2/Drc1 at start and
282 with Crb2 at broken DNA ^{44 46}. What argues however against Rad4 is its requirement for
283 the activation of Cds1 at stalled forks ⁴⁷ which is not affected by DBAN (figure 5a). A third
284 candidate is DDK kinase as it initiates the assembly of the replication complex at the end
285 of G1 (reviewed in ¹⁹) and terminates Chk1 activation by phosphorylating the Rad9 subunit
286 of the 9-1-1 complex ⁴⁸. We have however not found any evidence that the Rad9

287 phosphorylation pattern changes upon DBAN exposure (figure S1i). A fourth possibility is
288 provided by Mrc1 (Claspin) that associates with early replication origins at start²⁵ and
289 recruits human Chk1 to broken forks⁴⁹. A similar interaction between *S.pombe* Chk1 and
290 Mrc1 has not yet been reported. Finally, DBAN may act directly on Chk1 as indicated by
291 its hyper-phosphorylation (figure 5b). Chk1 is required in *S.pombe* and human cells at the
292 start point of the G1-S transition^{50 51} and at broken forks²⁶. Further work will however be
293 required to dissect these different possibilities that are of great interest as it still enigmatic
294 why human Chk1 occupies a dominant role in S phase whereas yeast Chk1 appears to act
295 mainly in G2 (figure 5e)³⁹.

296 DBAN is known to block a large number of enzymes *in vitro*⁷ but it is as yet unclear how it
297 interacts with proteins. Halogen atoms like bromine and chlorine have a positive charge,
298 known as the alpha-hole that can make electrostatic contacts with the protein backbone or
299 amino acid side chains⁵². While bromine interacts preferentially with the side chain of
300 arginine, chlorine prefers leucine⁵³. Whether this explains why TCAN is a stronger cell
301 cycle inhibitor than DBAN (figure 1) is difficult to tell as it is unclear to which protein they
302 bind. It is even not entirely safe to conclude that they bind to the same protein. DBAN and
303 TCAN behave in a similar way regarding the G1-S delay (figure 2) and the inhibition of
304 Chk1 phosphorylation (figure 5f). Both HANs differ however in their lethality when DNA
305 polymerase delta is mutated (figure 3d). Although this appears to contradict the earlier
306 notion that DBAN kills these mutant strains by blocking Chk1 phosphorylation at damaged
307 forks, it could be explained by the ability of TCAN to delay DNA replication (figure 4c). If
308 this delay were to prevent fork damage in pol delta mutant cells, the inhibition of Chk1
309 would not affect cell viability.

310 Although DNA replication stress is a good explanation for why DBAN (10µM) triggers a
311 second cycle delay (figure 1d), it would not provide an answer to why 20µM DBAN block

312 cells in the first G2 (figure 1f). The concentration dependency implies that DBAN has
313 either more than one target in cells with different affinities for the HAN or that DBAN
314 upregulates the expression or activity of a protein in a concentration dependent manner.
315 From the different options discussed earlier, the up-regulation of a phosphatase like PPA2
316 would connect the diverse cell cycle activities of DBAN and TCAN. Interestingly, induction
317 of PPA2 arrest cells in G2 independently of the DNA damage checkpoint when the
318 accessory protein Vpr of human immunodeficiency virus type 1 (HIV-1) is over-expressed
319 in *S.pombe*⁵⁴. Since this arrest shares a similar independence from the Rad3-Tel1
320 checkpoint as the G2 arrest produced by DBAN and TCAN (Figure 1e,f, S1f), it is quite
321 possible that both HANs delay cell cycle progression in G2 through a mechanism that
322 involves the activation of a phosphatase acting on the cell cycle machinery.

323 The final point to consider is whether DBAN, which is frequently found in water supply
324 systems², poses a serious cancer risk. The concentration of 10 μ M used in this study is
325 approximately 30 times higher than the WHO guideline of 0.35 μ M (70 μ g/L). It is therefore
326 unlikely that DBAN levels reach such high concentrations in tap water. The peak
327 concentration found in water supply systems in Western Australia, for example, was
328 0.13 μ M (26.6 μ g/L)⁵⁵, whereas the peak value in the United Kingdom was with 0.04 μ M
329 (8 μ g/L) much lower². It is however not yet clear whether haloacetonitriles accumulate over
330 a longer consumption period in the liver, gastrointestinal tract or the kidneys⁷. The latter
331 may explain why the consumption of chlorinated drinking water was linked with cancer³.

332 **Materials and Methods**

333

334

335 **Yeast Strains**

336 The genotype of the strains used in this study is *ade6-M210 leu1-32 ura4-D18*. Wild type
337 cells contained no additional mutation and the different mutant alleles are mentioned in the
338 text. The *cds1-HA₂His₆ [URA4+] ura4-D18* strain is described in ³⁵ and the *chk1-HA₃* strain
339 in ³⁷. Before cells were synchronised in G1 by nitrogen starvation, all auxotrophic markers
340 were crossed out.

341

342 **Cell synchronisation**

343

344 Cells were synchronised as described in ²⁹. HU was used at a final concentration of 15mM
345 for 3.5h at 30 °C in rich medium. For the G1 arrest, cells without auxotrophic markers
346 were first grown in minimal medium with nitrogen before being transferred to minimal
347 medium without nitrogen for 16h at 30°C. Lactose gradients were centrifuged for 8min at
348 800rpm. The *nda3.KM311* mitotic arrest was performed in rich medium as reported in ⁵⁶.

349

350 **Flow cytometry**

351

352 The DNA content was measured using a CUBE 8 (Sysmex) instrument as described in ²⁹.

353 The histograms were produced using the free Flowing Software

354 (<http://flowingsoftware.btk.fi/>)

355

356 **Phos-tag SDS page**

357

358 Phostag gels (6%) were prepared and run as reported in ⁵⁷.

359

360

361 **Antibodies**

362

363 Anti-HA antibody (BioSource, Covance MMS-101P-200), anti-Mrc1 antibody (ABCAM,

364 ab188269), anti-Cdc2 antibody (ab5467) and anti-Histone 2AX-S129-P antibody

365 (ab17576). Secondary mouse-HRP (Dako, P0447), secondary rabbit-HRP (Dako, P0217).

366

367 **Protein extracts**

368 Total protein extracts were prepared as described in ⁴¹

369

370 **Chemicals**

371 Bromoacetonitrile (Sigma Aldrich, 242489), dibromoacetonitrile (Alfa Aesar, A16994),

372 chloroacetonitrile (Sigma Aldrich, C19651), dichloroacetonitrile (Alfa Aesar, A10612),

373 trichloroacetonitrile (Sigma Aldrich, T53805), phostag-acrylamide (AlphaLabs, AAL-107,

374 300-93523), hydroxyurea (Formedium, HDU0025).

375

376 **Figure Legends**

377

378 **Figure 1. Dibromoacetonitrile (DBAN) arrests cell cycle progression in a**
379 **concentration dependent manner.** Wild type cells (*ade6-M210 leu1-32 ura4-D18*) were
380 synchronised in G2 by lactose gradient centrifugation²⁹ and released into rich medium
381 (3% glucose, 0.5% yeast extract, 100mg/L adenine) without (UT = untreated) or with 10µM
382 of the indicated haloacetonitriles (HANs). All HANs were diluted from a 12mM stock
383 solution in DMSO. **(a)** bromoacetonitrile (BAN), **(b)** chloroacetonitrile (CAN), **(c)**
384 dichloroacetonitrile (DCAN), **(d)** dibromoacetonitrile (DBAN), **(e)** trichloroacetonitrile
385 (TCAN), **(f)** DBAN at 10µM or 20µM, respectively. **(g)** DBAN affects cells in S phase which
386 triggers a second cycle delay.

387

388 **Figure 2. DBAN and TCAN delay G1-S transition**

389 **(a)** Wild type cells without auxotrophic markers were synchronised in G1 by nitrogen
390 starvation in minimal medium (3% glucose, 0.67% nitrogen base w/o amino acids and
391 ammonium sulphate) at 30°C²⁹. Cells were washed and released at T=0h into pre-
392 warmed minimal medium with ammonium sulphate. Samples were withdrawn at the
393 indicated time points and the DNA content was measured by flow cytometry²⁹. The dotted
394 lines indicate 1 copy of the chromosomes (1C, G1) and two copies (2C, G2), respectively.
395 **(b)** Comparison of the DNA content at the start of the experiment (0h, green histogram)
396 with the DNA content 4h after release (red histogram). Cells were released into medium
397 with 10µM of the indicated haloacetonitriles or 15mM hydroxyurea (HU). **(c)** Comparison
398 of the DNA content 8h post-release. The blue histogram is the DNA content of untreated
399 cells (UT) and the red histogram is the DNA content of cells treated with the indicated
400 chemicals. Nitrogen starvation arrests cells in G1 before start, whereas HU arrests cells in

401 early S phase.

402

403 **Figure 3. DBAN kills cells mutated in DNA polymerase delta**

404 **(a)** Model of the replication fork (adapted from²²). **(b-e)** Serial dilutions of the indicated
405 strains were applied to rich medium plates at 30°C. One plate was incubated at 37°C.
406 Incubation time: 3 days. The final concentration of DBAN was 10µM, but 20µM for TCAN.
407 The mutant alleles are: pol alpha (*swi7.H4*), Ctf4 (*mcl1-1*), pol delta (*cdc6.23*, *cdc27.P11*,
408 *cdc1.p13*), pol epsilon (*cdc20.M10*), S phase cell cycle inhibitor Mik1 (*mik1::ura4+*), cell
409 cycle inhibitor Wee1 (*wee1::ura4+*), Δ *cdm1* (non-essential pol delta subunit, *cdm1::ura4+*),
410 MCM2 (*cdc19.P1*), MCM4 (*cdc21-M68*), MCM5 (*nda4-108*), DDK/Cdc7 (*hsk1-1312*), Rad4
411 (TopBP1) (*rad4.116*), MAP kinase Sty1 (*sty1::ura4+*). Final concentration of BAN, CAN
412 and DCAN is 10µM.

413

414 **Figure 4. DBAN advances DNA replication in a pol delta mutant**

415 **(a)** Wild type cells (*ade6-M210 leu1-32 ura4-D18*) were grown in rich medium at 30°C and
416 synchronised in early S phase for 3.5h in 15mM hydroxyurea (HU)²⁹. HU was washed out
417 and cells were released into pre-warmed rich medium. The DNA content was measured at
418 the indicated times. The dotted lines indicate 1 copy of the chromosomes (1C, G1) and
419 two copies (2C, G2), respectively. The DNA content of nitrogen starved cells (no N) was
420 measured in a parallel experiment to have an internal standard. **(b-d)**. The indicated
421 mutant strains were HU-synchronised and released into rich medium with or without 10µM
422 DBAN. The green histogram is the DNA content of untreated cells, the red histogram is the
423 DNA content in the presence of DBAN.

424

425 Figure 5. DBAN suppresses Chk1 phosphorylation

426 **(a-b)** *cds1-His₆HA₂* and *chk1-HA₃* cells were grown in rich medium at 30°C and treated for
427 4h with 10µM DBAN, 12µM camptothecin (CPT), 12mM hydroxyurea (HU) or the
428 combination as indicated. Total protein extracts were loaded on a 6% phostag gel. H2AX
429 phosphorylation at S129 was detected after electrophoresis on a 20% acrylamide (37.5:1
430 acrylamide:bisacrylamide) gel, whereas the Chk1 shift was detected after electrophoresis
431 on a 10% (100:1 acrylamide:bisacrylamide) gel. The arrows indicate the smaller Cds1
432 band, Chk1-S345 phosphorylation and the hyper-phosphorylation of Chk1. **(c)** Model of
433 Chk1 activation by Rad3 at broken replication forks. CPT immobilises Topoisomerase 1
434 and the Rad9-Rad1-Hus1 ring aids Chk1 phosphorylation. Rad9 is also phosphorylated by
435 Rad3⁴⁰. **(d)** Drop test of the indicated strains. Chk1-D155E-HA3 is a kinase-dead mutant.
436 DBAN: 10µM; CPT: 12µM. **(e-f)** *chk1-HA₃* cells were HU synchronised (3.5h, 15mM HU,
437 rich medium) and released into medium without (UT) or with CPT (12µM), CPT (12µM) +
438 DBAN (10µM) or CPT (12µM) + TCAN (10µM). Total protein extracts were separated on a
439 8% (Mrc1) and 10% (Chk1) acrylamide gel. P = Phospho-Chk1-S345 shift band). **(g)**
440 *rad9-HA₃* cells were HU-synchronised and released into rich medium without (UT) or with
441 CPT (12µM), DBAN (10µM) or CPT (12µM) + DBAN (10µM). P = Phospho-Rad9 shift
442 band.

443

444 Figure 6. Model

445 **(a)** DBAN blocks G1-S transition. **(b)** DBAN prevents Chk1 phosphorylation by Rad3 at a
446 broken fork. The details of the model are discussed in the main text.

447

448 Supplementary Figure 1.

449 **(a-b)** Wild type cells (*ade6-M210 leu1-32 ura4-D18*) were synchronised by lactose

450 gradient centrifugation in G2 and released into rich medium without (UT) or with 10µM
451 DBAN (DB). (a) septated G1/S cells, (b) mitotic cells. (c) Checkpoint defective *rad3::ade6+*
452 *tel1::leu2+* cells. (d) Wild type cells were treated with 10µM of the indicated
453 haloacetonitriles for 3h at 30°C. Phosphorylated H2AX-S129 was detected using a
454 phosphospecific antibody. Cdc2 = loading control. (e) Concentration dependency of the
455 decline in H2AX phosphorylation. (f) The indicated strains were G2-synchronised and
456 exposed to 20µM DBAN. (g) *nda3.KM311* mutant cells were synchronised ³² at 20°C for
457 8h and released into rich medium at 30°C. The DNA content of the arrested cells (red) and
458 of cells 70 min post-release (green) is shown. The arrow indicates G2 cells. (h) *chk1-HA₃*
459 cells were treated with 0.05% MMS (methyl-methanesulfonate) or 12µM CPT with or
460 without 10µM DBAN for 3h at 30°C. (i) phos-tag analysis of the Rad9 protein samples
461 shown in figure 5g.

462

463 **Supplementary Figure 2.**

464 Wild type cells (*ade6-M210 leu1–32 ura4-D18*) were synchronised in early S for 3.5h with
465 15mM HU at 30°C and released into pre-warmed rich medium without a drug (a), with or
466 without 10µM DBAN (b), with or without 10µM TCAN (c). The DNA content of the
467 untreated cells (green) and of cells in the presence of the HAN (red) is shown. The dotted
468 line indicates cells with a 2C (G2) DNA content.

469

470 **Acknowledgements**

471 We are grateful to George Meyrick for matched funding in support of the KESS
472 (Knowledge Economy Skills Scholarships) Master project (F005714).

473

474 **Author Contributions**

475 TC wrote the manuscript, designed the study, performed most experiments and analysed
476 the data. JD performed some experiments. NF, CD and CF are co-applicants with TC on
477 the KESS grant and initiated the project. All authors reviewed the manuscript.

478

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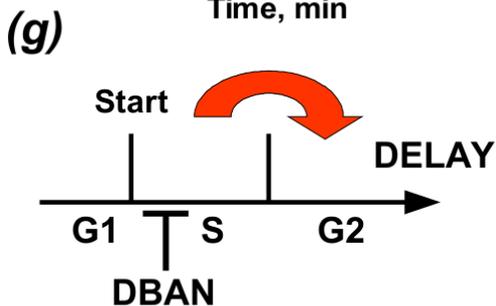
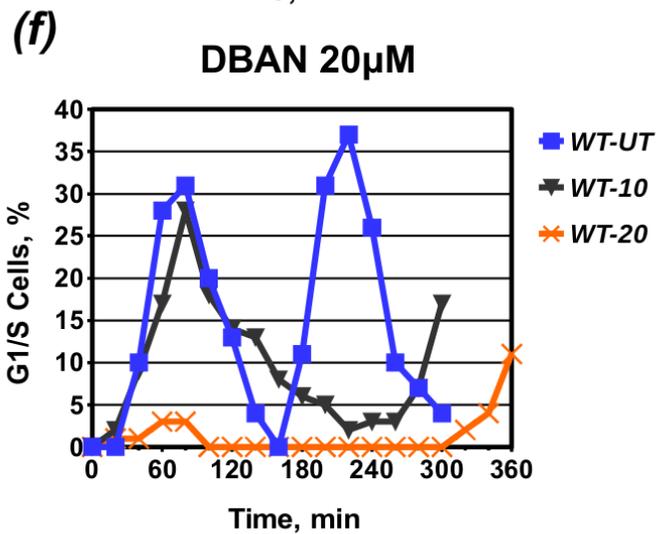
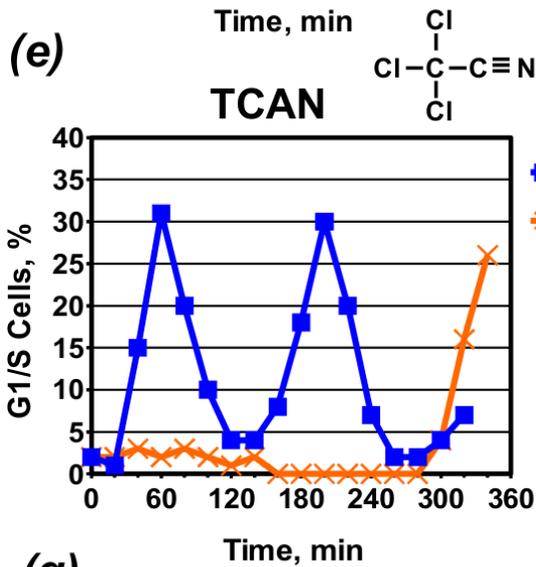
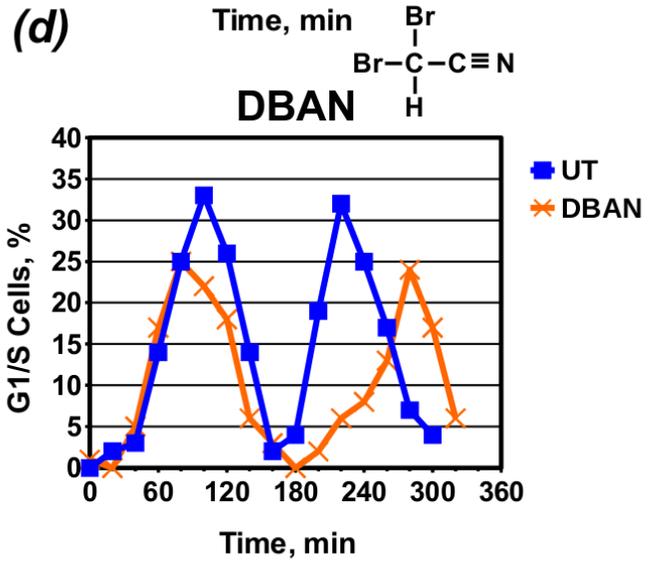
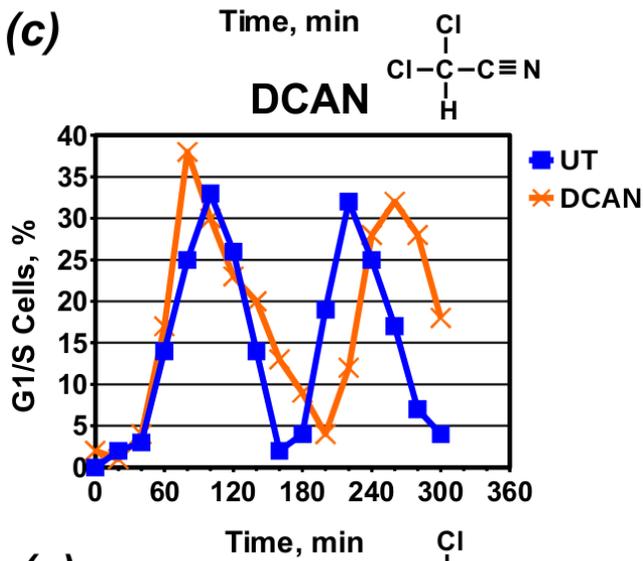
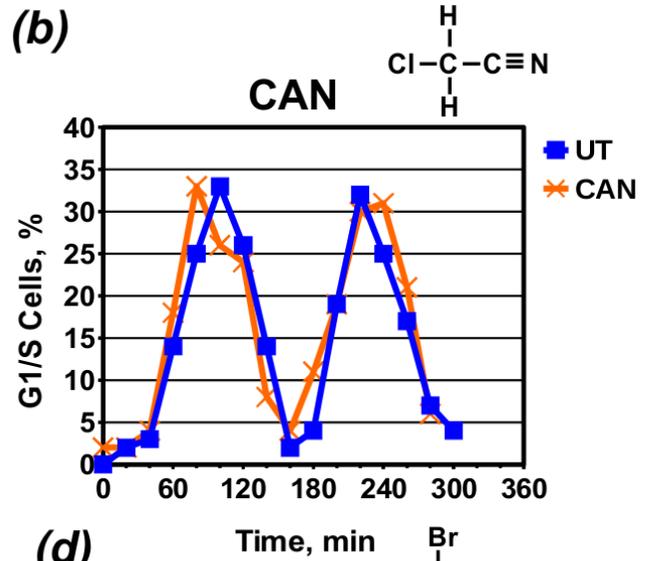
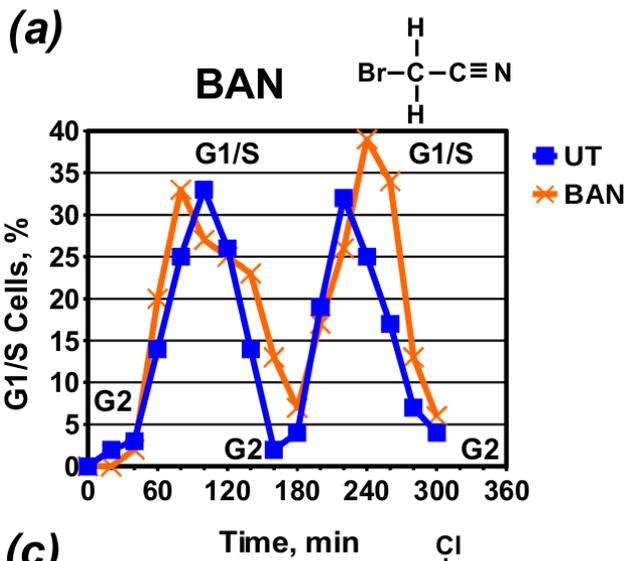
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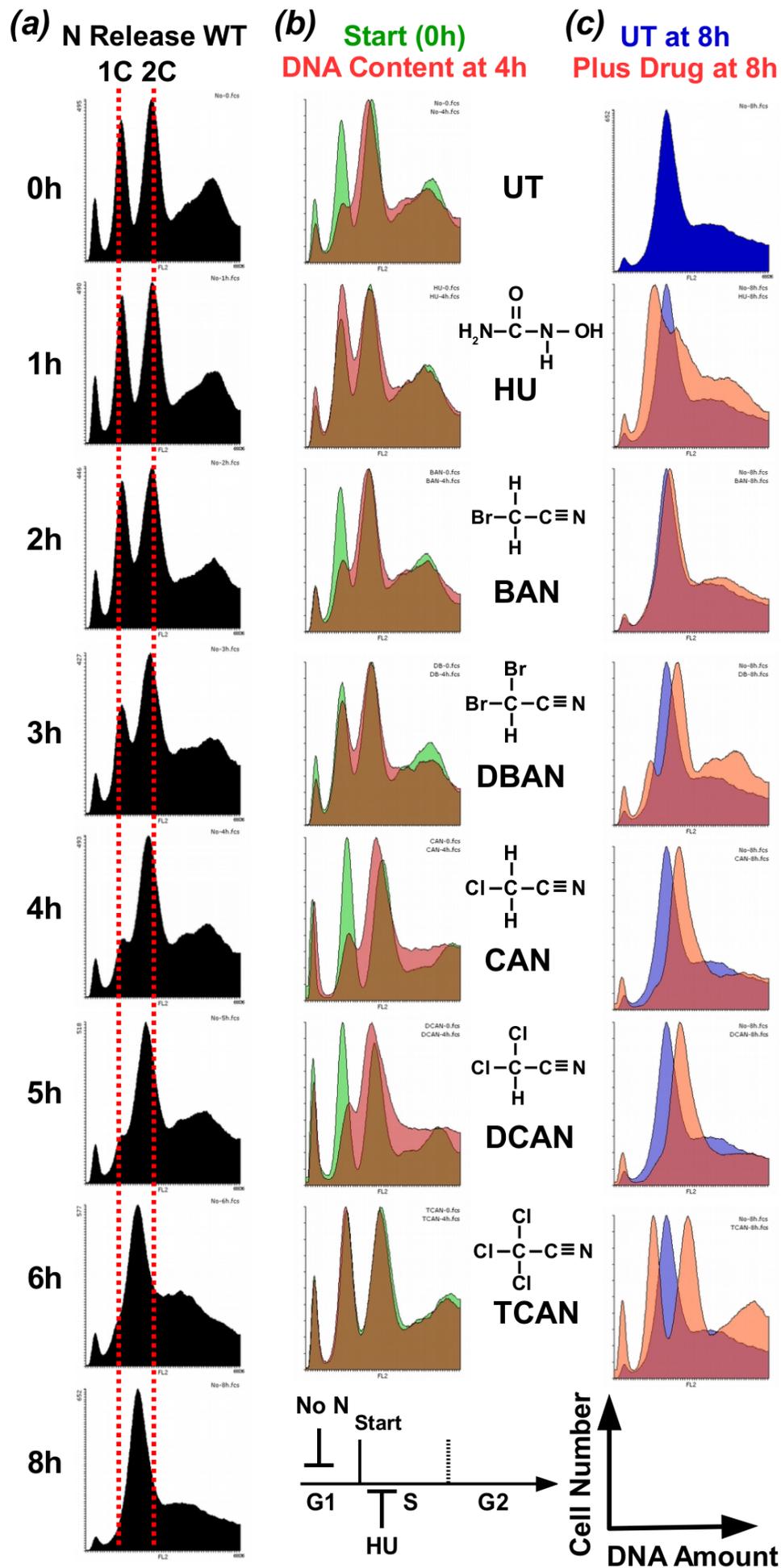
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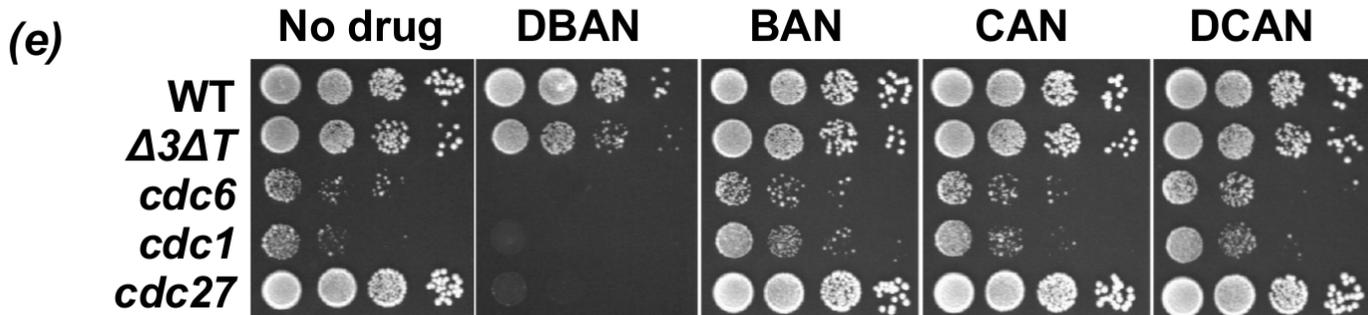
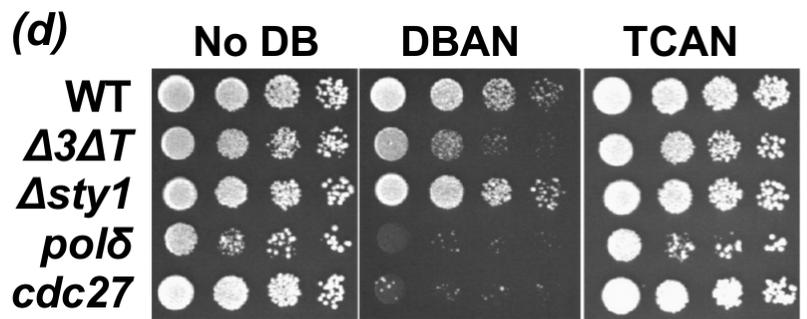
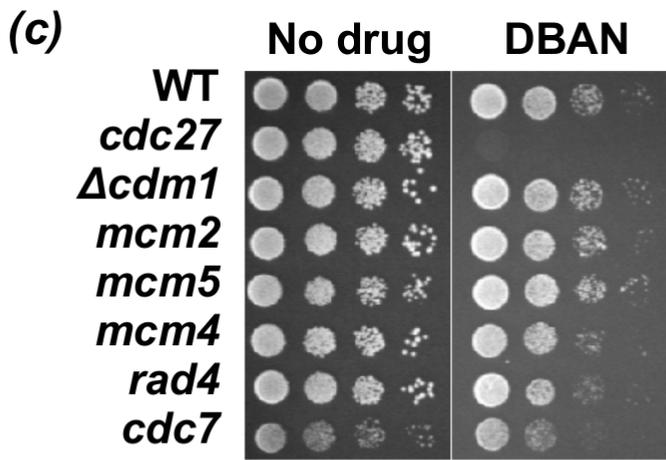
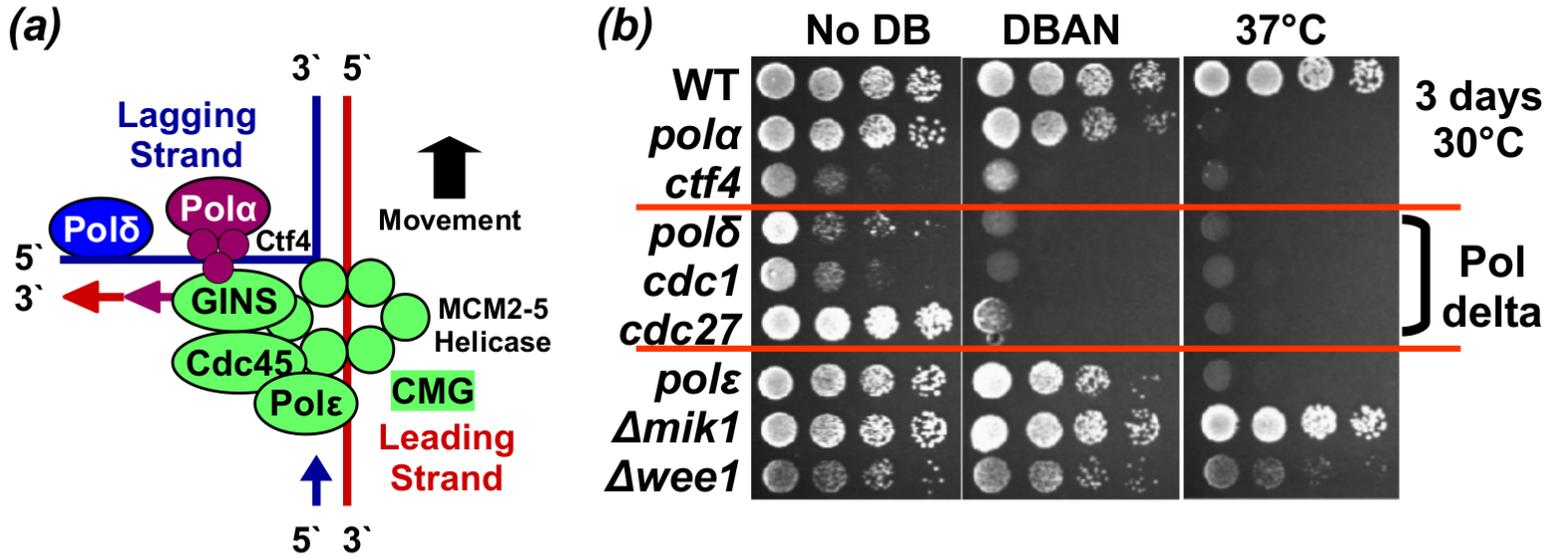
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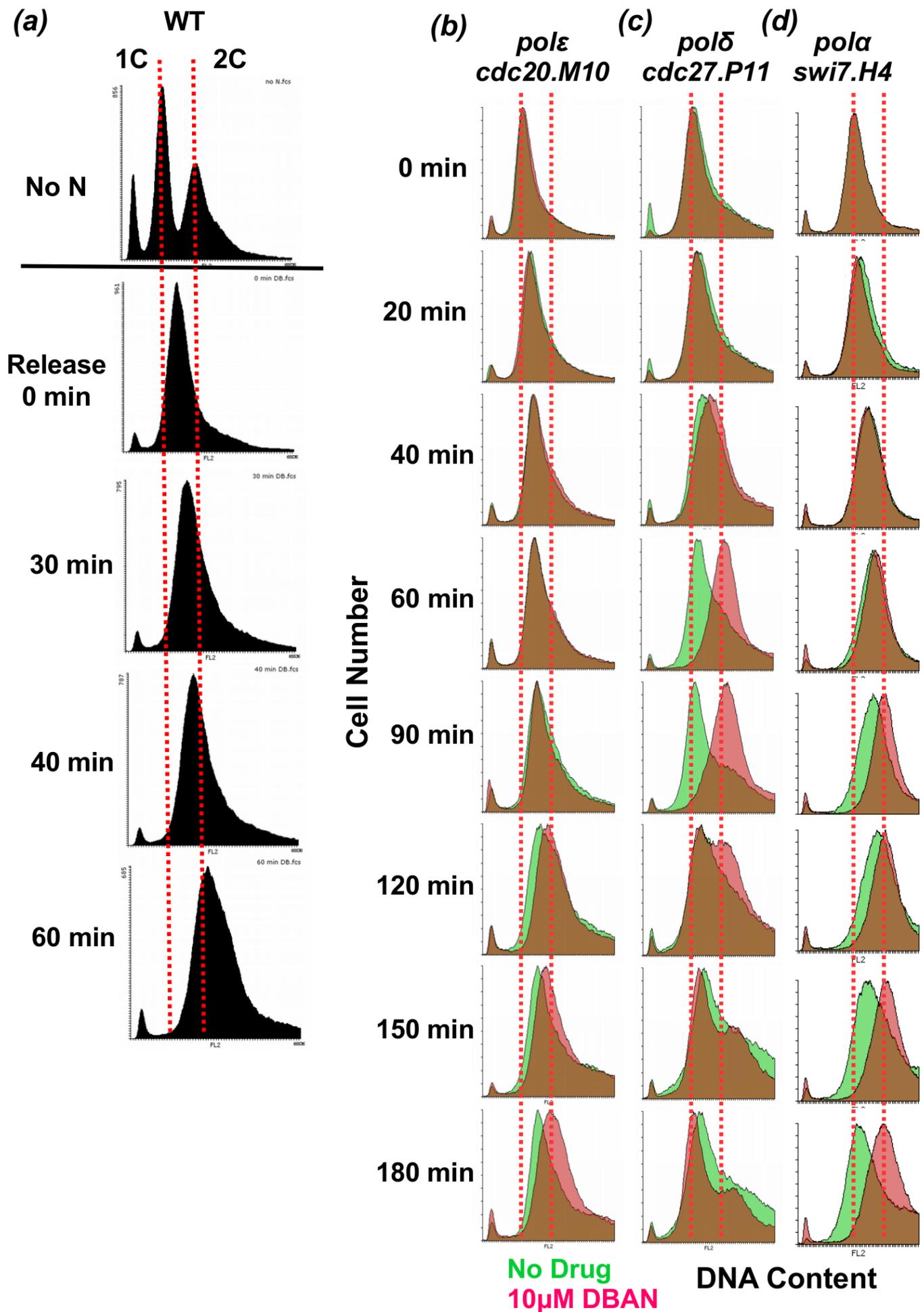
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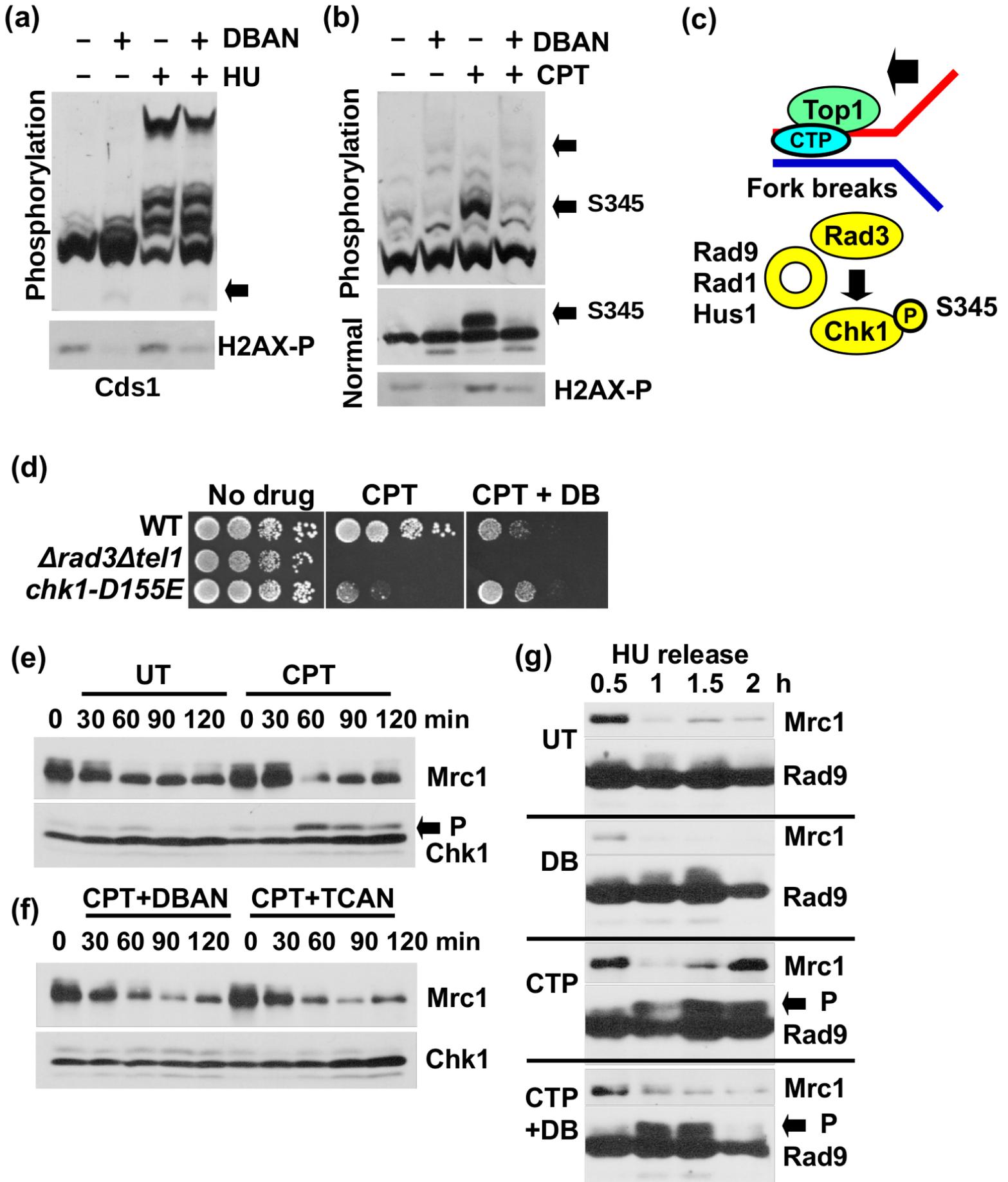


Caspari et al Fig 3 DNA polymerase delta mutants are DBAN sensitive





Caspari et al Fig 5 DBAN suppresses Chk1 activation



Caspari et al Figure 6

