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Title:

Crosstalk between repair pathways elicits Double-Strand Breaks in alkylated DNA: implications for the action of temozolomide.

Key words: alkylating agents / SN1 versus SN2 /O-alkylation versus N-alkylation/ unbiased discovery of DNA Binding proteins/ IDAP/ temozolomide / Mismatch Repair and Base Excision Repair crosstalk / protein-DNA pull-down assay / Mass Spectrometry protein identification / neighboring lesions / double-strand breaks

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Until now, the cytotoxic effect of SN1 alkylating agents was attributed to MMR acting on O⁶mG:T lesions that form following replication. Our paper highlights that O⁶mG:C lesion *per se* is a MMR target that can trigger DSB through coupling with BER.

48 **Abstract:**

49 Temozolomide (TMZ), a DNA methylating agent, is the primary chemotherapeutic drug used
50 in glioblastoma treatment. TMZ induces mostly N-alkylation adducts (N7-methylguanine and
51 N3-methyladenine) and some O⁶-methylguanine (O⁶mG). Current models propose that during
52 DNA replication, thymine is incorporated across from O⁶mG, promoting a futile cycle of
53 mismatch repair (MMR) that leads to DNA double strand breaks (DSBs). To revisit the
54 mechanism of O⁶mG processing, we reacted plasmid DNA with N-Methyl-N-nitrosourea
55 (MNU), a temozolomide mimic, and incubated it in *Xenopus* egg-derived extracts. We show
56 that in this system, mismatch repair (MMR) proteins are enriched on MNU-treated DNA and
57 we observe robust, MMR-dependent, repair synthesis. Our evidence also suggests that MMR,
58 initiated at O⁶mG:C sites, is strongly stimulated *in cis* by repair processing of other lesions,
59 such as N-alkylation adducts. Importantly, MNU-treated plasmids display DSBs in extracts, the
60 frequency of which increased linearly with the square of alkylation dose. We suggest that
61 DSBs result from two independent repair processes, one involving MMR at O⁶mG:C sites and
62 the other involving BER acting at a nearby N-alkylation adducts. We propose a new,
63 replication-independent mechanism of action of TMZ, that operates in addition to the well-
64 studied cell cycle dependent mode of action.

65

66

67 **Introduction:**

68 Alkylating agents, a class of important environmental carcinogens, have been widely
69 used in molecular biology to study fundamental repair processes and in the clinic to treat
70 cancer patients. Among the DNA adducts produced by methylating agents such as N-methyl-
71 N-nitrosourea (MNU) and temozolomide (TMZ), a clinically used mimic, the most abundant
72 are two N-alkylation adducts, at the N7 position of guanine (7mG: 70-75% of total alkyl
73 adducts) and the N3 position of adenine (3mA: 8-12%). Importantly, both reagents also
74 produce 8-9% O-alkylation adducts in the form of O⁶-methylguanine (O⁶mG). This feature
75 contrasts with another common methylating agent, methyl-methane sulfonate (MMS), which
76 forms a much lower level of O⁶mG (<0.3%) while producing similarly high proportions of 7mG
77 (81-83%) and 3mA (10-11%)(Beranek, 1990). For many years, the differences in O versus N
78 reactivities have been rationalized by differences in chemical reaction mechanisms; on one
79 side, compounds such as MMS, with very low O-reactivity were classified as an SN2 agent
80 (bimolecular nucleophilic substitution) while other agents, such as MNU and TMZ, with
81 increased O adduct formation were called SN1 agents (monomolecular nucleophilic
82 substitution). While this classification turned out not to be mechanistically accurate (Loechler,
83 1994), we will nevertheless use this nomenclature throughout this paper for the sake of
84 simplicity. The major N-alkylation (N-alkyl) adducts (7mG and 3mA) are repaired by base
85 excision repair (BER), using N-methylpurine-DNA glycosylase (MPG) also known as 3-
86 alkyladenine DNA glycosylase (AAG) and alkylpurine DNA N-glycosylase (APNG) (Chakravarti et
87 al., 1991; Lindahl, 1976). O-alkylation adducts (O⁶mG, O⁴mT) can be directly repaired by O⁶-
88 methylguanine-DNA methyl transferase (MGMT), a protein that transfers the methyl group
89 from these adducts to one of its cysteine residues (Demple et al., 1982; Olsson and Lindahl,
90 1980; Tano et al., 1990). In addition, alkylating agents also produce a variety of other minor
91 (1-2%) N-alkyl adducts, namely 1mA, 3mC, 3mT and 1mG that are directly demethylated by
92 AlkB homologs (Aas et al., 2003; Duncan et al., 2002; Falnes et al., 2002; Trewick et al., 2002).

93 In summary, SN1 and SN2 alkylating agents produce a diverse array of DNA adducts, but they
94 differ greatly in the amount of O⁶mG produced.

95 Agents such as MMS mostly induce N-alkyl adducts that lead to DSBs during S-phase
96 as a consequence of BER repair. Indeed, inactivation of the AAG glycosylase, the BER initiating
97 enzyme, suppresses DSB while inactivation of Pol β leads to their exacerbation (Simonelli et
98 al., 2017; Tang et al., 2011; Trivedi et al., 2005). In rodent cells, it was proposed that MMS-
99 induced DSBs arise when replication meets BER-induced single-strand breaks (Ensminger et
100 al., 2014). The toxicity of N-alkyl adducts was found to depend on the cell type. AAG-
101 mediated repair of N-alkyl adducts was found to mitigate toxicity in mouse ES cells and HeLa
102 cells, while repair was shown to cause toxic intermediates in retina and bone marrow cells
103 (Meira et al., 2009). In all cell type, O-alkyl adducts were found to be highly cytotoxic and
104 mutagenic. While the mutagenicity of O⁶mG is easily accounted for by its high propensity to
105 mispair with T during DNA synthesis (Bhanot and Ray, 1986; Loechler et al., 1984; Mazon et
106 al., 2010) its cytotoxicity is intriguing since O⁶mG *per se* does not interfere with DNA
107 synthesis. A seminal paper, published 50 years ago by Plant and Roberts (Plant and Roberts,
108 1971), noted that when synchronized HeLa cells are treated in G1 with MNU, they continue
109 through the first cell cycle almost normally and with little effect on DNA synthesis. On the
110 other hand, there is a dramatic effect on DNA synthesis in the second cell cycle after MNU
111 exposure. These data led the authors to surmise that cytotoxicity stems from a secondary
112 lesion that forms when DNA synthesis occurs across O⁶mG template adducts (Plant and
113 Roberts, 1971). It was demonstrated later that MNU-mediated inhibition of DNA synthesis, in
114 the first and second cycle, is due to the action of the mismatch repair machinery (MMR) that
115 acts on O⁶mG:T lesions that form upon DNA synthesis (Goldmacher et al., 1986; Kat et al.,
116 1993)(Noonan et al., 2012; Plant and Roberts, 1971; Quiros et al., 2010).

117 Indeed, O⁶mG:T lesions were found to be excellent substrates for MMR (Duckett et al.,
118 1999; Yoshioka et al., 2006). During MMR gap-filling, the O⁶mG:T mispair is reformed,
119 potentially leading to another round of MMR, thus entering so-called futile MMR cycles
120 (Kaina et al., 2007; Karran and Bignami, 1994; Olivera Harris et al., 2015; York and Modrich,
121 2006). The MMR cycling model has received experimental support *in vitro* (York and Modrich,
122 2006) and in *E. coli* (Mazon et al., 2010). Studies with synchronized cells have shown that the
123 critical events related to cytotoxicity occur in the 2nd cell cycle post treatment (Quiros et al.,
124 2010). However, as discussed in recent review articles, the precise mechanism by which MMR
125 leads to DSBs has yet to be established (Gupta and Heinen, 2019; Kaina and Christmann,
126 2019).

127 While most studies have been devoted to MNU-induced cell cycle effects, in the
128 present paper we wanted to investigate the early response to MNU treatment, i.e. in the
129 absence of replication. We addressed this question using *Xenopus* egg-derived extracts,
130 which recapitulate most forms of DNA repair (Wühr et al., 2014). Upon incubation in these
131 extracts, plasmids treated with MNU, exhibit robust repair synthesis in the absence of
132 replication. Repair synthesis occurs at O⁶mG:C lesions, depends on MMR, and involves an
133 excision tract of several hundred nucleotides. MMR events at O⁶mG:C sites are robustly
134 stimulated by additional processing at N-alkylation lesions, most likely via BER. Previous
135 studies have described activation of MMR in the absence of replication in cells treated by
136 SN1-methylating agents, a process termed noncanonical MMR (ncMMR) (Peña-Diaz et al.,
137 2012). Interestingly, we observed replication-independent induction of DSBs in MNU-treated
138 plasmids. The kinetics of DSB formation obeys a quadratic MNU dose-response suggesting the

139 involvement of two independent repair events. We propose that DSBs occur when the gap
140 generated at a O⁶mG adduct during MMR overlaps with a BER intermediate initiated at a N-
141 alkyl adducts in the opposite strand.

142 These data reveal a novel facet of MNU-induced damage to DNA that is replication
143 independent. Extrapolation of the *in vitro* data led us estimate that \approx 10 DSBs per cell can be
144 induced by a single daily dose of TMZ used in the clinic in the absence of replication.

145

146 **Results:**

147

148 1. Reaction conditions leading to similar levels of DNA alkylation:

149 Our goal is to determine the DNA proteome for distinct alkylating agents. For sake of
150 comparison, we needed to determine the reaction conditions for different alkylating agents
151 that lead to similar levels of total alkylation. As a proxy for total alkylation we monitored the
152 amount of N-alkyl adducts, namely 7mG and 3mA, that together represent > 80% of alkylation
153 for MMS and MNU. Estimation of the N-alkyl adduct level is achieved by converting these
154 adducts to single-stranded DNA breaks by a combination of heat depurination and alkali
155 cleavage treatment (Maxam and Gilbert, 1977)(Figure 1-figure supplement 1A). The resulting
156 plasmid fragmentation pattern were resolved and analyzed by agarose gel electrophoresis.
157 The reaction conditions were adjusted (by trial and error) as to generate a median fragment
158 size of 500 nt, corresponding to one alkylated base every 500 nucleotides on average (Figure
159 1-figure supplement 1A).

160

161 2. Identification of the proteins that specifically bind to DNA alkylation damage in

162 NucleoPlasmic Extracts (NPE):

163 In order to identify proteins binding to O⁶mG-containing base pairs in *Xenopus* egg-
164 derived extracts, we used a recently developed plasmid pull-down procedure, IDAP, for
165 Identification of DNA Associated Proteins (Isogawa et al., 2020; 2018). As outlined above,
166 MNU produces 20-25-fold more O⁶mG lesions than MMS (0.3% and 7-8% of total alkylation,
167 respectively), while the relative amounts of N-alkyl lesions produced by the two agents is
168 similar (> 80% of N7mG+N3mA) (Beranek, 1990). These agents react chemically with DNA
169 under neutral pH conditions, and we established *in vitro* reaction conditions that trigger
170 comparable levels of plasmid alkylation (see above and Fig 1-figure supplement 1A).

171 The pull-down procedure involves immobilization of plasmid DNA on magnetic beads
172 by means of a triple-helix forming probe (Figure 1A) (Isogawa et al., 2020; 2018). The same
173 amount of untreated or alkylated plasmids was coupled to magnetic beads and incubated in
174 NucleoPlasmic Extracts (NPE) derived from *Xenopus* eggs (Walter et al., 1998). The reaction
175 was stopped by dilution into a formaldehyde-containing buffer, which fixes protein-DNA
176 complexes. After washing the beads and reversing the cross-links, the recovered proteins
177 were visualized by silver staining following SDS-PAGE (Figure 1-figure supplement 1B). As a
178 negative control, mock conjugated beads (noDNA control lane) exhibit low protein
179 background illustrating efficient removal of non-specific proteins (Figure 1-figure supplement
180 1B). Proteins captured on the different plasmid samples were analyzed by label-free mass
181 spectrometry as described in Material and Methods. The MS data are presented in the form
182 of Volcano plots. When comparing MNU-treated to undamaged control plasmid, the MMR
183 proteins (labeled in red) were highly enriched in the MNU sample (Figure 1B). All six canonical
184 MMR proteins (MSH2, MSH3, MSH6, MLH1, PMS1 and PMS2) were specifically enriched on
185 MNU-plasmid. These proteins form the MutS α , MutS β , MutL α , and MutL β heterodimers

186 (Jiricny, 2006). Other proteins known to participate to MMR, RAD18, POL η , EXO1 and two
187 subunits of Pol delta (POLD2 and POLD3) were also specifically enriched on MNU-plasmid.
188 Previously, it was shown that purified MutS α does not bind to O⁶mG:C base pairs (Yoshioka et
189 al., 2006). Our present experiments involve extracts containing many proteins and there is
190 probably synergy between MutS α , MutL α (and other proteins) to achieve full MMR (Ortega
191 et al., 2021). Activation of MMR by a single O⁶mG:C lesion has been reported previously
192 (Duckett et al., 1999).

193

194 It was previously noted that upon oxidative stress, produced by hydrogen peroxide
195 treatment, RAD18 and Pol η are recruited to chromatin in a MSH2-MSH6 (MutS α) dependent
196 manner in human cells (Zlatanou et al., 2011). While MutS α , MutS β , and MutL α functionally
197 participate in MMR, the role of MutL β (MLH1-PMS1) remains unknown (Jiricny, 2006). No
198 MMR proteins were enriched on MMS-treated plasmids (Figure 1C). As MNU treatment
199 induces 20-30 times more O⁶mG adducts than MMS, we postulate that recruitment of MMR
200 proteins depends on O⁶mG. Comparison of proteins captured on MNU- versus MMS-treated
201 plasmids indeed reveals specific enrichment of MMR proteins. Proteins specifically recruited
202 at N-alkyl adducts (in green in Figure 1B and 1C) are absent in the MMS versus MNU Volcano
203 plot (Figure 1-figure supplement 1C), since N-alkyl adducts are equally present in both MMS
204 and MNU treated plasmids.

205 In addition, compared to lesion-free control plasmid, some proteins were enriched on
206 or excluded from both MMS and MNU-treated plasmids (Figure 1B and 1C, green labels). We
207 suggest that the recruitment or exclusion of these proteins depends on the abundant 7mG
208 and 3mA adducts formed by both MMS and MNU. The reason why BER proteins, normally
209 involved in the repair of these N-alkyl adducts, were not captured is unclear. One possibility is
210 that BER proteins interact too transiently with DNA to be efficiently captured.

211

212 3. Repair of alkylated plasmid DNA in NucleoPlasmic Extracts (NPE).

213 We next investigated the repair of DNA treated by the different alkylating agents in
214 NPE. Plasmid was alkylated with MMS, MNU, or ENU to a density of one lesion every \approx 500 nt
215 (Figure 1-figure supplement 1A). The alkylated plasmids were incubated in NPE in the
216 presence of α ³²P-dATP. These extracts contain high levels of geminin, an inhibitor of
217 replication licensing. Therefore, any observed DNA synthesis occurs independently of DNA
218 replication and corresponds to so-called "unscheduled DNA Synthesis" (UDS)(Figure 2A).
219 Undamaged plasmid exhibited a low level of background DNA synthesis, whereas MNU and
220 ENU treated plasmids sustained robust, time dependent UDS equivalent to 3-4% of the
221 synthesis needed for a full round of replication (Figure 2B). MMS-treated plasmid exhibited
222 UDS that was just two-fold above the background seen in undamaged plasmid (Figure 2B).
223 Given that the assay measures incorporation of α ³²P-dATP, long patch BER events (Sattler et
224 al., 2003) will be detected, while short patch BER events (1 nt patch) will only be detected at
225 3mA but not at 7mG adducts. The assay is clearly biased towards the detection of events such
226 as MMR that involve repair patches hundreds of nucleotides long.

227 We asked whether the observed UDS in MNU- and ENU-treated plasmids was MMR
228 dependent, as suggested by the mass spectrometry results. To test this idea, we depleted
229 MMR proteins from extracts using antibodies (Figure 2-figure supplement 1A), whose
230 specificity was previously validated (Kato et al., 2017; Kawasoe et al., 2016). Depletion of
231 MLH1 or PMS2 severely reduced UDS in MNU-treated plasmid, while no reduction was
232 observed in PMS1-depleted extracts (Figure 2-figure supplement 1B). This observation is

233 consistent with the fact that MutL α (composed of MLH1 and PMS2) is involved in canonical
234 mismatch repair whereas MutL β (composed of MLH1 and PMS1) is not (Jiricny, 2006).
235 Aphidicolin, an inhibitor of B-family DNA polymerases (Baranovskiy et al., 2014), decreased
236 incorporation on average 3.5-fold on MNU and ENU plasmids while it had a more modest
237 effect on MMS-treated plasmid (1.5-fold) (Figure 2-figure supplement 1C). These results
238 support the notion that UDS on MNU- and ENU-treated plasmids involves MMR, including a
239 gap filling event that most likely depends on DNA Pol δ , the only B family polymerase
240 detected in the MS analysis described above. Short-patch BER events are mediated by Pol β (X
241 family) that are insensitive to aphidicolin. The modest sensitivity of MMS-induced UDS to
242 aphidicolin is probably due to a fraction of BER events that belong to the long-patch BER
243 pathway mediated Pol δ/ϵ (Sattler et al., 2003).

244 We wanted to estimate the average amounts of DNA synthesis associated with MMR
245 at O⁶mG:C sites and BER at N-alkyl sites, respectively. At the 90 min time point (i.e. at near
246 plateau value), the difference in UDS between MNU- and MMS-treated plasmids, i.e.
247 attributable to repair at O⁶mG:C sites, was equivalent to \approx 3.1% of the input DNA (Figure 2B)
248 or \approx 270 nt (pBR322 plasmid is 4363 bp long). With an estimated \approx 1.7 O⁶mG adducts per
249 plasmid, the average repair patch per O⁶mG adduct is \approx 160 nt provided all O⁶mG lesions are
250 targeted by MMR. Evidence obtained with G:T and O⁶mG:T constructs (see below) indicates
251 that, under present experimental conditions, only about \approx 30% of O⁶mG are substrates for
252 MMR, suggesting that on average a MMR patch is \approx 500 nt long. Importantly, the MGMT
253 inhibitor Patrin-2 had no effect on UDS of MNU-treated plasmid, even at a dose of 200 μ M
254 (data not shown). Surprisingly, inhibition of MGMT by Patrin-2 was previously shown to occur
255 in *Xenopus* extracts (Olivera Harris et al., 2015). Two possibilities may account for the lack of
256 any measurable effect of MGMT inhibition: i) the number of MGMT molecules present in the
257 extract is small compared to the number of O⁶mG lesions introduced in the incubation mix or
258 ii) our batch of Patrin inhibitor is inactive. In all cases, if partial demethylation of O⁶mG by
259 MGMT occurs, the observed amount of UDS would be under-estimated. Thus, the conclusion
260 reached in the paper, namely that O⁶mG:C sites are substrates for MMR, remains correct.

261
262 With respect to N-alkyl adduct repair in MMS-plasmid, repair synthesis above the
263 lesion-free DNA control is equivalent to \approx 0.5% of input DNA (Figure 2B), corresponding to 43
264 nt total synthesis per plasmid. With \approx 17 N-alkyl adducts per plasmid, the average DNA
265 synthesis patch per adduct, in case all N-alkyl lesions are repaired, is \approx 2.6 nt, a value
266 consistent with a mixture of long (\approx 2-8 nt) and short patch (1nt) BER events at N-alkyl
267 adducts. In summary, the average DNA repair patch sizes at O⁶mG:C (\approx 500 nt) and N-alkyl (2-3
268 nt) sites are compatible with MMR and BER, respectively.

269 To learn more about UDS in this system, we analyzed repair products via gel
270 electrophoresis. Plasmid pBR322 treated with MMS or MNU was incubated in NPE,
271 supplemented or not with Aphidicolin in the presence of α ³²P-dATP, and analyzed on a
272 neutral agarose gel. As already noticed above (Figure 2-figure supplement 1C), addition of
273 Aphidicolin (Aph) led to more severe reduction in incorporation into MNU- (\approx 3.7-fold)
274 compared to MMS-treated plasmid (\approx 1.6-fold) (Figure 2C). We also note that in MNU-treated
275 plasmids, in the absence of Aph, open circular repair products were three-fold more
276 abundant than closed circular products (Figure 2C, ³²P image). This observation suggests that
277 MMR repair was complete in only \approx 25% of plasmid molecules while 75% of molecules
278 contained at least one nick (or a gap). Interestingly, there was a \approx 50% loss of total DNA in the
279 MNU + Aph lane compared to the other lanes, suggesting massive DNA degradation in NPE

280 due to polymerase inhibition by Aph. Indeed, the observed DNA degradation can specifically
281 be linked to repair events as the loss in radioactivity in MNU lanes -Aph versus + Aph is >70%
282 (Figure 2C, ³²P image). Under alkaline loading conditions (Figure 2D), repair products (³²P
283 image) in MNU-treated plasmids appeared mostly as a single-stranded linear band form. In
284 addition, there was a large smear (>25% of material) of shorter fragments. These results show
285 that most plasmids contain 1 nick and some contain several nicks. In the +Aph samples, the
286 open circular (oc) form, seen in the gel loaded under neutral conditions (Figure 2C), disappear
287 under alkali loading conditions (Figure 2D). This suggests that these oc molecules (Figure 2C)
288 contain many nicks that run as short fragments upon denaturation. In conclusion, MNU-
289 treated plasmids undergo robust repair synthesis that is more sensitive to aphidicolin
290 inhibition than MMS-treated plasmids.

291 We next examined O⁶mG-induced DNA synthesis in a different extract, namely High-
292 Speed Supernatant (HSS) of total egg lysate. Unmodified pBR322 (DNA0) or treated with MNU
293 to an extent of ≈1 N-alkyl adduct/500nt, were incubated in the presence of α³²P-dATP. Repair
294 synthesis was monitored at room temperature as a function of time using the spot assay
295 described above (Figure 2A). In HSS extract, MNU-treated plasmid does not exhibit significant
296 repair synthesis (Figure 2-figure supplement 2), in contrast to the robust repair synthesis seen
297 in NPE extract (Figure 2B). Although, HSS contains lower concentrations of most DNA repair
298 enzymes compared to NPE, HSS was shown to be proficient for MMR at a single O⁶mG
299 provided a nick is present in proximity (Olivera Harris et al., 2015). We reasoned that HSS
300 might not contain adequate concentrations of the DNA glycosylase AAG, which initiates BER
301 at N-alkyl sites. When HSS extract was supplemented with purified AAG glycosylase (150nM)
302 (NEB, Biolabs), robust repair synthesis is observed in MNU-treated plasmid (Figure 2-figure
303 supplement 2). These observations suggest the involvement of BER in stimulating MMR at
304 O⁶mG lesions.

306 4. MMR at single O⁶mG-containing base pairs is enhanced by the presence of N-alkylation 307 adducts.

308 Next, we explored a possible crosstalk between repair pathways acting on alkylated
309 DNA. In MNU-treated plasmid, there is on average one O⁶mG adduct for every 9-10 N-alkyl
310 adducts (Beranek, 1990). To investigate the repair response triggered by a single O⁶mG:C
311 lesion alone or in the presence of additional N-alkyl adducts, we implemented a
312 reconstitution experiment. For that purpose, a single O⁶mG:C construct (mGC) (Isogawa et al.,
313 2020) was treated with MMS to introduce ≈ 9-10 N-alkyl adducts per plasmid molecule,
314 generating plasmid mGC+MMS, that is expected to recapitulate adduct distribution found in
315 MNU-treated plasmids. Control plasmid GC was treated with the same concentration of
316 MMS, to generate GC+MMS. These *in vitro* manipulations did not affect plasmid topology as
317 all four constructs exhibit a similar migration pattern (Figure 3-figure supplement 1A).

318 Plasmid constructs GC and mGC and the corresponding two MMS-treated constructs
319 (GC+MMS and mGC+MMS) (Figure 3A), were incubated with NPE in the presence of α³²P-
320 dATP to monitor repair synthesis (i.e. UDS). We observed, incorporation of radioactivity
321 specifically attributable to the single O⁶mG:C lesion (compare mGC to GC in Figure 3B).
322 Activation of MMR by a single O⁶mG:C lesion has been reported previously (Duckett et al.,
323 1999). The specific involvement of MMR for O⁶mG dependent incorporation was re-assessed,
324 by incubating the single adducted O⁶mG:C construct in MLH1-depleted NPE extract;
325 radioactive incorporation above background was fully abolished in mGC plasmid (Figure 3-

326 figure supplement 1E). How MMR may get engaged in a repair reaction on a closed circular
327 template will be considered in the Discussion section.

328 Importantly, repair synthesis, due to the single O⁶mG:C lesion, is strongly enhanced by
329 the presence of MMS adducts (compare mGC+MMS to GC+MMS in Figure 3B). At the 2h time
330 point, incorporation, above background, due to the single O⁶mG, expressed in % replication
331 equivalent, represents 0.64% (difference between mGC and GC), while it amounts to 1.85% in
332 the presence of MMS lesions (compare mGC+MMS with GC+MMS). One can thus estimate
333 that, incorporation due to a single O⁶mG lesion, is stimulated about 2.9-fold (1.85/0.64) by
334 the presence in *cis* of MMS adducts (Figure 3B).

335 Finally, we wanted to compare the relative MMR repair efficiencies triggered by
336 O⁶mG:C and O⁶mG:T (or GT) mismatches (Figure 3A and B). These constructs were used as
337 single adducted constructs or after additional reaction with MMS similarly to the procedure
338 described for the corresponding GC or mGC constructs. The main observation is that GT
339 containing constructs trigger a much stronger MMR response than their GC counterparts
340 (Figure 3B). In the absence of MMS, at the 120' time point, the level of UDS in mGC
341 represents 23% of the level in mGT. In the presence of MMS, at the 120' time point, the level
342 of UDS in mGC+MMS represents 38% of the level in mGT+MMS. In conclusion, supposing that
343 100% of mGT mispairs are fully repaired, the extent of mGC repair would be in the range of
344 30%.

345

346 5. Nucleotide incorporation occurs in the vicinity of the single O⁶mG adduct.

347 The plasmids described above were incubated in α ³²P-dATP supplemented NPE for 2h,
348 purified plasmids and analyzed by agarose gel electrophoresis (Figure 3C). Covalently closed
349 circular (ccc) and relaxed forms (oc) were quantified in each lane (Figure 3C). In the presence
350 of MMS adducts, the single O⁶mG:C lesion contributes to a 2.8-fold increase in radioactive
351 incorporation compared to its contribution in the absence of MMS (Figure 3C) in good
352 agreement with the UDS data (Figure 3B).

353 We wanted to map the repair patches with respect to the O⁶mG adduct position by
354 restriction enzyme analysis. Digestion of the purified plasmids with *BmtI* and *BaeGI* restriction
355 enzymes, generates fragment S (589 bp) that encompasses the O⁶mG:C site and fragment L
356 (1525 bp) (Figure 3-figure supplement 1B). Following separation by agarose gel
357 electrophoresis, the DNA was imaged by ethidium bromide fluorescence and ³²P
358 autoradiography (Figure 3-figure supplement 1C). For each fragment, we determined its
359 specific activity by dividing the radioactivity signal by its amount as determined from the
360 ethidium bromide image (Figure 3-figure supplement 1D). As expected, the specific activities
361 of S and L fragments were similar in GC (random background incorporation: 0.125±0.015 AU
362 (arbitrary units)) and MMS treated (GC+MMS) (0.235±0.025 AU) control plasmids. In
363 GC+MMS, the specific activity was slightly higher than in control plasmid, probably reflecting
364 BER-mediated incorporation at randomly distributed N-alkyl adducts. In the two O⁶mG:C
365 containing plasmids (mGC and mGC+MMS), the S fragment exhibits a significantly higher
366 specific activity than the L fragment indicating that MMR activity is centered around the
367 O⁶mG:C site. In the absence of MMS, incorporation in mGC above background (dotted line in
368 Figure 3-figure supplement 1D), attributable to O⁶mG, amounts to 0.065 and 0.495 AU
369 (arbitrary units) for L and S fragments, respectively. Similarly, in the presence of random MMS
370 lesions (mGC+MMS), incorporation, above background (dotted line in Figure 3-figure
371 supplement 1D), attributable to O⁶mG amounts to 0.115 and 1,17 AU for L and S fragments,
372 respectively. These results clearly show that O⁶mGC induced repair essentially takes place

373 within the S fragment, with only modest spill-over into the L fragment (10-15%). This
374 observation appears to be good agreement with the estimated average MMR patch size
375 (~500 nt). Thus, MMS adducts do not modify the repair pattern, i.e. the relative distribution
376 of ³²P incorporation in S and L fragments, but they increase the frequency of repair centered
377 at O⁶mG sites. In conclusion, we show that stimulation of repair synthesis by N-alkyl adducts
378 specifically occurs in the vicinity of the O⁶mG adducts, illustrating that processing of N-alkyl
379 adducts enhances MMR activity.

380

381 6. MNU-treated plasmids undergo double-strand breaks during incubation in extracts.

382

383 Work in *E. coli*, provided elegant genetic evidence that the cytotoxicity of alkylating
384 agents forming O⁶mG adducts (such as N-methyl-N'-nitrosoguanidine and MNU), including
385 formation of replication-independent DSB, was strongly influenced by the status of the MMR
386 pathway (Karran and Marinus, 1982; Nowosielska and Marinus, 2008). We wondered whether
387 MNU can induce formation of DSBs independently of DNA replication. To increase the
388 sensitivity of our assay towards DSB detection, we used a larger plasmid, pEL97 (11,3 kb), and
389 treated it with MMS or MNU to introduce one alkylation event on average every 500 nt
390 (Figure 4-figure supplement 1). We also treated one sample with double the concentration of
391 MNU to achieve a 2-fold higher lesion density. Quantification of N-alkyl adducts by alkaline
392 cleavage and subsequent agarose gel electrophoresis led to the expected lesion density of
393 one N-alkyl adduct every 500 nt for MMS and MNU+, and one N-alkyl adduct every 250 nt
394 MNU++ (Figure 4-figure supplement 1C).

395 Alkylated and control plasmids (Figure 4-figure supplement 2A) were incubated in NPE
396 for 60' in the presence of α ³²P-dATP, resolved by agarose gel electrophoresis, and visualized
397 by ethidium bromide staining and ³²P imaging (Figure 4A). Both MMS and MNU caused
398 substantial conversion of the plasmid from supercoiled to open circular form, as expected
399 during repair synthesis. Consistent with our results above, MNU induced much more repair
400 synthesis than MMS. Strikingly, in both ethidium bromide and ³²P images, linear plasmid was
401 detected after exposure to MNU, but not MMS. For a two-fold increase in MNU exposure, the
402 linear plasmid band increased \approx 4-fold (Figure 4B). This quadratic dose-response strongly
403 suggests that DSBs occur as a consequence of two independent repair events at neighboring
404 lesions, for example a BER event at an N-alkyl adduct leading to a nick in one strand that is
405 encountered by a gap formed by an MMR event initiated at a O⁶mG site in the opposite
406 strand (Figure 5). To reveal single-strand breaks, the same samples were denatured prior to
407 native gel electrophoresis (Figure 4-figure supplement 2B). In the MNU++ sample, no linear
408 single-stranded DNA (ssDNA) is left, all the DNA molecules running as a smear centered
409 around the 3000 nt position (Figure 4-figure supplement 2B). The observed smear reveals
410 that the double-stranded DNA running as open circular plasmid molecules in the neutral
411 loading gel (Figure 4A), contain each on average 3-4 nicks per plasmid strand. The data reveal
412 that repair of MNU-treated plasmid in NPE causes SSBs, and that once the density of SSBs is
413 high enough, DSBs result.

414

415 **Discussion:**

416 With respect to the biological responses to SN1 alkylating agents most attention has
417 so far been devoted to responses that occur in the first or second cell cycle following
418 treatment as mentioned in the introduction (Noonan et al., 2012; Plant and Roberts, 1971;
419 Quiros et al., 2010).

420 In the present paper, we focus on early processing of DNA alkylation adducts by repair
421 pathways before the event of replication. Interestingly, we identify the formation of DSB as
422 the result of a putative crosstalk between repair pathways.

423

424 Late responses to SN1 agents:

425 Response of cells to SN1 methylating agents was shown to be initiated at O⁶mG:T
426 mispairs that form upon DNA replication of O⁶mG containing DNA template and shown to
427 involve the MMR machinery (Goldmacher et al., 1986) (Day et al., 1980; Karran et al., 1993;
428 Yarosh et al., 1983). The O⁶mG:T mispair is efficiently recognized by MutS α , the key MMR
429 initiator protein. Following removal of the nascent T residue across O⁶mG, T will be re-
430 inserted at high frequency during the MMR gap-filling step thus re-forming the initial O⁶mG:T
431 mispair. This iterative process, called “futile cycling”, has received experimental support
432 (Mazon et al., 2010; York and Modrich, 2006). However, it is not yet clear how these futile
433 cycles lead to DSBs (Ochs and Kaina, 2000), apoptosis and cell death (Gupta and Heinen,
434 2019; Kaina and Christmann, 2019). Two mutually non-exclusive models have been proposed,
435 i) a direct model where the encounter of the replication fork with the MMR intermediates
436 leads to fork collapse and to subsequent cytotoxic events; ii) a signaling model where the
437 MutS α complex acts as a sensor leading to ATR recruitment and subsequent initiation of the
438 ATR-Chk1 signaling pathway (Duckett et al., 1999; Yoshioka et al., 2006). However, presently
439 there is more evidence that the critical cytotoxic response to methylating agents is the
440 consequence of direct MMR processing rather than being mediated by a mere signaling
441 model (Cejka and Jiricny, 2008; Karran, 2001; Liu et al., 2010; York and Modrich, 2006).

442

443 Early responses to SN1 agents:

444 While all biological responses described above require replication of O⁶mG containing
445 DNA templates as the first step, we wanted to investigate the processing of MNU-alkylated
446 DNA in the absence of replication. Interestingly, we detect robust, MMR-dependent, UDS
447 upon incubation of MNU-treated plasmid in NPE. This observation reveals that, not only are
448 O⁶mG:C lesions recognized by MutS α as previously noted (Duckett et al., 1999; Karran et al.,
449 1993), but that the whole MMR repair process is engaged and proceeds to completion. We
450 would also like to stress the high sensitivity of the pull-down assay with respect to MMR
451 proteins capture. Indeed, the whole MMR machinery is enriched (Figure 1B) using a plasmid
452 that on average carries only 2-3 O⁶mG lesions/plasmid. In striking contrast, despite their
453 abundance, \approx 26 N-alkyl lesions/plasmid, N-alkyl lesions only recruit few specific proteins
454 (Figure 1C).

455 We wanted to investigate the potential effect that N-alkyl adducts may have on MMR
456 processing at O⁶mG:C base pairs. For that purpose, we compared a plasmid with a single site-
457 specific O⁶mG:C lesion to a plasmid additionally treated with MMS, an agent known to induce
458 essentially only N-alkyl adducts. The MMS treatment was adjusted as to produce the same
459 amount of N-alkyl adduct as generated by MNU. The single-adducted O⁶mG:C plasmid
460 triggered MMR mediated repair synthesis centered around the O⁶mG adduct. Interestingly,
461 the presence of randomly distributed N-alkyl adducts led to a 3-fold increase of the MMR
462 repair activity in the vicinity of the O⁶mG adduct. These data raise two questions, first, how
463 does the MMR machinery get engaged in ccc plasmid and how is MMR activity further
464 stimulated by N-alkyl adducts? In current models, functional engagement of MMR involves a
465 mismatch recognized by MutS α , subsequent recruitment of MutL α and PCNA (Jiricny, 2006).
466 Loading of PCNA by RFC normally requires a single-stranded nick but it was also shown to

467 occur, although less efficiently, on ccc DNA (Pluciennik et al., 2013; 2010). Under these
468 conditions, PCNA loading and MMR processing lack strand directionality. With respect to the
469 mechanism by which MMR activity, at a single O⁶mG:C lesion, becomes stimulated several
470 folds by the presence of N-alkyl adducts, we propose that processing of N-alkyl adducts by
471 BER creates repair intermediates (nicks) that stimulate PCNA loading. It was previously shown
472 that BER intermediates formed at oxidized purines or U residues can stimulate MMR
473 processing (Repmann et al., 2015; Schanz et al., 2009).

474
475 DSB form in MNU-treated DNA in the absence of replication: potential therapeutic
476 significance.

477 Interestingly, incubation of MNU-treated plasmid in extracts leads to DSBs (Figure 4)
478 that arise with a quadratic dose response suggesting the occurrence of two independent
479 repair activities taking place simultaneously in opposite strands at lesions that may be up to
480 several hundred nucleotides apart (see scenario in Figure 5). Similarly, *in vitro* processing of
481 neighboring G/U mismatches by BER and by non-canonical MMR was shown to lead to DSBs
482 (Bregenhorn et al., 2016).

483 The extent of DNA alkylation triggered by MNU *in vitro*, as deduced from our alkaline
484 cleavage determination, fits surprisingly well with the amount of alkylation induced by TMZ in
485 cell at equal concentrations (Moody and Wheelhouse, 2014) (Kaina, personal
486 communication). According to the model (Figure 5), formation of a DSB may occur when an
487 N-alkyl lesion is located within the repair track mediated by MMR at a O⁶mG:C site. In the
488 clinic, a daily dose of TMZ results in 50µM serum concentration and was shown to induce
489 5.2×10^4 and 7.3×10^5 O-alkyl and N-alkyl lesions per human genome, respectively (Kaina,
490 personal communication). We can estimate the number of events (per genome) where an N-
491 alkyl lesion is located within 500 nt on either side of a O⁶mG:C site. Given the N-alkyl lesion
492 density ($7.3 \times 10^5 / 6 \times 10^9 = 1.2 \times 10^{-4}$), the probability of presence of an N-alkyl lesion within an
493 MMR track is 0.12. In other words, among the 5.2×10^4 O⁶mG lesions, ≈6,240 are likely to
494 contain a N-alkyl lesion within a 1000 nt excision track. We will refer to such a lesion
495 configuration as a “Lesion Arrangement at-risk” for DSB formation.

496 Let us now estimate the level of DSB that may occur in a human genome, by
497 extrapolation of our *in vitro* data. In the present work, ≈6% of plasmid DNA (11.3 kb) treated
498 by MNU at 2mM exhibit a DSB (Figure 4). The observed amount of DSBs may in fact only
499 reflect a steady state level since efficient re-ligation mechanisms are known to operate in NPE
500 (Graham et al., 2016). As MNU and TMZ exhibit similar reactivities (Moody and Wheelhouse,
501 2014)(Kaina personal communication) a dose of 2mM MNU would lead to $3 \times 10^9 \times 0.06 /$
502 $11300 = 16,000$ DSBs per genome. In the clinic, the level of TMZ in the serum reaches up to
503 50µM, i.e. 40 times less than the 2mM dose used *in vitro*. Given the quadratic dose-response,
504 the estimated amount of DSBs per genome would be 1600 times less, i.e. ≈10. Lets note that
505 the conversion rate of a Lesion Arrangement at-risk into an actual DSB appears to be quite
506 low ($10 / 6,240 \approx 0.16\%$), reflecting the requirement for simultaneous occurrence of two repair
507 events (MMR and BER).

508 The alkylating agent Temozolomide (TMZ), a chemical mimic of MNU, is presently the
509 first-line and only anti-cancer drug in glioblastoma therapy (Moody and Wheelhouse, 2014).
510 The cytotoxic mode of action of alkylating agents such as TMZ is believed to result from
511 iterative MMR cycles. Iterative MMR cycles are deemed to lead to double-strand breaks (DSB)
512 via a mechanism that is not yet established (Ochs and Kaina, 2000). Indeed, it is not known
513 whether DSB's occur spontaneously at these sites or as a consequence of the replication fork

514 running into the MMR intermediates. Induction of these putative DSB's is presently thought
515 to be the primary mode-of-action of TMZ when administered to patients with glioblastoma.
516 Understanding both early and late cellular responses to MNU/TMZ appears thus to be critical.
517 During cancer treatment, a dose of TMZ is delivered concomitantly with a radiotherapy
518 session daily, for 6 weeks (for a recent review see Strobel et al., 2019). As estimated above, a
519 daily dose of TMZ, may lead to a ≈ 10 DSBs/cell resulting from BER/MMR crosstalk, a number
520 comparable to the number of DSBs induced by 0.5-1 Gy of IR. Moreover, it was established
521 empirically, that the treatment TMZ plus radiotherapy exhibits supra-additive cytotoxicity as
522 long as TMZ administration *precedes* radiotherapy (Bobola et al., 2010). Our data may provide
523 some rationale for this empirically determined regimen. Indeed, the single-stranded DNA
524 stretches formed at early time points during MMR processing at O⁶mG:C sites (step 3 in
525 Figure 5) constitute preferential targets for the conversion of the numerous single-stranded
526 breaks induced by ionizing radiations (IR) into DSBs, thus providing an explanation for the
527 observed supra-additivity in the treatment when TMZ *precedes* IR. A commonly used
528 radiotherapy session involves an IR dose of 2 Gy that predominantly induces ≈ 2000 single-
529 strand breaks and ≈ 40 DSBs/cell.

530 As the majority of cells in a glioblastoma tumor are not proliferating, insight into
531 attacking non-dividing cells might be very useful in treating this almost always fatal tumor.
532 This pre-replicative mechanism for TMZ cytotoxicity will need to be investigated in cellular
533 systems. In conclusion, the present work offers a novel mechanistic insight into the
534 cytotoxicity of TMZ via induction of DSBs, at early time points following exposure, before
535 replication. This early response comes in complement to the late, replication and cell cycle
536 dependent, responses that have been described over the years.

537
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541

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545

546

547 **Material and Methods.**

548

549 Plasmids: alkylated plasmids as used in the present paper are outlined below:

550

name	Size (kb)	Assay
pAS200.2	2,1	Site-specific O ⁶ mG lesion
pBR322	4,3	Random alkylation / UDS repair assay
pAS04	6,5	IDAP pull-down assay / MS analysis of bound proteins
pEL97	11,3	Random alkylation / UDS assay / post incubation analysis

551

552 Alkylation reactions are conducted as indicated in Fig 1-figure supplement 1, at a plasmid
553 concentration of 10ng/ μ l in CE buffer (citrate 10mM pH7, EDTA 1mM) + 10% DMSO final.

554 Alkylation reactions are terminated by addition of STOP buffer (5x: 1.5M sodium acetate, 1M
555 mercapto-ethanol) followed by ethanol precipitation. The DNA pellet is washed with ethanol
556 90% and re-dissolved in TE at 50ng/μl.

557

558 Cleavage reactions at 7-alkylG and 3-alkylA adducts. Alkylated plasmids (50ng in 10μl of CE
559 buffer) are first incubated for 90°C during 15' at pH7 (PCR machine). Following addition of 1μl
560 of NaOH 1N, the sample is further incubated at 90°C for 30'. Following addition of 2μl of
561 alkaline 6x loading buffer (NaOH 300mM, EDTA 6mM, Ficoll (Pharmacia type 400) 180mg/ml,
562 0.15% (w/v) bromocresol green, 0.25% (w/v) xylene cyanol), the cleaved plasmid samples are
563 loaded on a neutral agarose gel. (Figure 1-figure supplement 1A)

564

565 NPE and HSS Xenopus extracts: Two type of extracts derived from *Xenopus laevis* eggs are
566 used throughout the paper, namely NucleoPlasmic Extracts (NPE) and High-Speed
567 Supernatant (HSS) as described previously (Lebofsky et al., 2009).

568

569 Western Blot: Antibodies against Mlh1, Pms2 and Pms1 as previously described (Kato et al.,
570 2017; Kawasoe et al., 2016). For western blotting primary antibodies used at 1:5000 dilution.

571

572 Single adducted plasmids. Covalently closed circular plasmids containing a site-specific
573 O⁶mG:C base pair (plasmid mGC) and the corresponding lesion-free control (plasmid GC)
574 were constructed. We also constructed similar plasmids with a single GT or a single O⁶mG:T
575 mismatch located at the same position (plasmids GT and mGT, respectively). All constructs
576 were derived from plasmid vector pAS200.2 (2.1 kb) (Isogawa et al., 2020).

577

578 Plasmid immobilization on magnetic beads and pull-down procedure.

579 Alkylated plasmids samples (250 ng of each -MMS, -MNU and -ENU), as well as a non-
580 alkylated control sample (DNA0) were immobilized on magnetic beads at a density of 10 ng
581 plasmid / μl of M280 bead slurry using a triple helix-based capture methodology (Isogawa et
582 al., 2018). The TFO1 probe used here is 5' Psoralen – C6 – TTTTCTTTCTCCTCTTCTC– C124 –
583 Desthiobiotin (20 mer) with C124: hexaethylene glycol x6. Underlined C is for 5mC; it was
584 synthesized by using DNA/RNA automated synthesizer and purified with conventional
585 methods (Nagatsugi et al., 2003).

586

587 Immobilized plasmid DNA are incubated in NPE extract (final volume 16μl) for 10 min
588 at room temperature under mild agitation. To monitor non-specific protein binding to beads,
589 we included a negative control (noDNA sample) containing the same amount of M280 beads
590 treated under the same conditions but in absence of plasmid DNA. Reactions were stopped
591 by addition of 320μl of a 0.8% HCHO solution to cross-link the proteins-DNA complexes for 10
592 min at room temperature. The beads were subsequently washed at RT with 200μl of 100mM
593 NaCl containing buffer (ELB buffer), re-suspended in 70μl of Extract Dilution buffer and
594 layered on top of a 0.5M sucrose cushion in Beckman Coulter tubes (Ref: 342867). The beads
595 were quickly spun through the cushion (30 sec at 10 000 rpm), the bead pellet is re-suspended
596 into 40μl of ELB sucrose and further analyzed by PAGE or by MS.

596

597 PAGE / silver staining:

598 An aliquot of each incubation experiments, corresponding to 30 ng of immobilized plasmid
599 was treated at 99°C for 25 min in a PCR machine to revert HCHO cross-linking in LLB, 50 mM

600 DTT. Samples were loaded on a 4-15% PAGE (Biorad pre-cast) gel at 200 volts for 32min and
601 stained using the silver staining kit (silver StainPlus, Biorad).

602

603 Incorporation of $\alpha^{32}\text{P}$ -dATP into DNA: spot assay.

604 Plasmids are incubated in nuclear extracts supplemented with $\alpha^{32}\text{P}$ -dATP; at various time
605 points, an aliquot of the reaction mixture is spotted on DEAE paper (DE81). The paper is
606 soaked in 100ml 0.5M Na_2HPO_4 (pH \approx 9), shaken gently for 5' before the buffer is discarded;
607 this procedure is repeated twice. Finally, the paper is washed an additional 2 times in 50ml
608 ethanol, air dried and analyzed by ^{32}P imaging and quantification. The extent of DNA repair
609 synthesis is expressed as a fraction of input plasmid replication (i.e. 10% means that the
610 observed extent of repair synthesis is equivalent to 10% of input plasmid replication). This
611 value is determined knowing the average concentration of dATP in the extracts (\approx 50 μM) and
612 the amount of added $\alpha^{32}\text{P}$ -dATP.

613

614 Mass Spectrometry. Label-free mass spectrometry analysis was performed using on-bead
615 digestion. In solution digestion was performed on beads from plasmid pull-downs. We added
616 20 μl of 8 M urea, 100 mM EPPS pH 8.5 to the beads, then 5mM TCEP and incubated the
617 mixture for 15 min at room temperature. We then added 10 mM of iodoacetamide for 15min
618 at room temperature in the dark. We added 15 mM DTT to consume any unreacted
619 iodoacetamide. We added 180 μl of 100 mM EPPS pH 8.5. to reduce the urea concentration to
620 <1 M, 1 μg of trypsin, and incubated at 37 $^\circ\text{C}$ for 6 hrs. The solution was acidified with 2%
621 formic acid and the digested peptides were desalted via StageTip, dried via vacuum
622 centrifugation, and reconstituted in 5% acetonitrile, 5% formic acid for LC-MS/MS processing.

623 All label-free mass spectrometry data were collected using a Q Exactive mass
624 spectrometer (Thermo Fisher Scientific, San Jose, CA) coupled with a Famos Autosampler (LC
625 Packings) and an Accela600 liquid chromatography (LC) pump (Thermo Fisher Scientific).
626 Peptides were separated on a 100 μm inner diameter microcapillary column packed with \sim 20
627 cm of Accucore C18 resin (2.6 μm , 150 \AA , Thermo Fisher Scientific). For each analysis, we
628 loaded \sim 2 μg onto the column. Peptides were separated using a 1 hr gradient of 5 to 29%
629 acetonitrile in 0.125% formic acid with a flow rate of \sim 300 nL/min. The scan sequence began
630 with an Orbitrap MS^1 spectrum with the following parameters: resolution 70,000, scan range
631 300–1500 Th, automatic gain control (AGC) target 1×10^5 , maximum injection time 250 ms,
632 and centroid spectrum data type. We selected the top twenty precursors for MS^2 analysis
633 which consisted of HCD high-energy collision dissociation with the following parameters:
634 resolution 17,500, AGC 1×10^5 , maximum injection time 60 ms, isolation window 2 Th,
635 normalized collision energy (NCE) 25, and centroid spectrum data type. The underfill ratio
636 was set at 9%, which corresponds to a 1.5×10^5 intensity threshold. In addition, unassigned
637 and singly charged species were excluded from MS^2 analysis and dynamic exclusion was set to
638 automatic.

639 Mass spectrometric data analysis. Mass spectra were processed using a Sequest-based in-
640 house software pipeline. MS spectra were converted to mzXML using a modified version of
641 ReAdW.exe. Database searching included all entries from the *Xenopus laevis*, which was
642 concatenated with a reverse database composed of all protein sequences in reversed order.
643 Searches were performed using a 50 ppm precursor ion tolerance. Product ion tolerance was
644 set to 0.03 Th. Carbamidomethylation of cysteine residues (+57.0215Da) were set as static
645 modifications, while oxidation of methionine residues (+15.9949 Da) was set as a variable
646 modification. Peptide spectral matches (PSMs) were altered to a 1% FDR (Elias and Gygi,

647 2010; 2007). PSM filtering was performed using a linear discriminant analysis, as described
648 previously (Huttlin et al., 2017), while considering the following parameters: XCorr, ΔC_n ,
649 missed cleavages, peptide length, charge state, and precursor mass accuracy. Peptide-
650 spectral matches were identified, quantified, and collapsed to a 1% FDR and then further
651 collapsed to a final protein-level FDR of 1%. Furthermore, protein assembly was guided by
652 principles of parsimony to produce the smallest set of proteins necessary to account for all
653 observed peptides.

654

655

656 **Legend to Figures:**

657 **Figure 1.** Pull-down of proteins that bind to alkylated- versus untreated-plasmid DNA.

658 **A.** Experimental workflow. plasmid DNA (pAS04, 6.5kb) was treated with alkylating agents
659 under conditions leading to a similar extent of N-alkylation (\approx one alkaline cleavage site every
660 500 nt, (Figure 1-figure supplement 1A). Immobilized plasmid DNA were incubated in
661 *Xenopus* NucleoPlasmic Extracts (NPE) for 10 min at room temperature under mild agitation.
662 The reaction was stopped by addition of formaldehyde (0.8% final) to cross-link the proteins-
663 DNA complexes. The beads were processed and analyzed by PAGE or by MS as described in
664 Material and Methods.

665 **B.** Relative abundance of proteins captured on MNU-treated versus untreated DNA0. Proteins
666 captured on equal amounts of MNU- or untreated plasmid were analyzed by label-free MS in
667 triplicates. For all proteins, average spectral count values in the MNU-treated plasmid sample
668 were divided by the average spectral count values in the DNA0 sample. The resulting ratio is
669 plotted as its \log_2 value along x-axis. The statistical significance of the data is estimated by the
670 p value in the Student test and plotted as the $-\log_{10}p$ along y-axis. Proteins enriched on MNU
671 versus untreated plasmid DNA appear in the right-side top corner and essentially turn out to
672 be MMR proteins labelled in red (Figure 1B). Data shown are analyzed using Xenbase data
673 base.

674 **C.** Relative abundance of proteins captured on MMS-treated versus untreated DNA0. Proteins
675 captured on equal amounts of MMS- or untreated plasmid were analyzed by label-free MS in
676 triplicates. The data are analyzed and plotted as in panel B for MNU using Xenbase data base.
677 Proteins (labeled in green in Figure 1B and 1C) are found enriched or excluded in both MMS
678 versus DNA0 and MNU versus DNA0 plasmids. We suggest these proteins are recruited or
679 excluded from binding to DNA by the abundant class of N-alkylation adducts that both MMS-
680 and MNU-treated plasmids share in common (\sim 27 N-alkyl adducts per plasmid).

681

682 **Figure 1-figure supplement 1.**

683 **A.** Alkylation reaction conditions and alkaline cleavage. The alkylation reaction conditions
684 (concentration, reaction temperature and time), were adjusted by successive trials until an
685 average of one N-alkyl adduct per 500 nt was reached. The desired level of alkylation (\approx 1 N-
686 alkyl adduct / 500nt) was attested using the alkaline cleavage procedure, followed by neutral
687 agarose gel electrophoresis (for details, see Material and Methods).

688

689 **B.** PAGE analysis and silver staining of the captured proteins. Proteins captured on equal
690 amount of immobilized plasmid DNA incubated in NPE (as described in Figure 1A) were
691 resolved by denaturing gel electrophoresis and silver stained. Samples, corresponding to \approx 30
692 ng of immobilized input plasmid DNA, were loaded on a 4-15% PAGE (Biorad pre-cast) gel, run
693 at 200 volts for 32min and silver stained. Lanes DNA0, MMS, MNU and ENU display a complex
694 pattern of proteins (total amount of proteins per lane estimated at 100-200 ng). To monitor
695 non-specific protein binding to beads, we include a negative control containing the same
696 amount of M280 beads in the absence of plasmid DNA (noDNA). Importantly, in the noDNA
697 lane only a low amount of residual protein is visible indicating efficient removal of non-
698 specifically protein binding during the washing procedure.

699

700 **C.** Relative abundance of proteins captured on MNU- versus MMS-treated plasmid. Proteins
701 captured on equal amounts of MNU- or MMS-treated plasmid were analyzed by label-free MS

702 in triplicates. The data are analyzed and plotted as described in Figure 1B using Xenbase data
703 base. Proteins enriched on MNU versus MMS-treated DNA appear in the right-side top corner
704 and essentially turn out to be MMR proteins labelled in red (Figure 1B).

705
706

707 **Figure 2.** DNA repair synthesis in alkylated and undamaged control plasmid DNA in NPE
708 extracts.

709 **A.** Outline of the spot assay. Plasmids were incubated in nuclear extracts supplemented with
710 $\alpha^{32}\text{P}$ -dATP; at various time points, an aliquot of the reaction mixture was spotted on DEAE
711 paper (see Material and Methods). The dot blot that is shown for sake of illustration only.

712 **B.** Plasmid DNA pBR322 (4.3kb) samples, modified to a similar extent with -MMS, -MNU and -
713 ENU, were incubated in NPE supplemented with $\alpha^{32}\text{P}$ -dATP at room temperature;
714 incorporation of radioactivity was monitored as a function of time using the spot assay
715 described above (Figure 2A). Undamaged plasmid DNA0 was used as a control. At each time
716 point, the average values and standard deviation from three independent experiments were
717 plotted. The y-axis represents DNA repair synthesis expressed as a fraction of input plasmid
718 replication (i.e. 10% means that the observed extent of repair synthesis is equivalent to 10%
719 of input plasmid replication). This value was determined knowing the average concentration
720 of dATP in the extract (50 μM) and the amount of added $\alpha^{32}\text{P}$ -dATP.

721 **C.** MMS and MNU-treated plasmids were incubated in NPE, supplemented or not, by
722 aphidicolin (150 μM final). After 1 h incubation, plasmids were purified and analyzed by
723 agarose gel electrophoresis under neutral loading conditions. The gel was imaged by
724 fluorescence (left: ethidium bromide image) and by autoradiography (right ^{32}P image). The
725 number below each lane indicates the total amount of signal per lane (expressed in AU).
726 Aphidicolin treatment decreases incorporation into MNU-treated plasmid close to 4-fold,
727 while it affected incorporation into MMS-treated plasmid only 1.6-fold.

728 **D.** Samples as in C. Gel loading is performed under alkaline conditions to denature DNA
729 before entering the neutral agarose gel allowing single-stranded nicks present in DNA to be
730 revealed. The number below each lane indicates the amount of signal per lane (AU).

731

732 **Figure 2-figure supplement 1.** Involvement of Mismatch repair in repair synthesis and effect of
733 aphidicolin.

734 **A.** Analysis by WB of the NPE extracts depleted for MMR proteins. Antibodies against Mlh1,
735 Pms2 and Pms1 as previously described (Kato et al., 2017; Kawasoe et al., 2016). Extracts
736 were depleted, with the respective antibodies, for three rounds at 4C° at a ratio beads:
737 antibody: extract = 1 :3 :5.

738

739 **B:** Depletion experiments show that $\alpha^{32}\text{P}$ -dATP incorporation into MNU-plasmid is mediated
740 by Mismatch Repair. NPE was depleted by antibodies against Pms1, Pms2, Mlh1 or mock
741 depleted. Upon incubation at room temperature in the different NPE extracts, incorporation
742 of $\alpha^{32}\text{P}$ -dATP in MNU- plasmid was monitored by the spot assay as a function of time. At each
743 time point, the average values and standard deviations from two independent experiments
744 were plotted. As expected from previous data (Figure 2B), robust incorporation was observed
745 for MNU-plasmid incubated in mock-depleted extract. In contrast, radioactive dATP
746 incorporation was severely reduced when the plasmid was incubated in extracts depleted
747 with antibodies against Mlh1 or Pms2. These data strongly suggest that the incorporation
748 seen in mock depleted NPE is mediated by MMR, as both Mlh1 and Pms2 assemble into the

749 functional MMR complex MutL α . In contrast, depletion with antibodies against Pms1 did not
750 affect incorporation kinetics as Pms1 is reported not to function in MMR (Jiricny, 2006).

751
752 **C.** Effect of aphidicolin: alkylated plasmids (pEL97: 11.3kb) were incubated in NPE
753 supplemented or not by aphidicolin (150 μ M final) in the presence of 32 P-dATP for 1h at RT.
754 Analysis was performed by spot assay as described in Material and Methods. The y-axis
755 represents the percentage of radioactive DNA synthesis expressed in % of full plasmid
756 replication. Addition of Aphidicolin to NPE (150 μ M final) more severely reduces
757 incorporation in MNU+, MNU++ and ENU plasmids, compared to control and MMS plasmids.
758 Fold reduction in incorporation under aphidicolin conditions is shown.

759
760 **Figure 2-figure supplement 2.** Repair synthesis in HSS extracts.
761 Plasmid treated with MNU (\approx 1 N-alkyl adduct/500nt), was incubated in HSS extracts in the
762 presence of α 32 P-dATP; repair synthesis was monitored at room temperature as a function of
763 time using the spot assay. Both DNA0 and MNU plasmids only exhibit background
764 incorporation levels. Robust UDS is observed in MNU-plasmid upon addition of hAAG
765 glycosylase (150nM). Average and standard deviation of two independent experiments.

766
767 **Figure 3.** Stimulation of MMR at a single O 6 mG site by N-alkyl adducts *in cis*.
768 **A.** Covalently closed circular (ccc) plasmids (pAS200.2, 2,1 kb) containing a site-specific
769 O 6 mG:C base pair (plasmid mGC) and the corresponding lesion-free control (plasmid GC)
770 were constructed (Isogawa et al., 2020). Similarly, plasmids with a site specific GT or a
771 O 6 mG:T mismatch were constructed. All 4 constructs were treated with MMS in order to
772 introduce random N-alkyl (7mG and 3mA) adducts, generating plasmids GC+MMS,
773 mGC+MMS, GT+MMS and mGT+MMS. We adjusted the MMS reaction conditions as to
774 introduce \approx 9 adducts per plasmid (i.e. one N-alkylation adduct every \approx 500 nt). The resulting
775 proportion of O-alk and N-alkyl adducts mimics the proportion in MNU-treated plasmid. The
776 single O 6 mG adduct and the randomly located N-alkyl adducts are represented by a star and
777 red dots, respectively.

778 **B.** Plasmids described above were incubated in NPE supplemented with α 32 P-dATP at room
779 temperature; incorporation of radioactivity was monitored as a function of time using the
780 spot assay. The y-axis represents the percentage of DNA repair synthesis with respect to input
781 DNA (i.e. 10% means that the observed extent of repair synthesis is equivalent to 10% of
782 input plasmid replication). Overall, incorporation into GT and mGT plasmids is higher than
783 incorporation in their GC and mGC counterparts. Incorporation attributable to repair at the
784 O 6 mG:C lesion is increased close to 3-fold due to the presence of random N-alkyl lesions
785 introduced by MMS treatment. The stimulatory effect of random N-alkyl lesions on GT and
786 mGT repair is observed but is slightly less pronounced than for mGC.

787 **C.** The same plasmids were incubated for 2h incubation in NPE, purified, resolved by agarose
788 gel electrophoresis and revealed by ethidium bromide fluorescence and 32 P autoradiography.
789 The total amount of signal per lane is indicated (AU). As expected, the amount of plasmid
790 extracted from each incubation mix is relatively constant, as quantified below the ethidium
791 bromide image. Increase in repair at the O 6 mG:C lesion due to MMS-treatment (2.8-fold) is in
792 good agreement with data in Figure 3B.

793
794 **Figure 3-figure supplement 1.** Mapping repair synthesis in the vicinity of a single O 6 mG adduct.
795 **A:** Agarose gel electrophoresis of the native form of GC, mGC, GC+MMS and mGC+MMS

796 **B.** Restriction map of pAS201, the two single cutter enzymes *BmtI* and *BaeGI* yield a short S
797 (589bp) and long L (1525bp) fragment, respectively. Fragment S contains the single O⁶mG
798 adduct.

799 **C.** Plasmids GC, mGC, GC+MMS and mGC+MMS were incubated in NPE in the presence of
800 $\alpha^{32}\text{P}$ -dATP and extracted after 2h. The purified plasmids were digested with restriction
801 enzymes *BmtI* and *BaeGI*. Digested plasmids were analyzed by agarose gel electrophoresis
802 and visualized by ethidium bromide staining and by ³²P-imaging.

803 **D.** The specific activity (SA) of a given fragment (S or L) was quantified as the amount of ³²P
804 incorporated divided by the amount of DNA deduced from the Ethidium bromide
805 fluorescence image (expressed in arbitrary units AU). In control plasmid GC, a similar SA value
806 was observed for both fragments as expected for residual background incorporation;
807 similarly, in MMS treated plasmid (GC + MMS), the SA value is similar for S and L fragments.
808 The slightly increased SA value in GC+MMS compared to GC is compatible with repair
809 synthesis by BER at randomly located MMS-induced lesions. For plasmids containing a single
810 O⁶mG:C lesion (mGC and mGC+MMS), there is a robust increase in SA of the short compared
811 to the long fragments. Incorporation, above background, due to O⁶mG, in the absence of
812 MMS, amounts to 0.065 and 0.495 AU for long and short fragments, respectively (signal
813 above dotted line in S3D). Similarly, in the context of MMS lesions, incorporation, above
814 background, due to O⁶mG amounts to 0.115 and 1,17 AU for long and short fragments,
815 respectively (signal above dotted line in S3D). Taken together these results clearly show that
816 O⁶mGC-mediated repair specifically takes place in the S fragment, with only modest spill-over
817 into the L fragment (10-15%).

818 **E.** Plasmid with a single O⁶mG:C lesion site (mGC) and control plasmid (GC) were incubated in
819 NPE depleted with anti Mlh1 antibodies and mock treated NPE. Incorporation was measured
820 into whole plasmids. The increase in incorporation into mGC compared to GC observed upon
821 incubation in NPE fully disappeared when Mlh1 was depleted. This experiment shows that the
822 increase in incorporation observed in mGC compared to incorporation in control GC plasmid
823 can specifically be attributed to mismatch repair activity at the single O⁶mG:C lesion

824

825 **Figure 4.** Double-strand breaks occur in MNU-treated plasmid during incubation in extracts.

826 **A.** Analysis by AGE of alkylated plasmids (pEL97: 11,3kb) incubated in NPE in the presence of
827 $\alpha^{32}\text{P}$ -dATP. Plasmid pEL97 was treated with MMS, MNU+ and ENU as to introduce \approx one
828 alkylation event on average every 500 nt on average. For MNU, a plasmid with twice the level
829 of alkylation (MNU++, 1 lesion every 250 nt) was also produced (Figure 4-figure supplement
830 1). Alkylation of these plasmids essentially not affected their migration on agarose gels (Figure
831 4-figure supplement 2A). After 2h incubation, the reaction was stopped and a known amount
832 of pBR322 (10 ng) plasmid was added as an internal standard. Ethidium bromide image: in the
833 different lanes the internal standard band, pBR (ccc), appears to be of similar intensity (1158
834 +/- 95 AU), assessing reproducible DNA extraction. For the alkylated plasmids, incubation in
835 NPE led to massive conversion from ccc to relaxed plasmid. ³²P image: Little incorporation of
836 ³²P-dATP is seen in DNA0 and in MMS-treated plasmid compared to MNU and ENU treated
837 plasmids as shown by the relative incorporation levels normalized to 1 for untreated plasmid
838 (DNA0). As expected, the MNU++ sample exhibits about twice the amount of incorporated
839 radioactivity compared to MNU+. In both Ethidium bromide and ³²P images, a small amount
840 of linear plasmid is seen mostly in the MNU++ sample. This band is also visible in the MNU+
841 and ENU lanes although at a weaker intensity.

842 **B.** Quadratic dose-response for DSB formation. When the % of linear form (linear/(linear +
843 oc)), is plotted as a function of the square dose of MNU (mM^2) for untreated, MNU+ and
844 MNU++ plasmids, we observed a straight line ($y = 1,4173x - 0,0288$; $R^2 = 0,9999$).

845

846 **Figure 4-figure supplement 1.** Estimation of N-alkylation levels of modification by MMS and
847 MNU.

848 A. Alkaline fragmentation of alkylated plasmid (pEL97, 11.3 kb) DNA is analyzed by agarose gel
849 electrophoresis. B. Densitometry of the ethidium bromide-stained agarose gel. C. Estimation
850 of the average number of alkali cleavage sites per plasmid strand. The data reveal that the
851 average number of cleavage sites per plasmid strand is 21-23 for both MMS and MNU+
852 reaction conditions (the average distance between two sites is ≈ 510 nt). For MNU++, the
853 average distance between two cleavage sites is ≈ 254 nt.

854

855 **Figure 4-figure supplement 2.** Fragmentation of alkylated plasmid as analyzed on AGE loaded
856 under alkaline conditions.

857 **A:** Alkylation did not affect plasmids topology except for ENU treatment that increases
858 relaxation as seen in the AGE image.

859 **B:** Analysis by AGE, under alkaline loading conditions, of alkylated plasmids (pEL97: 11,3kb)
860 incubated in NPE in the presence of $\alpha^{32}\text{P}$ -dATP (same samples as in Figure 4A). Loading under
861 alkaline conditions allowed single-stranded nicks to be revealed. In the different lanes, an
862 internal standard band, pBR (ccc), was introduced before the extraction procedure in order to
863 assess consistent recovery of DNA during extraction (as seen by ethidium bromide staining).
864 For the alkylated plasmids, incubation in NPE led to massive conversion of plasmid DNA into a
865 smear with a low amount of DNA present as a single-stranded linear band (doublet). For the
866 MNU++ sample, no linear ss DNA was seen, all DNA was converted into a smear. The
867 smearing can best be seen in the ^{32}P image. We suggest that the ss DNA fragments that form
868 the smear arise between a gap caused by a MMR event at a O^6mG sites and an uncompleted
869 BER event at a neighbor lesion. It cannot be excluded that some fragments result from MMR
870 attempts at two O^6mG lesions. The intense ^{32}P labelling of the fragments in the smear result
871 from incorporation of up to several hundred nt during a single MMR repair synthesis.
872 Relative ^{32}P incorporation normalized to 1 for DNA0 are indicated below the ^{32}P -image.

873

874 **Figure 5:** Simultaneous repair of two closely spaced MNU-induced lesions may lead to a DSB.
875 Such a situation occurs when an N-alkyl lesion located within $\approx 500\text{nt}$ of an O^6mG lesion are
876 processed simultaneously (“Lesion Arrangement at-risk”). Note that the MMR excision track
877 can occur on either strand as described for noncanonical MMR (Peña-Díaz et al., 2012).

878 Reaction of MNU with double-stranded DNA induces N-alkylation adducts, mostly 7mG and
879 3mA shown as * and O-alkylation adducts (O^6mG), at a 10:1 ratio approximatively. Step 1: a
880 BER event is initiated at an N-alkyl adduct, creating a nick. Step 2: concomitantly, a MMR
881 event takes place, in the opposite strand, at a nearby at a $\text{O}^6\text{mG}:\text{C}$ site. Step 3: the MMR
882 machinery extends the nick into a several hundred nt long gap by means of Exo1 action. Step
883 4: The two independently initiated repair events lead to a DSB, if the MMR gap reaches the
884 BER initiated nick before resealing.

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892

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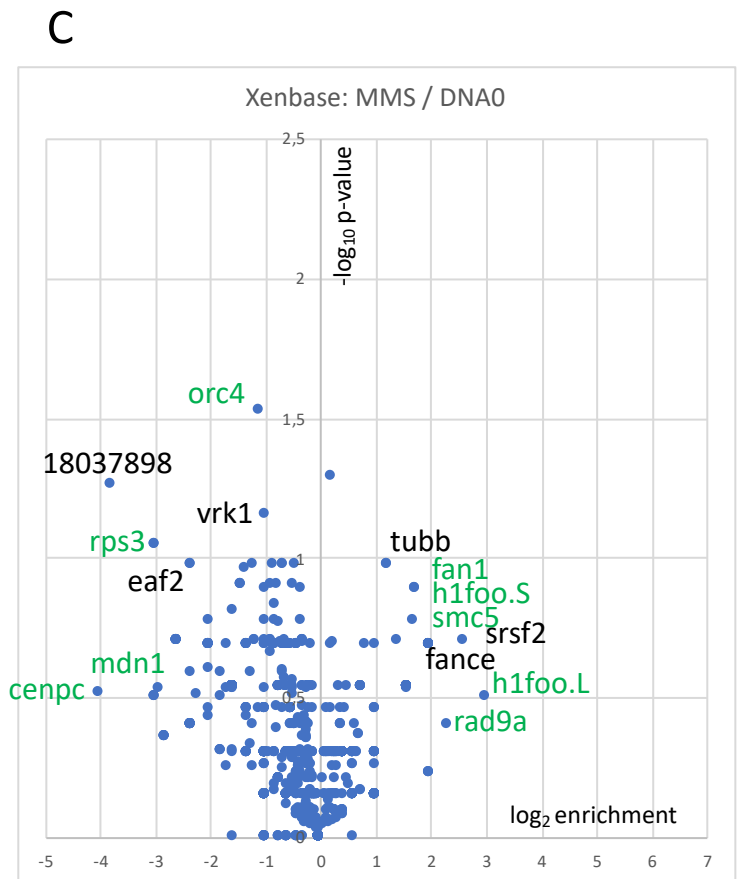
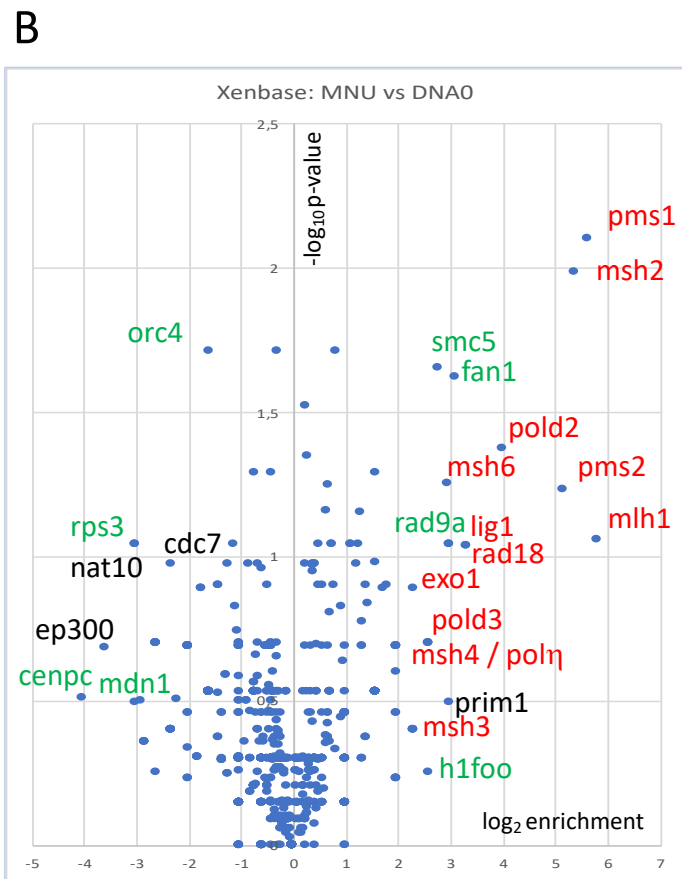
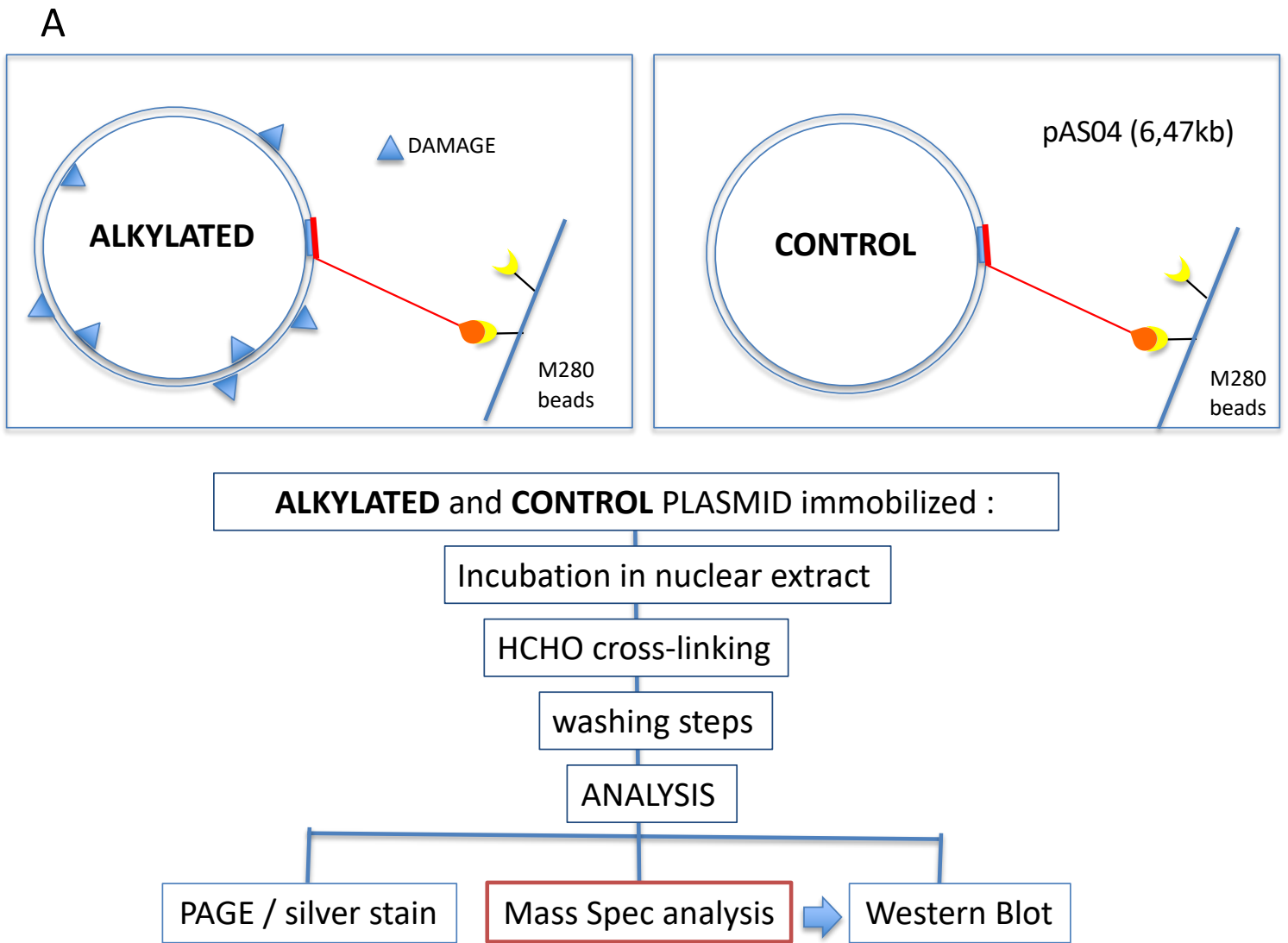
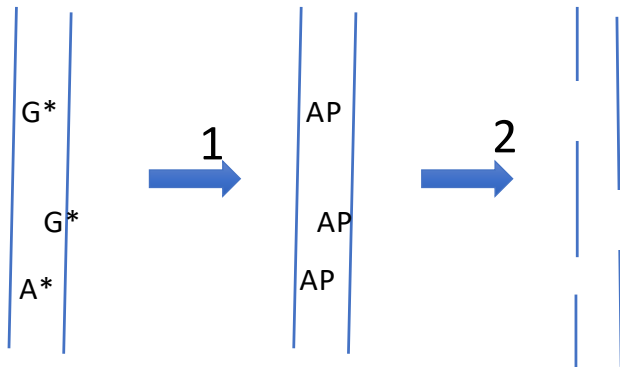


Figure 1

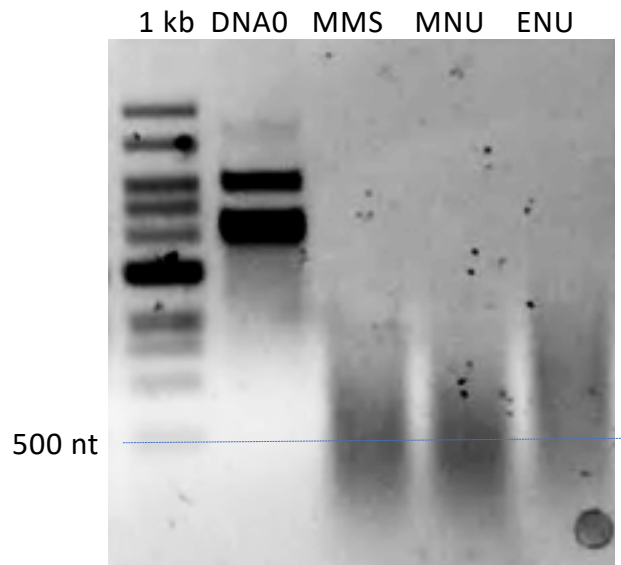
A.

0.65 ml tube	DNA0	MMS: 10mM final	MNU: 1mM final	ENU: 30mM final
pBR322 1ug in CE buffer	90ul	90ul	90ul	90ul
Alkylating agent in DMSO	10ul DMSO	10ul MMS at 100mM	10ul MNU at 10mM	10ul ENU at 300mM
Temp / time	50°C / 40 min	40°C / 30min	50°C / 16 min	50°C / 40 min

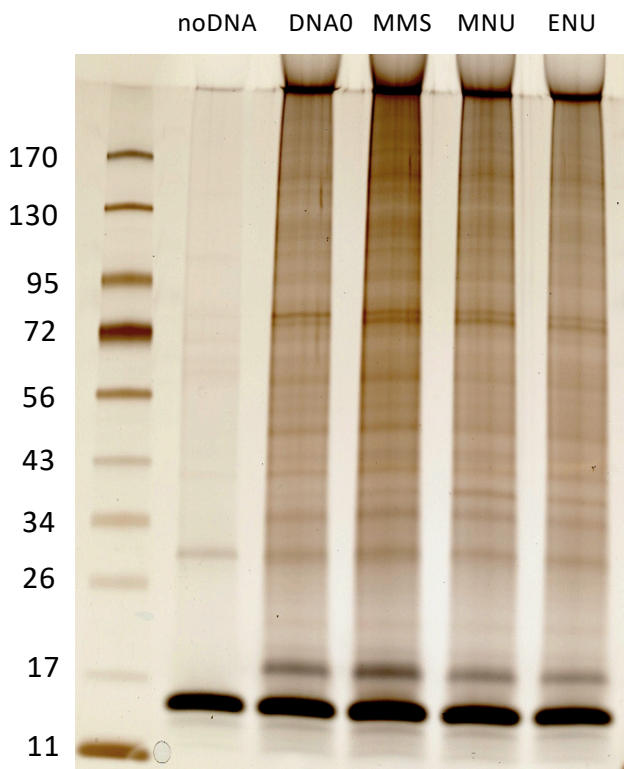
Alkylation reaction conditions leading to an average of one N- alkylation adduct every 500 nt.



Cleavage at 7mG (G*) and 3mA adducts (A*):
 1. Heat-induced depurination at pH7 (90°C, 15')
 2. Heat-induced strand cleavage at pH13 (90°C, 30')



B.



C.

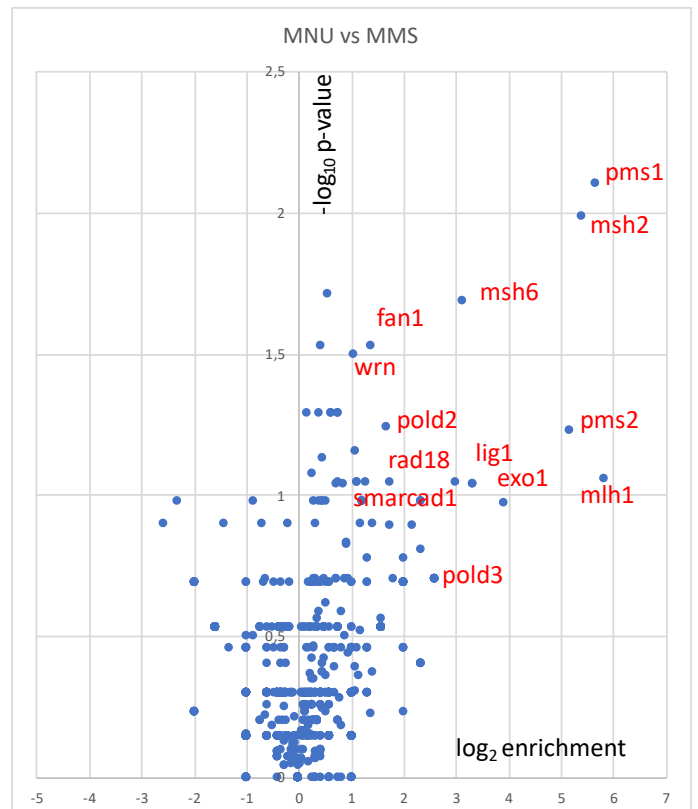
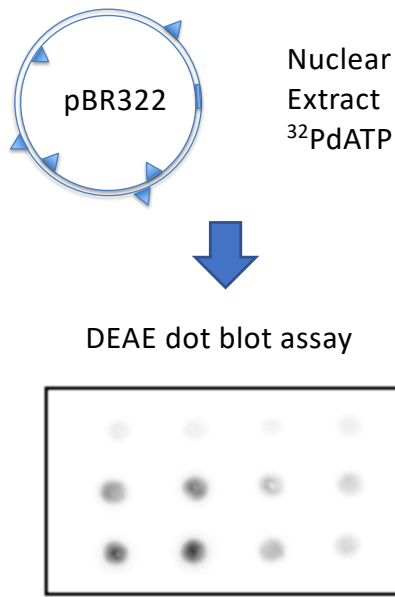
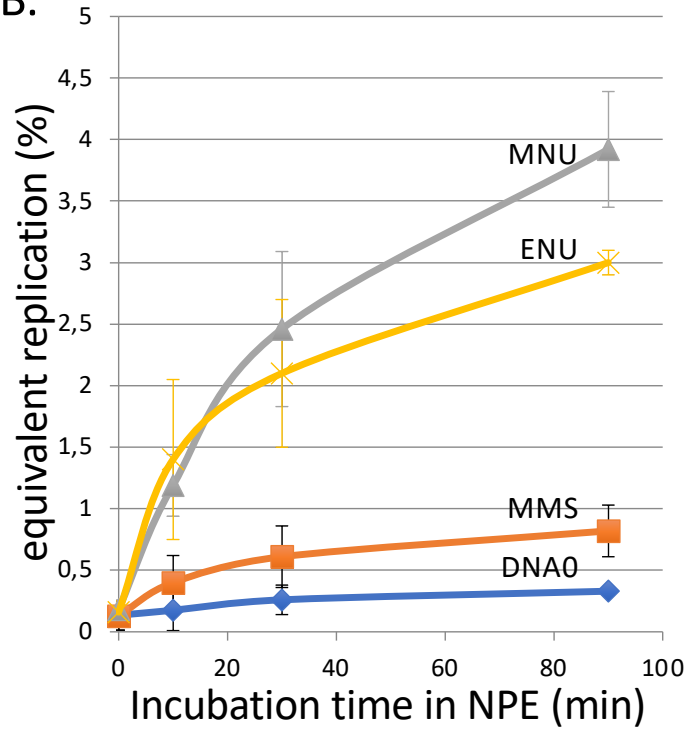


Figure 1-figure supplement 1

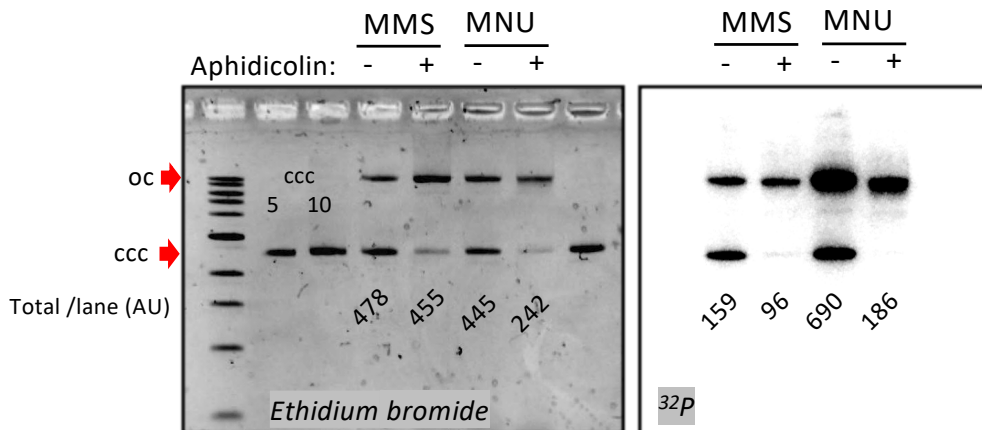
A.



B.



C.



D.

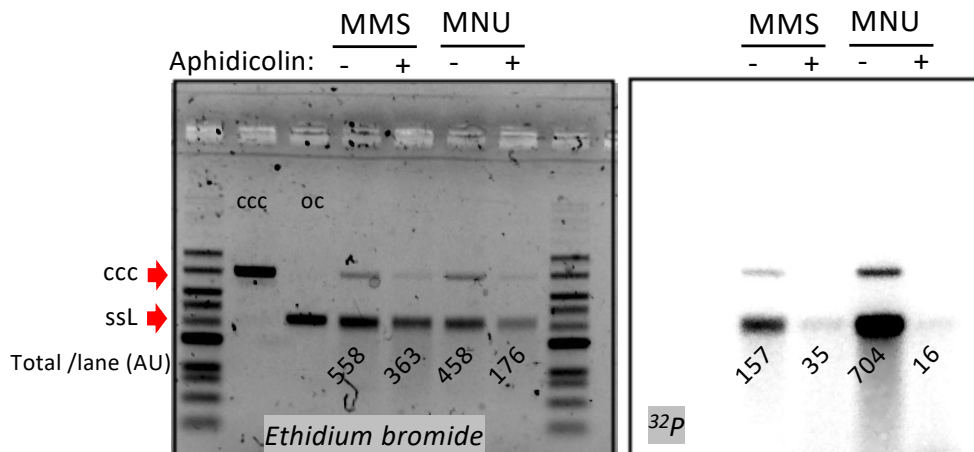
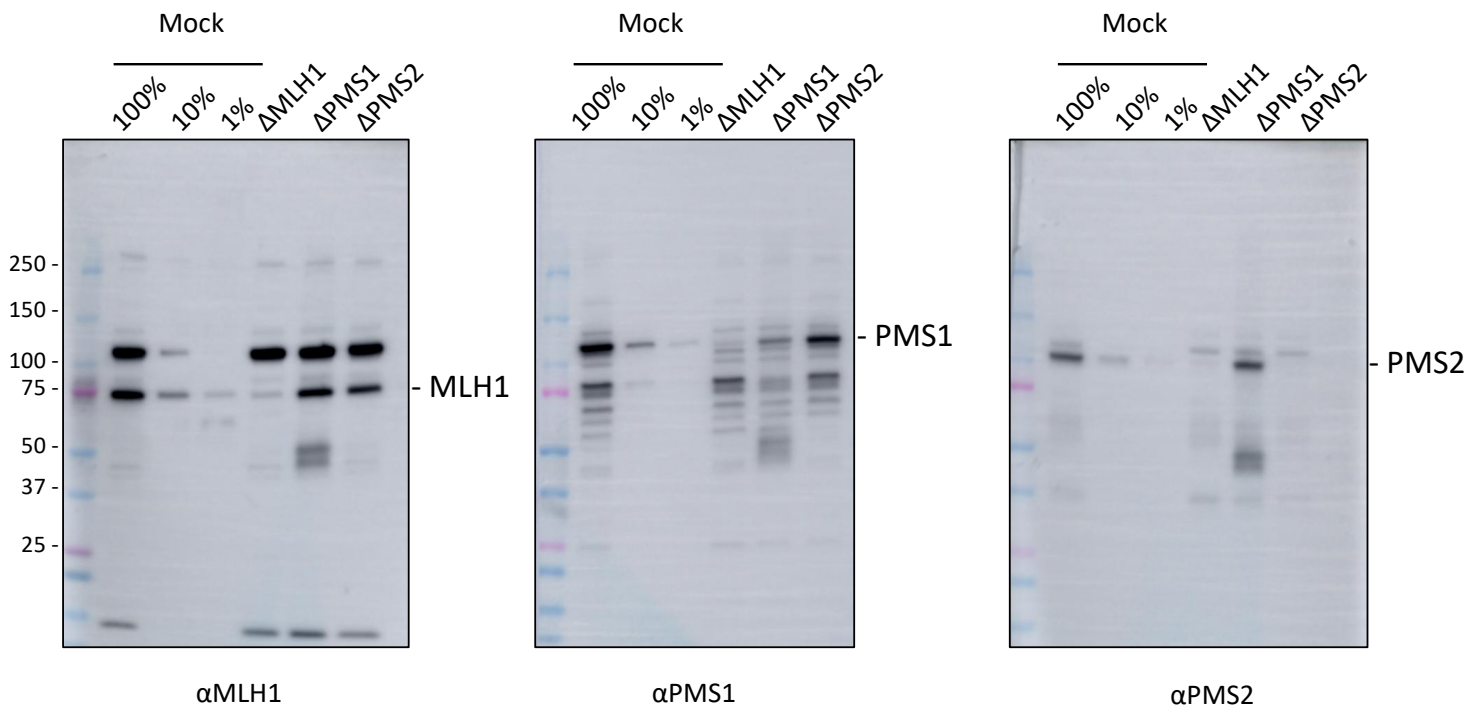
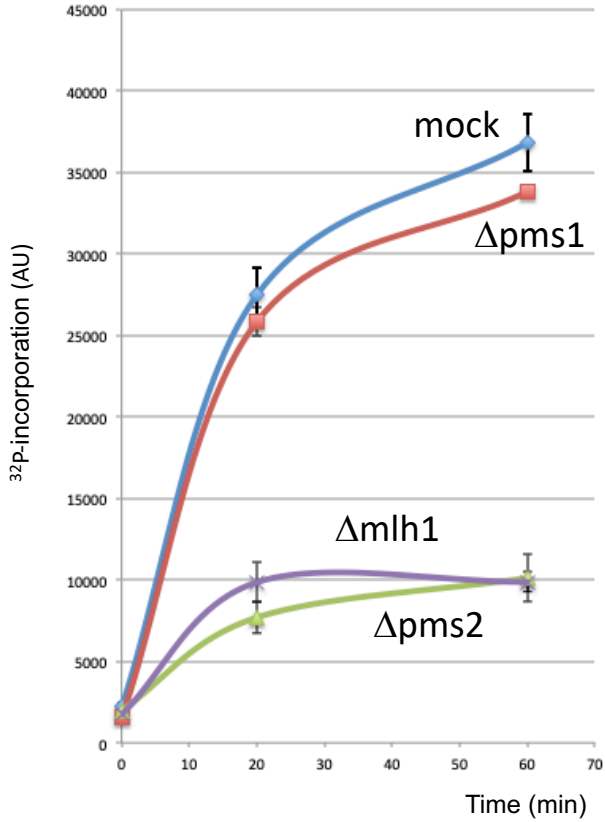


Figure 2

A.



B.



C.

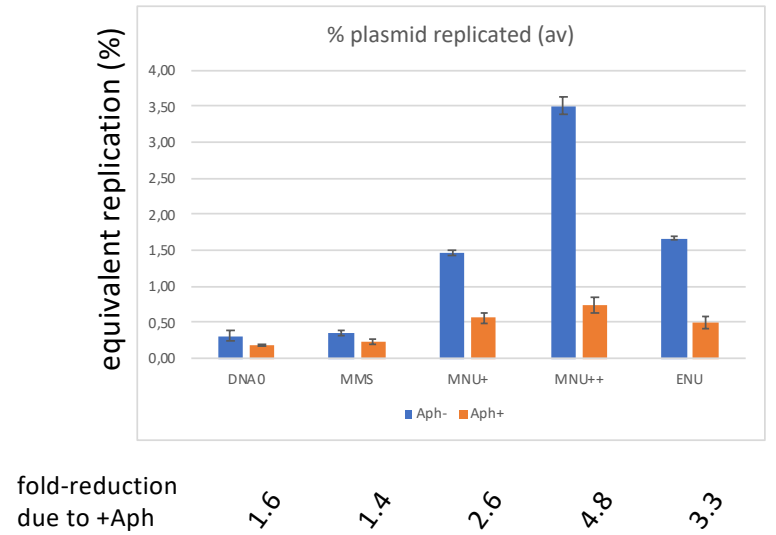


Figure 2-figure supplement 1

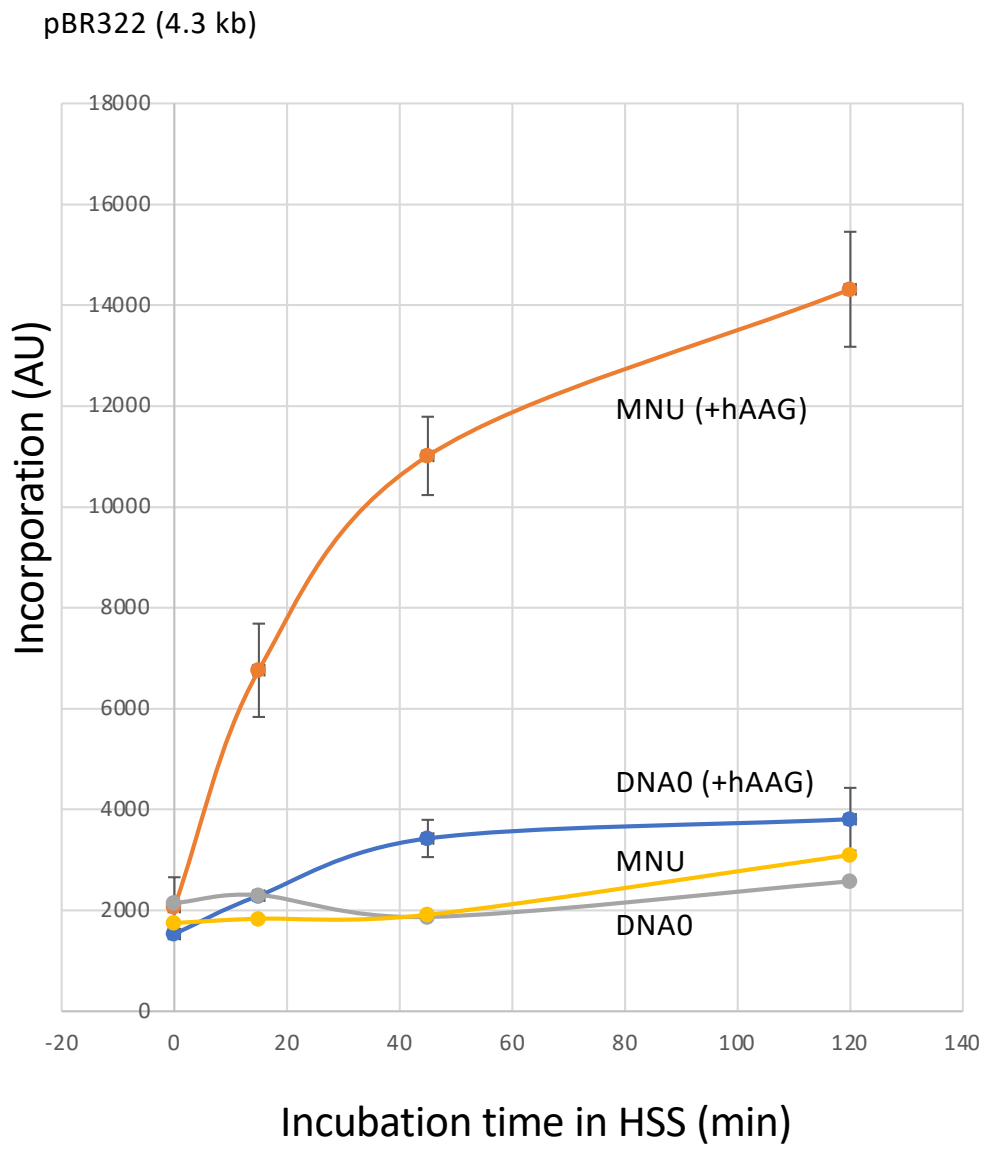


Figure 2-figure supplement 2

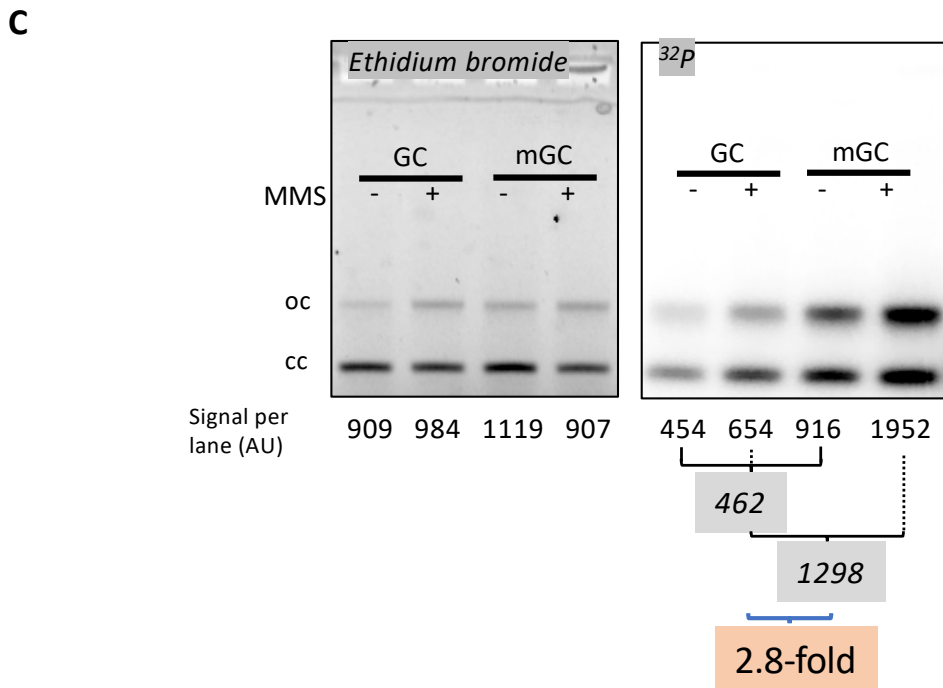
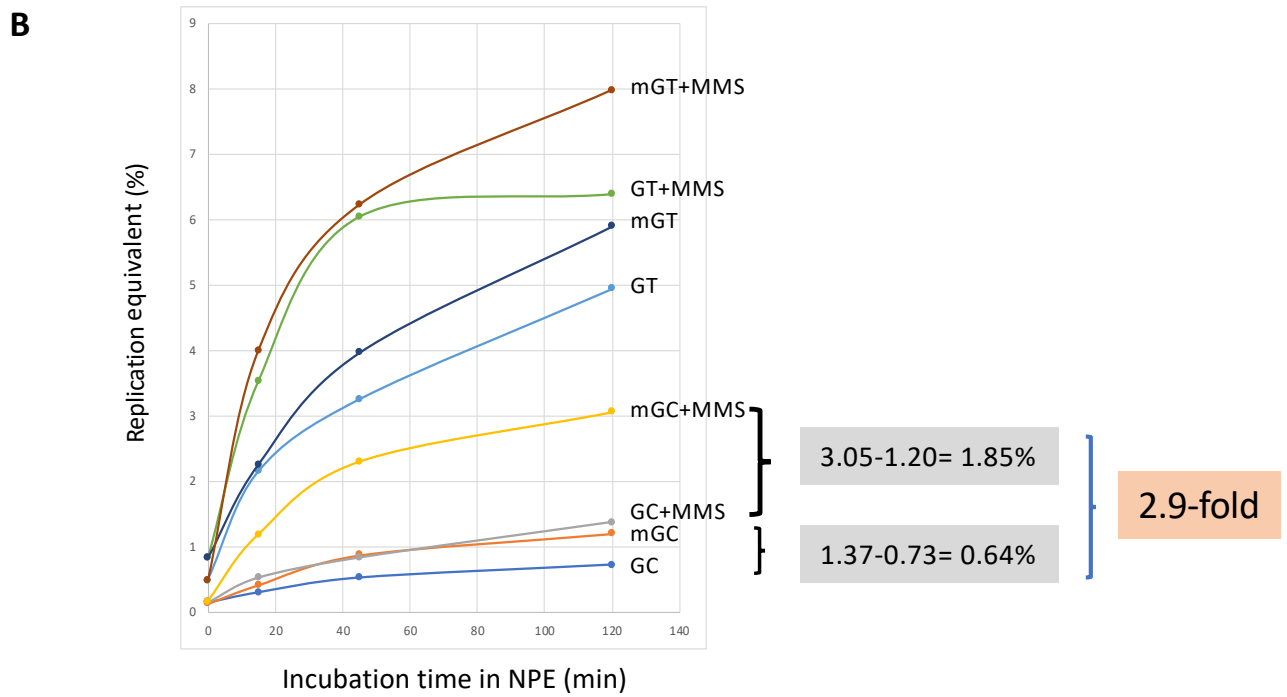
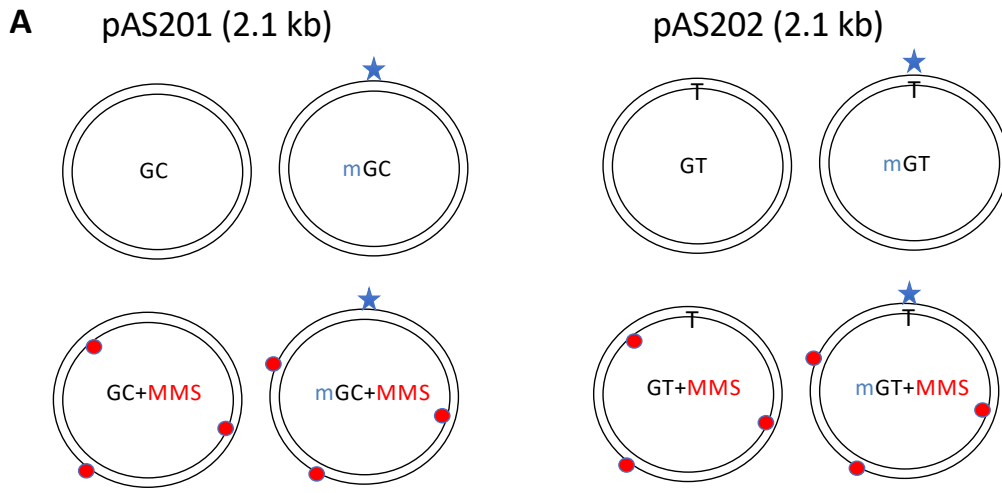


Figure 3

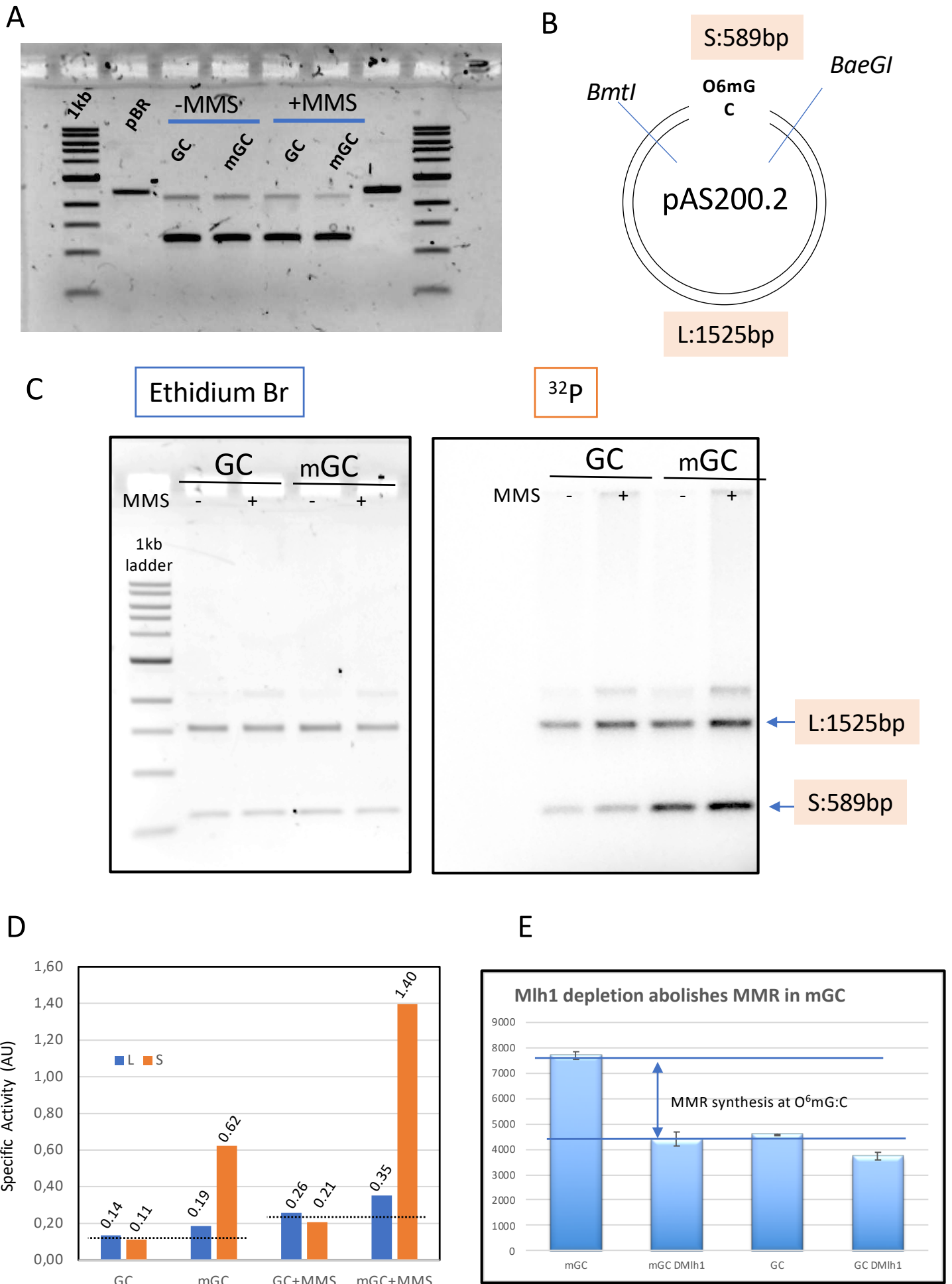
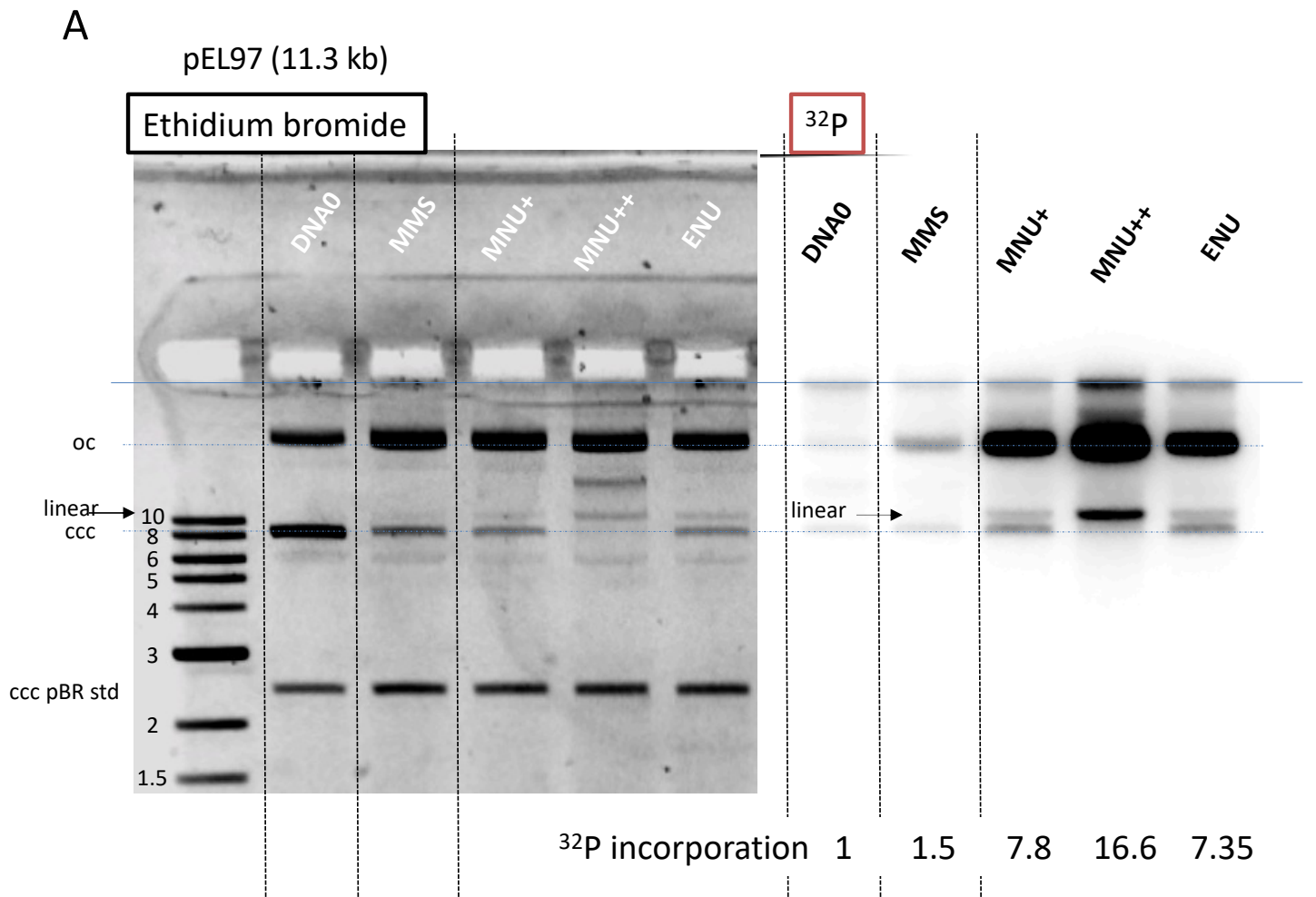


Figure 3-figure supplement 1



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B

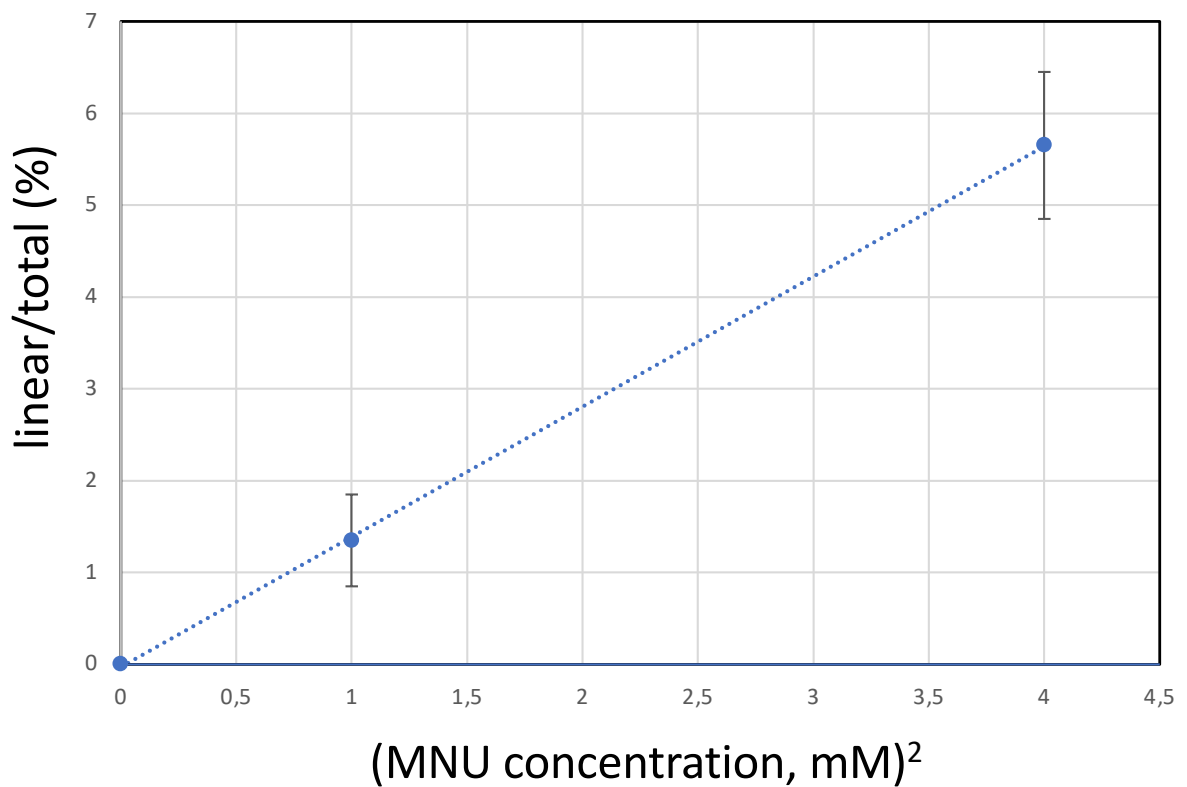
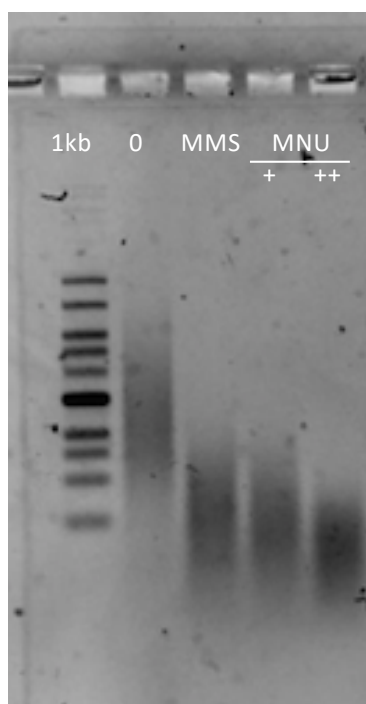


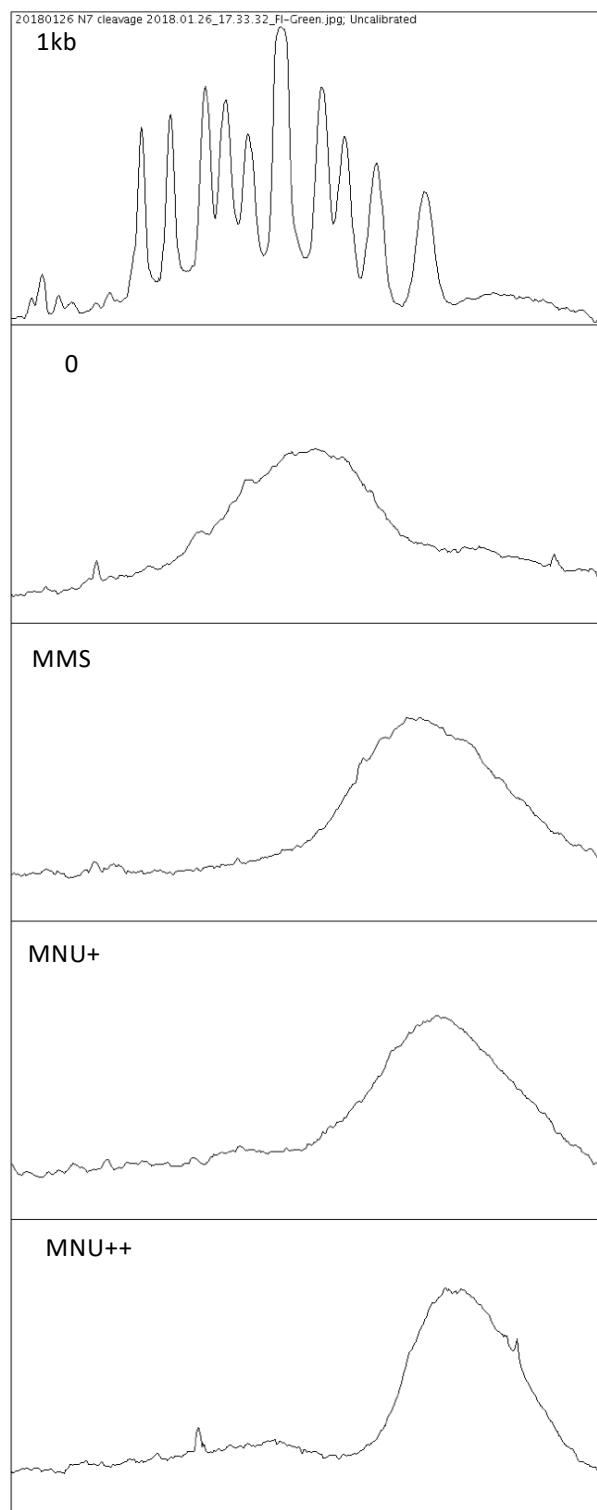
Figure 4

pEL97 (11.3 kb)

A



B



C

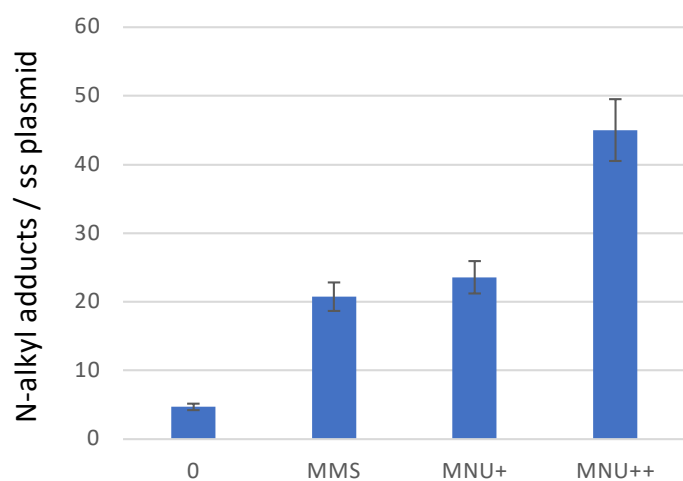
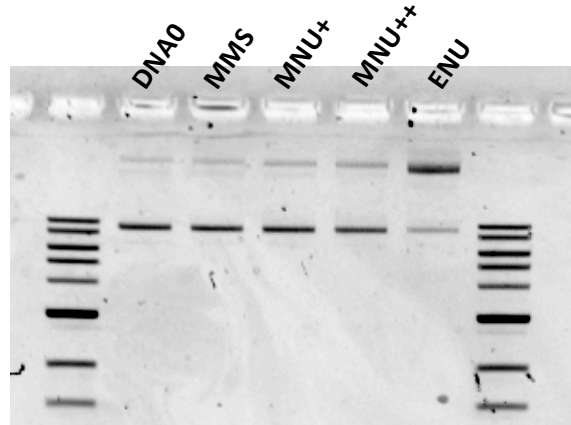


Figure 4-figure supplement 1

pEL97 (11.3 kb)

A



B

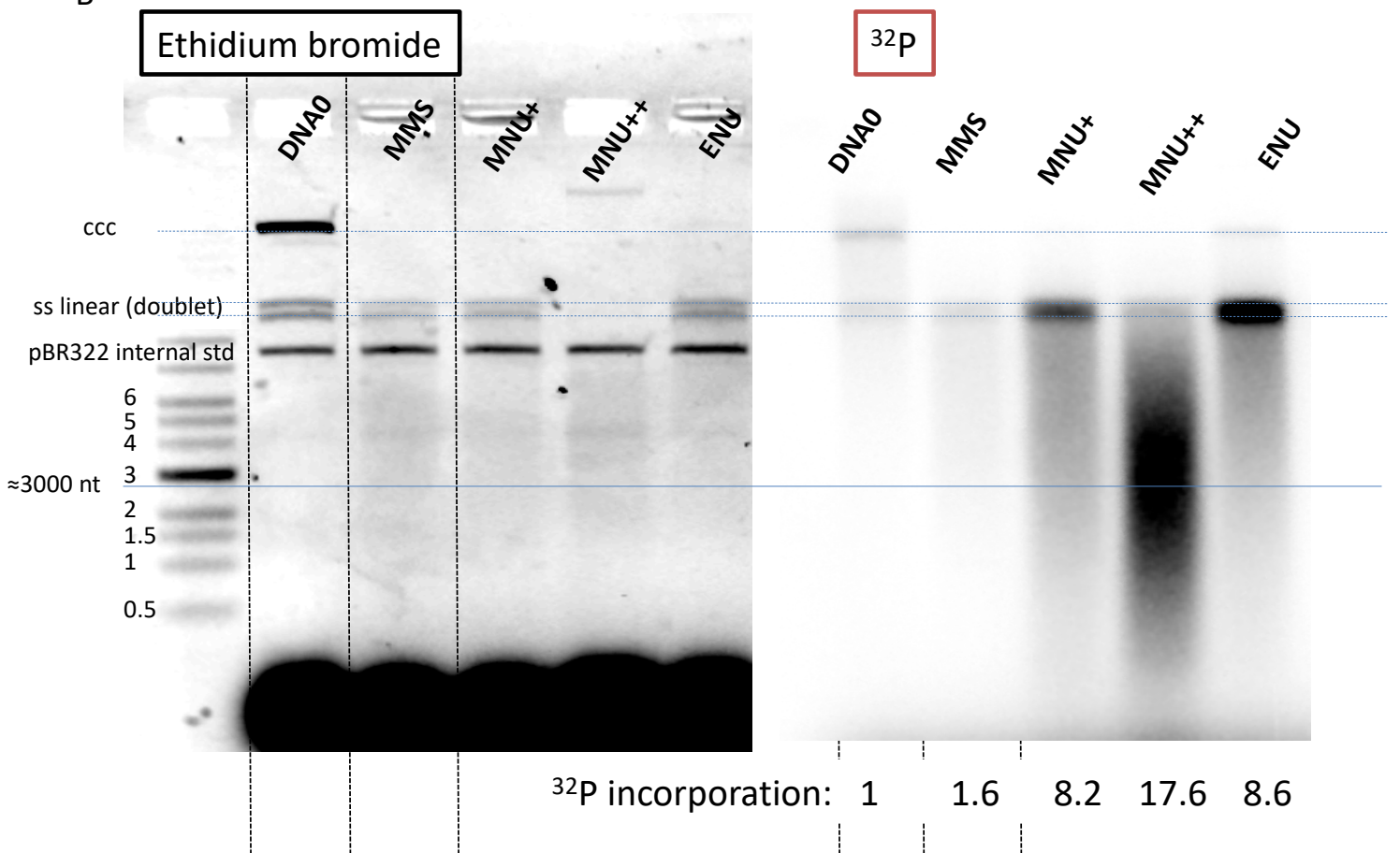


Figure 4-figure supplement 2

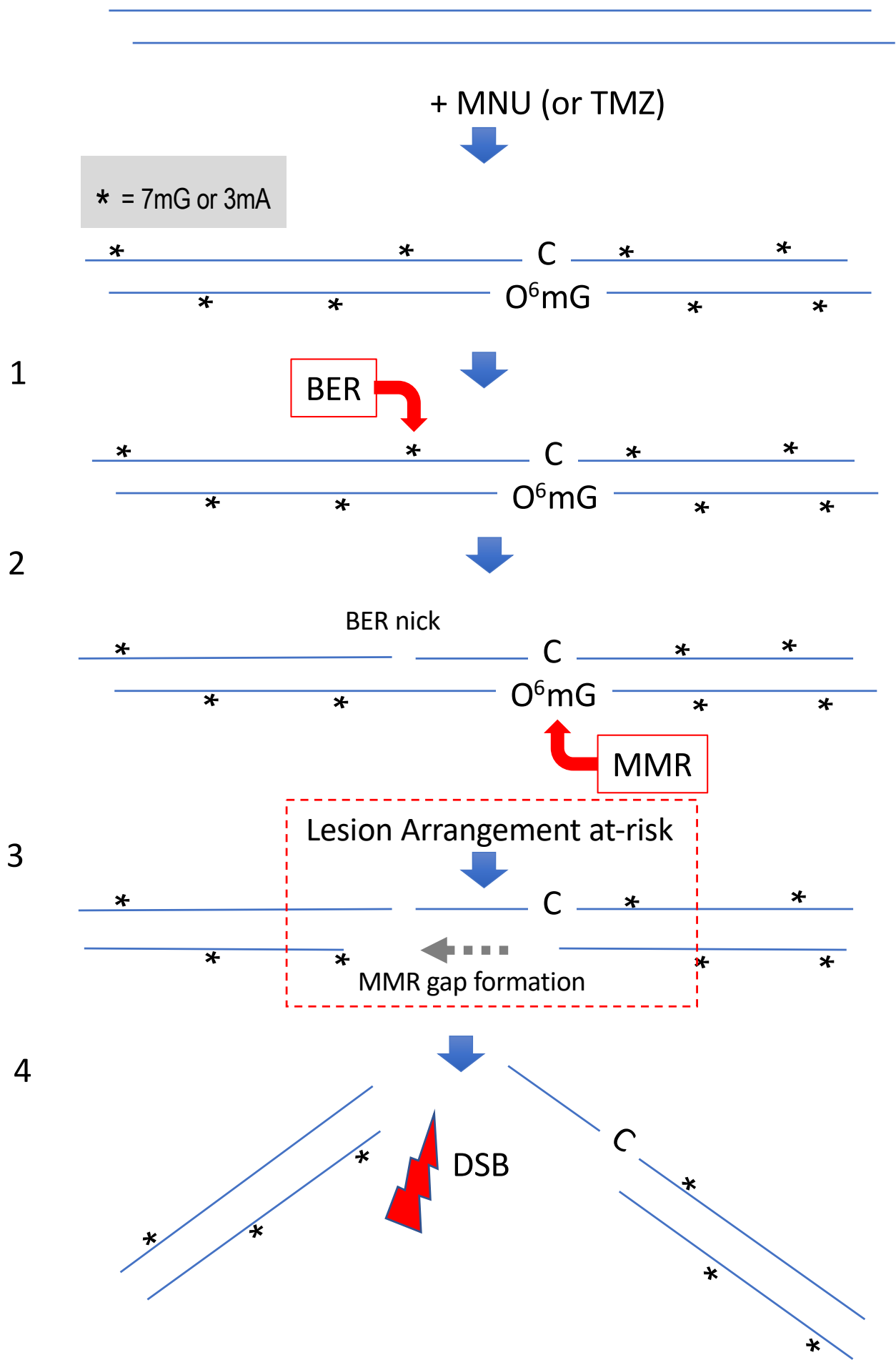


Figure 5