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Interplay between base excision repair activity and toxicity of 3-methyladenine DNA glycosylases in an *E. coli* complementation system

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ABSTRACT

DNA glycosylases carry out the first step of base excision repair by removing damaged bases from DNA. The *N*3-methyladenine (3MeA) DNA glycosylases specialize in alkylation repair and are either constitutively expressed or induced by exposure to alkylating agents. To study the functional and evolutionary significance of constitutive *versus* inducible expression, we expressed two closely related yeast 3MeA DNA glycosylases – inducible *Saccharomyces cerevisiae* MAG and constitutive *S. pombe* Mag1 – in a glycosylase-deficient *Escherichia coli* strain. In both cases, constitutive expression conferred resistance to alkylating agent exposure. However, in the absence of exogenous alkylation, high levels of expression of both glycosylases were deleterious. We attribute this toxicity to excessive glycosylase activity, since suppressing *sp*Mag1 expression correlated with improved growth in liquid culture, and *sp*Mag1 mutants exhibiting decreased glycosylase activity showed improved growth and viability. Selection of a random *sp*Mag1 mutant library for increased survival in the presence of exogenous alkylation resulted in the selection of hypomorphic mutants, providing evidence for the presence of a genetic barrier to the evolution of enhanced glycosylase activity when constitutively expressed. We also show that low levels of 3MeA glycosylase expression improve fitness in our glycosylase-deficient host, implying that 3MeA glycosylase activity is likely necessary for repair of endogenous lesions. These findings suggest that 3MeA glycosylase activity is evolutionarily conserved for repair of endogenously produced alkyl lesions, and that inducible expression represents a common strategy to rectify deleterious effects of excessive 3MeA activity in the absence of exogenous alkylation challenge.

1. Introduction

DNA glycosylases remove damaged bases from DNA by catalyzing hydrolysis of the *N*-glycosidic bond. The resulting abasic site is processed by enzymes that cleave the sugar phosphate backbone (endonucleases), process the resulting free ends (lyases, phosphatases), fill in the resulting gap (DNA polymerases), and ligate the DNA sugar phosphate backbone (DNA ligases). Collectively, this form of DNA repair is known as base excision repair (BER).

*N*3-methyladenine (3MeA) glycosylases constitute a class of glycosylases that specialize in repair of alkylation damage. *Escherichia coli* contains two 3MeA glycosylases in its genome: constitutively expressed *tagA* and the inducible *alkA* [1,2]. The TAG protein acts almost exclusively on 3MeA lesions, while AlkA has a broad substrate range for a multitude of endogenously- and exogenously-produced non-bulky lesions in addition to 3MeA [3–5]. *alkA* mutants are sensitive to exogenous alkylating agents, whereas *tagA* mutants have only moderately increased sensitivity [6–8].

The fission yeast *Schizosaccharomyces pombe* contains a constitutive 3MeA glycosylase, *sp*Mag1 [9], which plays a relatively minor role in alkylation resistance [10–12]. By contrast, the *Saccharomyces cerevisiae* ortholog, *sc*MAG, is inducible, repairs a broader set of alkylation lesions than *sp*Mag1, and its deletion has a profound effect on *S. cerevisiae* alkylation resistance, similar to *E. coli alkA* [4,13–16]. Thus, constitutively expressed 3MeA glycosylases tend to have narrower substrate specificity and less overall impact on alkylation resistance than inducible ones.

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BER requires the coordinated action of several enzymes working in succession. Some BER intermediates, notably incompletely processed nicks, are highly cytotoxic. Therefore, minimizing the formation of these intermediates, which requires a high level of control at the expression and the protein interaction levels, is essential [17]. Alterations in 3MeA glycosylase expression or activity can be deleterious. *sc*MAG overexpression, for example, results in a strong mutator phenotype and compromised viability in both *S. cerevisiae* and *E. coli* strains [18,19]. The observed cytotoxicity in this case is most likely attributable to DNA nicks that are generated by endonuclease processing of abasic sites, as these can easily become DNA strand breaks, which are highly cytotoxic [20].

To gain insight into the selective pressures driving the evolution of inducible and constitutive 3MeA glycosylases, we examined the effect of ectopically expressing *sp*Mag1 and *sc*MAG in a 3MeA glycosylase-deficient strain of *E. coli*. We provide several lines of evidence suggesting that, in the absence of exogenous alkylating agents, *sp*Mag1 has only a positive effect on the fitness of its *E. coli* host strain when present at low levels. Increasing *sp*Mag1 activity or expression, on the other hand, increases protection from exogenous alkylating agents but decreases survival in the absence of exogenous alkylation. These effects are more pronounced in *sc*MAG (an inducible 3MeA glycosylase), which is more deleterious than *sp*Mag1 in the absence of exogenous alkylating agents but that also confers more robust protection to exogenous alkylation. These findings suggest that inducible 3MeA glycosylases represent an evolutionary solution to increase glycosylase activity for protecting cells from exogenous alkylation while minimizing the negative effects inducible glycosylases have on organismal fitness.

2. Methods

2.1. Plasmids and bacterial strains

All constructs were cloned into the pLitmus 28i vector (New England Biolabs), which carries carbenicillin resistance as a selectable marker, using the *XhoI* and *HindIII* restriction enzymes. *S. cerevisiae* MAG (RefSeq NM 001179032.1) was cloned using the primers AAAAAACTCGAGATGGGTTCTTCTCAC (F) and AAAAAAAAGCTTTTAGGATTTCACGAA (R) and *S. pombe* Mag1 (RefSeq NM 001019417.2) using the primers AAAAAACTCGAGATGGGTTCTTCTCAC (F) and AAAAAAAAGCTTTCAGTGTTTCTTCGG (R), using previously described *sp*Mag1 and *sc*MAG plasmids as PCR templates [13]. All constructs were transformed into an F′ (fertility plasmid) positive MV1932 strain of *E. coli*, which is an alkylation-sensitive *alkA tag* derivative of AB1157. For the concatemer formation assay and growth experiments a F′-negative strain was used. The AB1157 strain was used as an alkylation resistant control, although a substantial fraction of the MV1932 genome is known to derive from Hfr KL16 [21].

2.2. Viability assay

Cultures of MV1932 cells containing the appropriate constructs were grown to saturation overnight in the presence of 100 μg/ml carbenicillin, and then diluted to an optical density (OD) of 0.15. Cultures were then grown to saturation again (approximately 18 h) in the presence of 100 μg/ml carbenicillin. Growing cultures to saturation twice helped balance substantial differences in growth rate observed in the initial inoculation. Cultures were then serially diluted to an appropriate cellular density and then plated on Petri dishes containing 100 μg/ml carbenicillin.

2.3. Concatemer formation assay

500 ng of pLitmus 28i plasmid DNA extracted from F′-negative MV1932 *E. coli* cells containing the appropriate constructs were either digested with *SacI*, which linearizes the plasmid, or left undigested and the products separated by electrophoresis on a 1% agarose gel for 2 h at 120 V and 400 mA. Formation of concatemers was identified in the form of high molecular weight bands on the gel that disappear upon restriction as previously described in [22].

2.4. Measurement of expression

GFP was introduced in the same multiple cloning site of the pLitmus vector used for expression of *sp*Mag1 and *sc*MAG, using *SnaBI* and *HIndIII* by standard PCR amplification with restriction site adapters followed by ligation. This reporter plasmid was transformed into MV1932 cells in parallel with the other constructs and colonies were grown in liquid culture under three conditions: LB, LB with 1 mM IPTG, and LB with 1% glucose. GFP fluorescence was measured by flow cytometry. 1 ml of overnight culture was centrifuged, resuspended in PBS, and GFP fluorescence was measured using a Cytopeia Influx cytometer with 200 mW Coherent 488 laser, and 531/40 PMT. Single viable cells were gated based upon forward and side scatter parameters. For each growth condition, 10,000 cells were analyzed. A pLitmus vector with no insert was used as a control.

2.5. Growth curves

Cultures of MV1932 cells transformed with the appropriate constructs were grown as described for the viability assay, but in this case in the presence or absence of 1% glucose. Cultures were diluted down to an OD of 0.15 in a 96-well plate and their growth shaking at 37°C was monitored hourly by OD using a Molecular Devices Versamax microplate spectrophotometer.

2.6. MNNG gradients

pLitmus 28i vectors containing *sp*Mag1 and *sc*MAG were transformed into competent MV1932 cells and cells grown in the presence of 100 μg/ml carbenicillin. Monocultures of these constructs were grown to saturation overnight, followed by dilution and growth to exponential phase. 50 μl of culture at exponential phase was mixed with 2 ml of top agar and stamped onto an MNNG (methylnitronitrosoguanidine)-containing gradient using the thin side of a sterile glass microscope slide. Gradients were poured as previously described [23]. In brief, square Petri dishes (Fisher Scientific) were placed on a slope of 10° while 25 ml LB agar containing 400μM MNNG was poured onto the plate. Once the agar had solidified, the Petri dish was set flat and a layer of 25 ml LB agar without MNNG was poured. The antibiotic carbenicillin was added to both layers of the gradient to promote plasmid retention as well as discourage contamination. 1 M MNNG stock solution was prepared by suspending MNNG powder into 100% DMSO. A 400 μM MNNG gradient was prepared by mixing 10 μl of 1 M MNNG with 25 ml LB agar. Cells on MNNG gradients were grown at 37 °C for 30 h. Gradient growth was measured in centimeters and the highest point of growth marked at the end of continuous culture growth. Length of growth on the gradient was proportional to protection against MNNG toxicity. To confirm that all cultures were at similar levels of viability, cultures were plated at a 10^{-6} dilution on Petri dishes containing carbenicillin and colonies counted. 0.05% Methyl methanesulfonate (MMS) gradients were run in the same way as MNNG gradients except that the stock solution came as 99% pure liquid (Aldrich).

2.7. Random mutant spMag library generation

*sp*Mag1 cDNA was subjected to random mutagenesis using the GeneMorph II Random Mutagenesis Kit (Agilent Technologies). 100 ng of target DNA was amplified through 25 cycles of PCR using the suggested PCR program outlined in the GeneMorph II manual with a T_m of 55 °C. Exact 18mer primers of the 5′ and 3′ end of the *sp*Mag1 cDNA were used to perform the mutagenic PCR. The mutant PCR product was gel purified using the Nucleospin extract II kit (E&K Scientific) and incorporated into the pLitmus 28i vector using megaprimer PCR, which uses a wild-type template and a mutant PCR amplicon as primer (megaprimer) to synthesize the desired mutant construct and subsequently eliminates the template through *DpnI* digestion [24]. We used 250 ng of PCR product as the megaprimer and 50 ng of the template plasmid (pLitmus 28i containing wild-type *sp*Mag1). The PCR product was then digested with 10 U of *DpnI* for 1 h and transformed into competent Top 10 cells. Cells were plated on pre-warmed Petri dishes containing carbenicillin and grown overnight at 37 °C. The resulting semi-lawn was washed with 2 ml of LB broth and subsequently mini-prepped to recover our library. Overall, our library contained plasmid DNA from >25,000 individual colonies. After construction, the library was transformed into Top 10 cells and plated at a low colony density (∼100 colonies/Petri dish). 143 random colonies from a few transformations were sent to Sequetech Corporation (Mountain View, CA) for sequencing to determine the spectrum of the random library. We obtained on average 1.7 mutations per *mag1* gene. By type, our sequences broke down as follows: 51% had at least one non-synonymous mutation, 20% had at least one indel or a stop codon, 19% had only synonymous mutations, and 11% corresponded to wild-type.

2.8. Genetic selections

To evolve *sp*Mag1 in the absence of methylation challenge, the *sp*Mag1 library was transformed into MV1932 cells and plated at a high density of transformant colonies (>100,000). The plate was then washed with 2 ml of LB broth. 10 μl aliquots of the wash were inoculated into 5 separate test tubes containing 5 ml LB broth and 100 μg/ml carbenicillin. Cultures were grown to saturation phase over a 24 h period. The next day, 10 μl of the saturated culture were used to inoculate a culture containing fresh LB and carbenicillin. This process was repeated for 5 days, and on the final day cultures were diluted 10^6-fold and plated on Petri dishes containing 100 μg/ml carbenicillin. Random colonies from each of the five independent experiments were sequenced (Sequetech Corporation, Mountain View, CA). The sequencing results are summarized in Table 1.

To evolve *sp*Mag1 under MNNG selective pressure, we transformed our library into MV1932 cells, ran our cultures on MNNG gradients as described above and picked 28 colonies growing above the threshold of continuous growth for wild-type *sp*Mag1.

2.9. 7MeG, εA and 3MeA repair assays in vitro

*sp*Mag1 proteins (wild-type and K126N E130G N175D mutant) were purified as previously described [13]. Briefly, proteins were overexpressed in *E. coli* as N-terminal His-tagged constructs and purified by affinity chromatography. The His-tags were removed by protease cleavage and the native proteins purified by ion exchange chromatography to yield >95% pure protein. Proteins were stored in 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mM DTT and 0.05 mM EDTA.

In vitro base excision activity of 3MeA, 7MeG and εA was performed as described previously [13]. Briefly, 7MeG and εA excision was measured by alkaline cleavage of the abasic site product generated from *sp*Mag1 incubation with the 25mer oligonucleotide d(GACCACTACACCXTTTCCTAACAAC)/d(GTTGTTAGGAAACGGTGT-AGTGGTC), in which X denotes the position of 7MeG or εA. The percent repair was calculated as the fraction product remaining after 4 h. 3MeA excision was measured by base released from calf thymus DNA treated with *N*-methyl-*N*-nitrosourea (MNU) [25]. Percent repair of 3MeA lesions was calculated as the amount of enzymatically removed 3MeA compared to the maximal amount of 3MeA present in the substrate, as measured by treatment with 5 N HCl. All glycosylase reactions were incubated for 4 h at 30 °C in 50 mM HEPES buffer (pH 7.4), 100 mM KCl, 10 mM DTT, and 2 mM EDTA.

2.10. Statistics

Data are expressed as mean ± standard deviation (SD). Data were analyzed using the Dunnett's test of means to test specific hypotheses comparing the performance of various mutants *versus* an appropriate control, using JMP software (Version 10.0, 2012, SAS Institute). A *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. Expression of yeast glycosylases modulates the viability of MV1932 E. coli cells

The MV1932 (*tagA ada*) strain of *E. coli* is used as a complementation system for 3MeA glycosylases, since these cells lack *tagA* and are unable to express *alkA* [26]. We investigated the phenotypic impact of expressing two yeast glycosylases, constitutively expressed *S. pombe* Mag1 and inducible *S. cerevisiae* MAG, in this glycosylase-deficient strain of *E. coli* to gain insight into the functional significance of the different expression and activity profiles of constitutive *versus* inducible glycosylases.

In the absence of alkylating agent challenge, ectopic yeast glycosylase expression in MV1932 cells resulted in substantial toxicity, most noticeably in saturated cultures, which produced elevated levels of metabolic stress and endogenous DNA damage [27,28]. Expression of both *sp*Mag1 and *sc*MAG decreased cell viability,

Table 1
*sp*Mag1 viability selection results. List of *sp*Mag1 non-synonymous mutants obtained from each individual selection experiment.

Sequence	Number of clones obtained from each independent selection experiment					Total
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	
Wild-type	2	2	4	2	5	15
L122M	1	0	0	1	0	2
K126N E130G N175D	1	3	2	2	3	11
N42Y V165A	1	0	1	0	0	2
W208stop	1	2	1	2	0	4
I147V T151A A206S	0	0	2	0	1	3
R149K	1	0	0	0	1	2
T13I Y39C I108N	0	1	0	0	0	1
E6V I173F	0	0	0	1	0	1

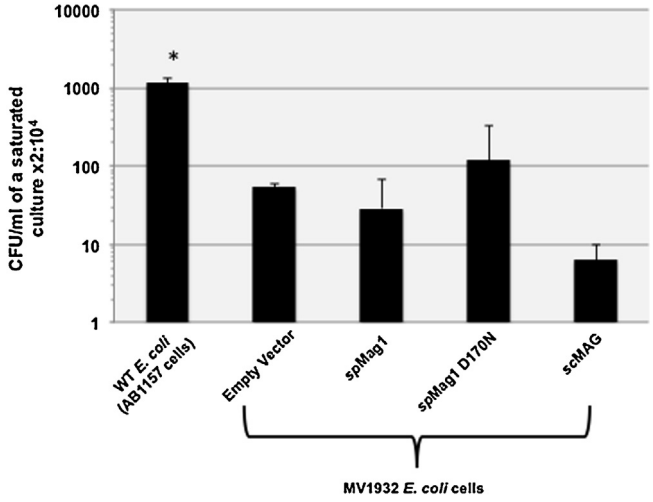

Fig. 1. Effect of expressing yeast 3MeA glycosylases on the viability of a glycosylase-deficient strain of *E. coli*. Viability of cells transformed with *sc*MAG and *sp*Mag1 constructs grown in liquid culture to stationary phase, in the presence of carbenicillin to ensure plasmid retention. All the constructs are expressed in the pLitmus 28i vector. In all cases, the host is the glycosylase-deficient strain of *E. coli* MV1932, except wild-type *E. coli* (WT), which is the AB1157 strain carrying an empty pLitmus 28i vector. Error bars represent standard deviation of triplicate experiments. The AB1157 strain of *E. coli* showed a highly statistically significant difference in viability relative to the empty vector MV1932 strain control ($p < 0.0001$ according to the Dunnett's test), but the differences between the other samples and the empty vector control did not reach statistical significance.

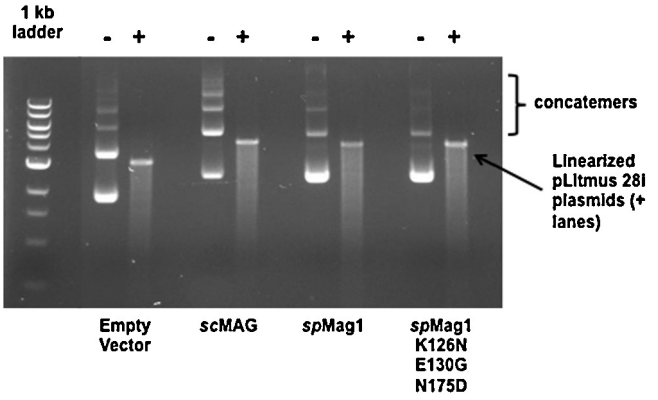

Fig. 2. Concatemer formation associated with plasmid propagation in MV1932 glycosylase-deficient *E. coli*. pLitmus 28i plasmid DNA extracted from MV1932 *E. coli* cells containing either the empty vector, *sp*Mag1, or *sc*MAG were linearized with the restriction enzyme *SacI* and run on a 1% agarose gel for 2 h. The *SacI*-digested empty vector is 3.2 kb long while the digested *sp*Mag1 and *sc*MAG1-containing vectors are 4.0 kb and 4.2 kb, respectively. Concatemer formation is observed as bands of high molecular weight that collapse upon *SacI* restriction.

with *sc*MAG appearing to have a stronger effect (Fig. 1). Interestingly, the complete absence of 3MeA glycosylase activity was also cytotoxic (Fig. 1), suggesting that optimal fitness depends on a window of 3MeA glycosylase activity. The *sp*Mag1 D170N mutation, which substantially decreases *sp*Mag1 enzymatic activity without abolishing it [13], moderately increased survival of MV1932 cells over both an empty vector and wild-type *sp*Mag1 (Fig. 1). This further supports the interpretation that both a complete absence and an excess of 3MeA glycosylase activity have negative effects on viability.

3.2. Cytotoxicity is likely due to DNA damage

We hypothesized that the toxicity associated with both a lack and an excess glycosylase activity may be caused by accumulation of BER intermediates, which typically occurs as a result of imbalances in expression of individual components in this pathway [29]. Consistent with this hypothesis, both *sc*MAG and *sp*Mag1 expression, as well as the absence of 3MeA glycosylase expression (empty vector), lead to increased plasmid concatemer formation (Fig. 2). By contrast, expression of a hypomorphic mutant of spMag1 that has no apparent deleterious effect on cell viability (K126N E130G N175D) (Fig. 4a) produced minimal concatemerization. Concatemer formation is considered an indicator of double-strand repair, albeit an indirect one.

3.3. SpMag1 toxicity can be reversed by decreased expression

To confirm that excessive *sp*Mag1 glycosylase activity is deleterious in our complementation system, we decreased spMag1 expression, which is under the control of the *lac* promoter, by adding 1% glucose to LB media, and followed growth by monitoring optical density. Decreased expression in the presence of glucose was confirmed by flow cytometry using a GFP transcriptional fusion construct as a reporter (see Section 2.4, Fig. 3a). In the absence of glucose, growth curves paralleled our viability results: AB1157 exhibited maximal growth, cells not expressing any *sp*Mag1 (empty vector or GFP) or cells expressing WT spMag1 showed poor growth, and cells expressing spMag1 D170N showed intermediate growth (Fig. 3b). As an additional control for possible non-specific effects of recombinant 3MeA glycosylase expression, we also included the hypomorphic mutant of *sp*Mag1 mentioned previously (K126N E130G N175D). Fig. 3c and d show that reducing *sp*Mag1 expression through addition of glucose to the media completely reversed the observed delay in growth of the wild-type and of the D170N mutant. This reversal is specific for *sp*Mag1, as it is not observed in the empty vector or GFP-expressing controls.

3.4. spMag1 mutants exhibiting reduced toxicity can be evolved

To gain additional insight into the nature of the observed toxicity, we selected *sp*Mag1 mutants with improved fitness in the absence of any exogenous alkylation challenge. To do this, we generated a random mutant library of *sp*Mag1 using error-prone PCR and selected for increased viability by passing cultures carrying our random mutant libraries five times. Our selection was performed in five parallel runs. We sequenced a total of 45 colonies, 7–10 per independent experiment. Table 1 below lists each of the *sp*Mag1 mutants that we identified, by experiment. We identified a high frequency (31%) of the wild-type sequence with no enrichment relative to the unselected library (30%), suggesting the wild-type sequence likely represents carryover from the unselected library. In the remaining sequences, we found a striking degree of convergent evolution, with six mutants appearing more than once independently and one (K126N E130G N175D) appearing a total of 11 times and in all five selections. All six mutants increased viability relative to wild-type *sp*Mag1, confirming their selective advantage. In all cases, the increase in viability was substantial, representing at least a 10-fold increase in cell viability (Fig. 4a).

3.5. spMag1 mutants with enhanced viability are hypomorphic for alkylation repair

We established an *in vivo* alkylation resistance assay to monitor the repair capacity of the mutant glycosylases described above. The assay consists of exposing MV1932 *E. coli* cells to a gradient of the alkylating drug MNNG and measuring the distance of continuous growth by our cultures in centimeters (Figs. 4b,c and 5). MNNG is an S_N1 agent that generates a variety of cytotoxic lesions. To determine if our assay was specific for the repair of particular alkyl lesions, we tested three glycosylases differing in their substrate

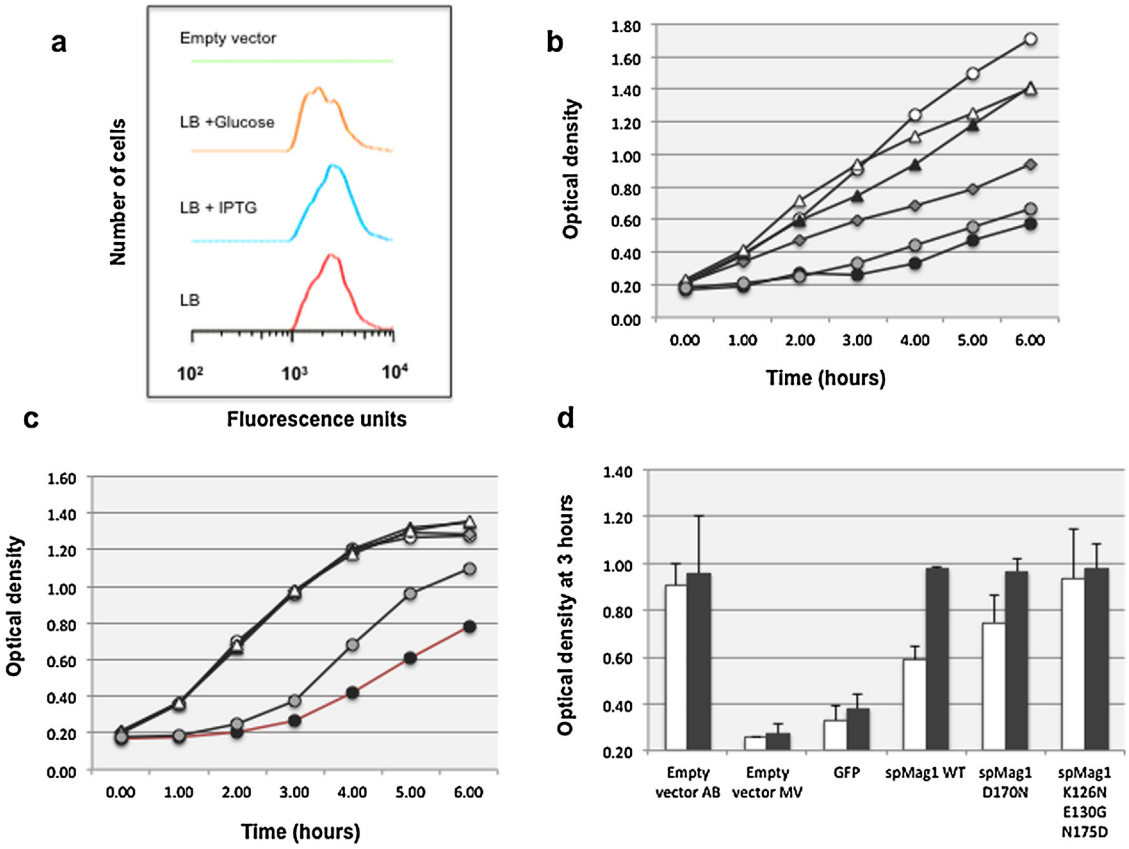

Fig. 3. *sp*Mag1 toxicity is due to excessive 3MeA glycosylase activity. (a) Monitoring of recombinant expression. The "cycle 3" variant of GFP [41] was cloned into pLitmus (the vector used to express our yeast glycosylases) as a reporter for expression. This construct was transformed into MV1932 cells and cells were grown to saturation under three different conditions: regular LB ("LB"), LB with 1% glucose ("LB+ glucose"), or with 1 mM IPTG (LB + IPTG). Fluorescence was measured by flow cytometry and the results were expressed as number of cells (*y*-axis) with number of fluorescence units (*x*-axis). (b and c) Effect of spMag1 activity on growth. Cultures of MV1932 cells containing the appropriate constructs were grown in the absence (b) or presence (c) of 1% glucose. Cultures were diluted to an OD_{600} of 0.15 in a 96-well plate and their growth monitored hourly. Data represent averages of triplicate samples. Open circles, AB1157 cells expressing an empty vector; gray diamonds, wild-type *sp*Mag1; open triangles, *sp*Mag1 K126N E130G N175D mutant; black triangles, *sp*Mag1 N170D mutant; gray circles, pLitmus-GFP; black circles, empty vector. (d) Optical density of cells grown for 3 h in LB media containing 0% (white bars) or 1% glucose (dark gray bars). Error bars represent the standard deviation from triplicate samples. AB = AB1157 cells; MV = MV1932 cells.

specificities. *E. coli* TAG repairs 3MeA almost exclusively, *sc*MAG repairs a broad range of substrates, and *sp*Mag1 repairs several methylpurine lesions but with less efficiency than the other two [10,13,16,30,31]. We found that in this assay resistance to MNNG toxicity closely paralleled the reported 3MeA repair capacity of the glycosylase expressed, with comparable levels of MNNG protection for both *E. coli* TAG and *sc*MAG and decreased resistance for *sp*Mag1 (Fig. 5a). Since 3MeA is made by both S_N1 and S_N2 agents with similar frequency [32], we predicted the S_N2 alkylating agent methyl methanesulfonate (MMS) would produce similar results as MNNG. This is indeed the result we found (Fig. 5b). Thus, these results suggest that our alkylation resistance assay in MV1932 cells largely detects 3MeA repair, although the contribution of other alkyl lesions cannot be excluded. We also confirmed that our observed protection against MNNG- and MMS-induced lesions was mediated by base excision repair by showing that in all cases mutations inactivating or decreasing glycosylase activity (TAG E38A, *sc*MAG D209N, and *sp*Mag1 D170N) abolished protection (Fig. 5).

3.6. Characterization of selected spMag1 mutants

Using our MNNG *in vivo* alkylation resistance assay, we estimated the 3MeA repair capacity of the six mutants of *sp*Mag1 described above. We found that in all cases our mutants exhibited a decreased capacity to protect MV1932 cells from MNNG generated lesions (Fig. 4b and c). This result strongly suggests that minimizing *sp*Mag1 toxicity in MV1932 cells involves attenuating the alkylation repair activity of the protein. To investigate the role of each individual mutation, we ran every mutation listed in Table 1 through a SIFT (Sorting Intolerance from Tolerance) algorithm. SIFT analysis estimates the detrimental potential of a given substitution at a given position based on a combination of phylogenetic and biophysical considerations [33,34]. A SIFT substitution probability score indicates the likelihood of the null hypothesis (no functional change), so that values below 0.05 are considered likely to affect protein function. The results of this analysis (Table 2) predict that all *sp*Mag1 mutations except 151A, 165A, and 206S do not interfere with enzymatic function. All of the mutants, with the exception of L122M, had at least two mutations, and the impact on the ability of these enzymes to repair MNNG-induced lesions likely involves epistatic interactions (see Section 4).

3.7. Biochemical characterization of the most frequently selected mutant of spMag1

We next characterized the base excision activity of our most frequently selected *sp*Mag1 mutant, K126N E130G N175D, using purified protein *in vitro*. We looked at repair of three substrates: 3MeA, 7MeG and 1,N^6-ethenoadenine (εA). Repair of 7MeG and εA was quantified using oligonucleotide excision assays. Because 3MeA is a relatively labile lesion that cannot be synthesized into an oligonucleotide, 3MeA repair was assayed by mass spectrometric

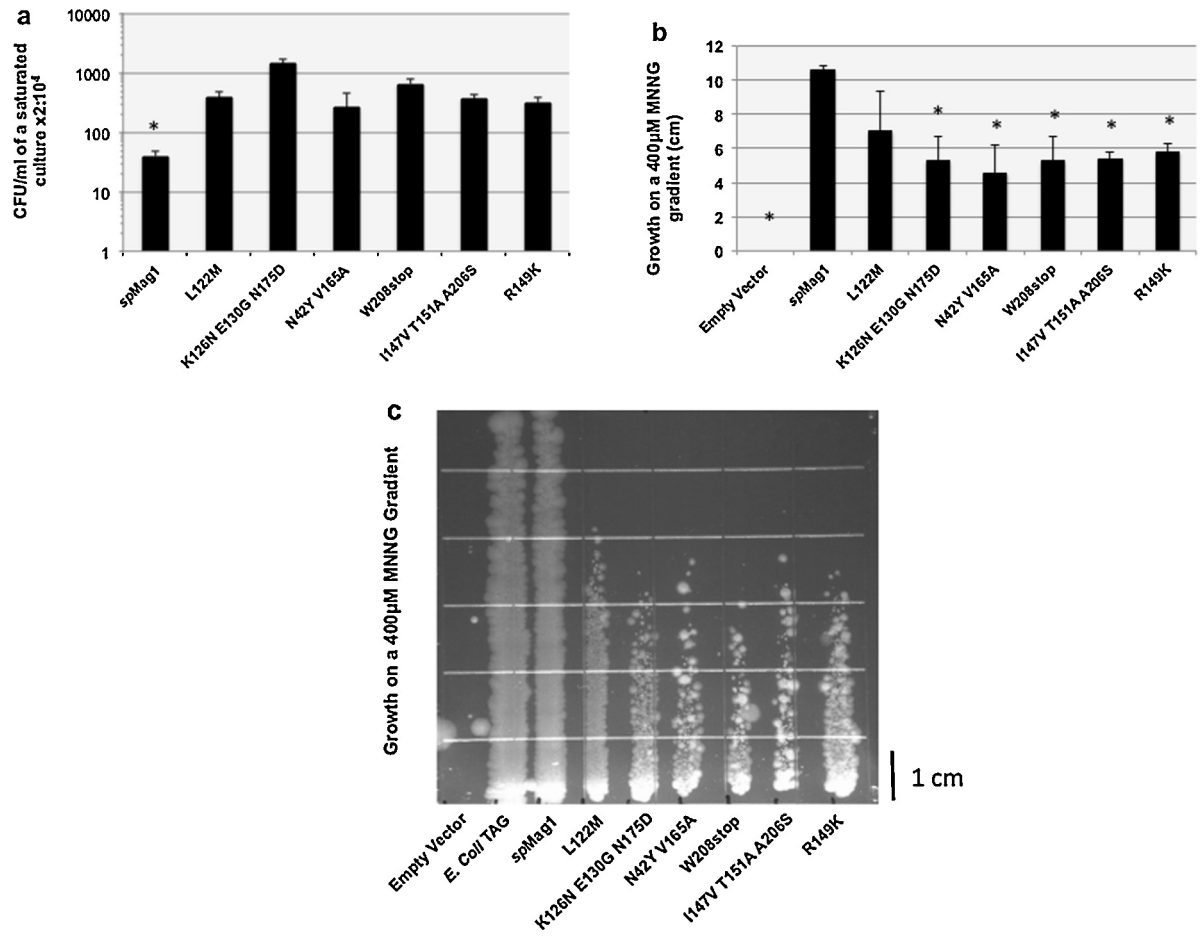

Fig. 4. Impact of expressing selected *sp*Mag1 mutants on cell viability and MNNG resistance. The six mutants that were independently selected more than once in five parallel directed evolution experiments for increased viability in the absence of exogenous alkylating agents were characterized. These mutants were expressed using the pLitmus 28i vector in MV1932 *E. coli* cells. (a) Viability of cells grown in the presence of carbenicillin to stationary phase and plated on LB plates with 100 μg/ml carbenicillin. According to Dunnett's test of means, all selected mutants show a statistically significant increase in viability relative to wild-type *sp*Mag1 ($p < 0.05$). (b) MNNG resistance. Growth (in cm) on a gradient containing the alkylating drug MNNG at a concentration of 400 μM. Error bars represent standard deviation of duplicate experiments. According to the Dunnet's test of means, all the mutants tested grew significantly less than wild-type *sp*Mag1 along an MNNG gradient ($p < 0.001$) (asterisk), with the exception of L122M ($p = 0.3$). (c) One representative MNNG gradient of those quantified in panel b.

quantitation of 3MeA lesions released from calf thymus DNA that had been treated with the methylating agent MNU (*N*-methyl-*N*-nitrosourea) [25]. We found that the *sp*Mag1 K126N E130G N175D mutant retained base excision activity toward all three substrates, but its level of activity was decreased for all (Fig. 6). Overall, our genetic and biochemical analyses suggest that in order to obtain optimal fitness levels in the absence of exogenous alkylation challenge, in MV1932 cells, *sp*Mag1 activity needs to be reduced to avoid toxicity, although not abolished completely.

Table 2
SIFT substitution for *sp*Mag1 mutants exhibiting enhanced viability. Probability of non-synonymous mutations being functional based on SIFT algorithm. 95 homologous sequences included in the analysis.

Position	Mutant	Substitution observed	SIFT substitution probability
N42	N42Y V165A	Y	0.06
L122	L122M	M	0.13
K126	K126N E130G N175D	N	0.58
E130	K126N E130G N175D	G	0.37
I147	I147V T151A A206S	V	0.29
R149	R149K	K	0.35
T151	I147V T151A A206S	A	0
V165	N42Y V165A	A	0
N175	K126N E130G N175D	D	0.2
A206	I147V T151A A206S	S	0.01

3.8. *spMag1 selection under MNNG challenge*

We next forced evolution to maintain robust glycosylase activity by performing selections of our random mutant spMag1 library in the presence of the methylating agent MNNG. After transformation of our library into MV1932 cells, we ran our cultures on MNNG gradients and picked 28 colonies showing maximal resistance. We observed an enriching trend for clones bearing the wild-type sequence or synonymous mutations, with 50% of the clones we selected for MNNG resistance having no changes at the amino acid level, up from 30% in our unselected library (Pearson's chi-squared *p*-value = 0.081). This is a clear indication that MNNG treatment favored the retention of wild-type levels of activity in order to prevent alkylation-induced cytotoxicity. This result is consistent with our alkylation resistance assays showing that *sp*MAG1 protects cells against MNNG (Figs. 4 and 5). In addition, we found one-point mutant (S60P) in 21% of the selected clones and another point mutant (M45V) in 4% of them. We characterized the effect of these two *sp*Mag1 mutations on viability (Fig. 7a) and on MNNG resistance (Fig. 7b), and found that both mutants increased average viability in saturated cultures relative to wild-type *sp*Mag1. However, neither mutant showed increased levels of MNNG resistance. In fact, S60P showed a clear decrease in protection against MNNG-generated lesions. The fact that we selected a mutant hypomorphic for glycosylase activity (S60P) following a selection for increased

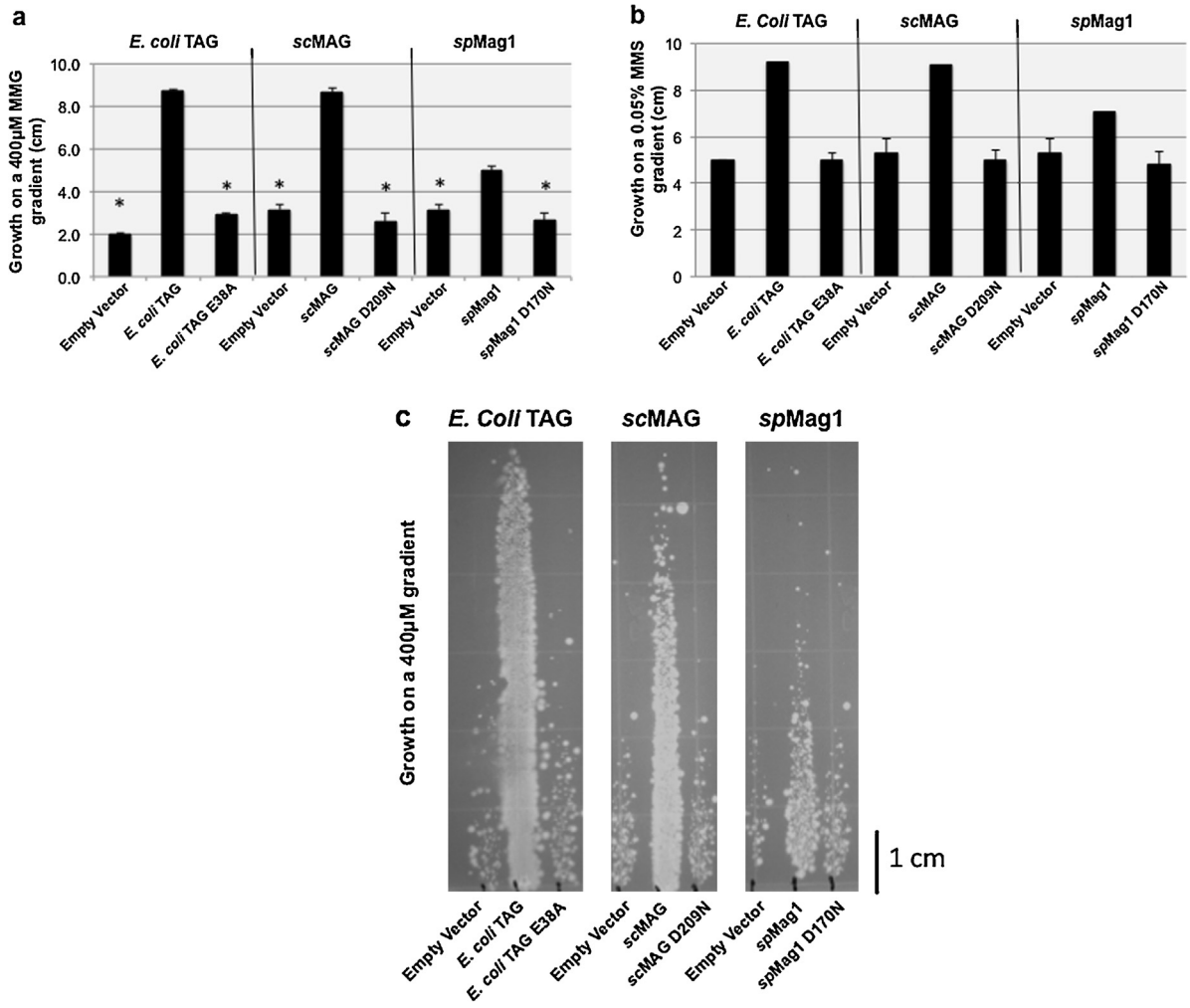

Fig. 5. Impact of 3MeA glycosylase expression on resistance against DNA alkylating agent-induced cytotoxicity. Measure of continuous growth (in cm) of *E. coli* MV1932 cells complemented with the *E. coli* TAG, or yeast *sp*Mag1 and *sc*MAG glycosylases along a 400 μM MNNG (a) and 0.05% MMS (b) gradient. Catalytically inactive mutants (TAG E38A, *sc*MAG D209N, *sp*Mag1 D170N) are included as negative controls. All glycosylases were expressed using the pLitmus 28i vector. Error bars represent standard deviation of three independent experiments. According to Dunnett's test of means, both empty vector and inactive mutants TAG E38A, *sp*Mag1 D170N, and *sc*MAG D209N grew significantly less than their wild-type counterparts along an MNNG gradient ($p < 0.0001$), and *sp*Mag1 grew significantly less than *sc*MAG ($p < 0.0001$). (c) Representative MNNG gradient, quantified in panel A.

repair indicates that in our *E. coli* complementation system protection from MNNG cytotoxicity through enhanced glycosylase activity is insufficient to offset the deleterious effects of constitutive 3MeA glycosylase expression.

4. Discussion

4.1. Comparison to previous reports

Posnick and Samson previously showed that constitutive expression of two 3MeA glycosylases, *E. coli* TAG and *S. cerevisiae* MAG, had a negative impact on the viability of E. coli cells[19]. They also observed that *sc*MAG expression was the more deleterious of the two. These results are consistent with our data, which show that ectopic expression of *sp*Mag1, a constitutively active 3MeA glycosylase like *E. coli* TAG, is less deleterious than constitutive expression of *sc*MAG, a normally inducible glycosylase.

Our results differ from those of Posnick and Samson in one key aspect, which is that we observed protection from, rather than sensitization to alkylating agent challenge. This is most likely due to the fact that Posnick and Samson used a host strain of *E. coli* that produced endogenous levels of both *E. coli* TAG and *E. coli* AlkA, whereas we used an *E. coli* strain virtually devoid of endogenous glycosylase activity. In their case, additional 3MeA glycosylase expression likely produced an imbalance in glycosylase activity relative to other BER players. Upon exposure to exogenous alkylating agents, this imbalance would lead to a build-up of cytotoxic intermediates [17]. By contrast, in our glycosylase-deficient strain, 3MeA glycosylase activity should be limiting for BER, and therefore it makes sense that providing cells with this activity increases resistance to exogenous alkylation.

4.2. Optimal fitness depends on a window of spMag1 activity

The present work indicates that in our *E. coli* complementation system and in the absence of exogenous alkylation, optimal fitness is restricted to a narrow window of 3MeA glycosylase activity. Complete absence of 3MeA glycosylase activity is deleterious. Empty vector MV1932 cells consistently showed decreased viability relative to wild-type *E. coli* and our selections consistently selected against indels and stop codons, with the exception of W208, which is at the C-terminus of the protein and therefore unlikely to abolish activity completely. Conversely, an excess of 3MeA glycosylase activity was equally or even more deleterious to

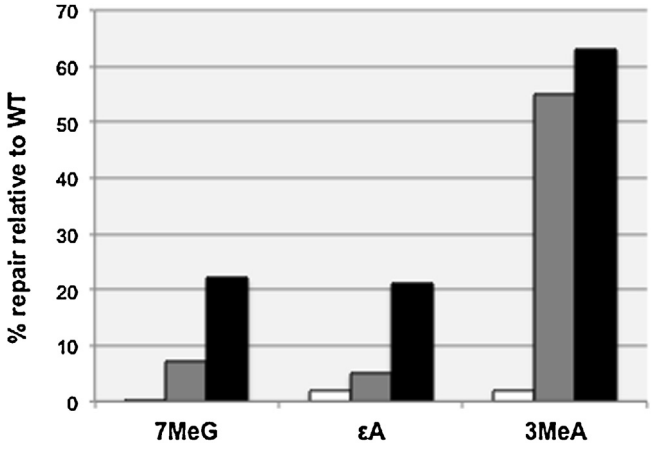

Fig. 6. *In vitro* repair of individual lesions by purified *sp*Mag1. Percent repair of 7MeG, εA, and 3MeA lesions by wild-type (black bars) and by the K126N E130G N175D triple mutant (gray bars) of *sp*Mag1 relative to a no enzyme control (white bars). The results show one representative experiment. Repair of 7MeG and εA was measured using a 25mer oligonucleotide containing a single 7MeG or εA lesion, and the % repair is defined as the fraction of abasic site product generated after a 4-h reaction. Repair of 3MeA was measured by release of 3MeA lesions from MNU-treated calf thymus DNA; percent 3MeA repair is defined as the amount of 3MeA liberated by enzymatic treatment relative to acid-catalyzed, non-enzymatic 3MeA depurination.

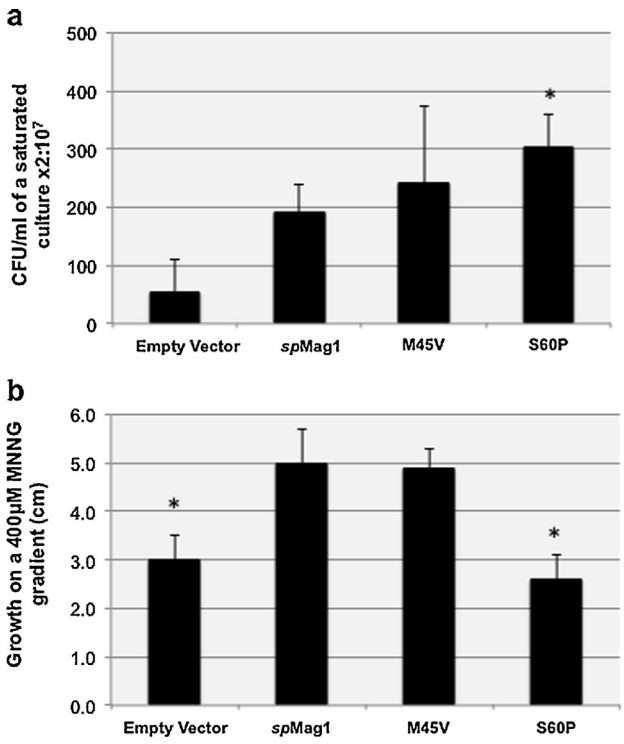

Fig. 7. Protection profile and viability of MNNG-selected *sp*Mag1 mutants. *E. coli* MV1932 cells expressing two *sp*Mag1 mutants selected for improved resistance to alkylation damage in the presence of the alkylating agent MNNG. These mutants were expressed from the high copy pLitmus 28i vector. (a) Viability of cells grown to stationary phase without the presence of alkylation challenge. Error bars represent the standard deviation of triplicate experiments. According to the Dunnett's test of means, the only statistically significant difference we found is between our empty vector control and S60P ($p < 0.004$). (b) Growth (in cm) against a 400 μM MNNG gradient. Dunnett's test of means indicate that our empty vector control ($p < 0.005$) and the S60P *sp*Mag1 mutant ($p < 0.002$) grew significantly less than wild-type *sp*Mag1 along a gradient of MNNG (asterisk).

cell viability. This is evidenced by the following three observations: (1) high expression of *sp*Mag1 had a negative impact on MV1932 viability and growth; (2) *sc*MAG, which confers higher resistance to MNNG-generated lesions in our *in vivo* alkylation protection assay, exhibited a stronger cytotoxicity than *sp*Mag1; and (3) mutants of *sp*Mag1 with attenuated activity toward MNNG-induced lesions *in vivo* and with lower activity *in vitro* showed increased viability in the absence of alkylation damage. Taken together, these observations suggest that, in the absence of exogenous alkylation, *sp*Mag1 expression only has a positive effect on the fitness of its *E. coli* host strain when present at low levels of activity. Decreased activity can result from changes in enzymatic activity or from other factors affecting the steady state concentration of the enzyme such as alterations in folding, stability, or efficiency of translation.

4.3. Endogenous 3MeA is likely a critical endogenous cytotoxic lesion

The *sp*Mag1 K126N E130G N175D mutant evolved independently in all five experiments and therefore was subject to very strong positive selection. Although this triple mutant still retains activity toward all lesions tested biochemically, its level of activity is less than that of wild-type *sp*Mag1. Interestingly, our biochemical experiments showed that the ability of *sp*Mag1 K126N E130G N175D to remove 7MeG and εA lesions was affected to a greater extent than its ability to remove 3MeA lesions. This result is consistent with our *in vivo* alkylation resistance assay, which showed that all *sp*Mag1 mutants selected for increased fitness in the absence of alkylation challenge retained the ability to protect MV1932 *E. coli* cells from MNNG- generated 3MeA.

The retention of 3MeA activity relative to other substrates by our mutants is remarkable. It may reflect a functional selection for repair of endogenous 3MeA lesions, which suggests that 3MeA may represent a significant endogenous cytotoxic lesion (at least in this strain of *E. coli*). This could account for the narrow substrate specificity profile consistently seen in constitutively expressed 3MeA glycosylases [13,30,31,36–39]. Alternatively, the observed retention of 3MeA activity could be due to the relaxed structural requirements of 3MeA repair given the unstable nature of this lesion [35]. In this scenario, the increased viability associated with *sp*Mag1 K126N E130G N175D could be unrelated to 3MeA repair. For example, this mutant may have decreased generation of abasic sites associated with εA or 7MeG.

4.4. spMag1 selected mutants: structure-functional considerations

We found a high degree of convergent evolution when we evolved mutants with improved fitness in the absence of exposure to exogenous alkylation. Six different non-synonymous mutants were independently found in more than one experiment. This high level of convergent evolution may be due to a narrow window of optimal glycosylase activity, to structural constraints that modulate the activity of *sp*Mag1, or to a combination of both.

Of the recurring mutants that we obtained, only one was a single mutant: L122M. Based on the 3D structure of *sp*Mag1 [13] (Fig. 8a), substitutions at position 122 likely impact the position of the helix–hairpin–helix DNA binding motif, which would explain the apparent decrease in 3MeA repair capacity observed for this mutant. All other mutants had more than one mutation. Based on our SIFT analysis, we found two likely cases of positive epistasis, *i.e.* two cases in which deleterious mutations appear to be partially compensated by additional mutations. The first case is the N42Y V165A double mutant. The V165A mutation had a highly significant (<0.05) SIFT score of zero. V165 lies buried and immediate adjacent to the active site (Fig. 8b), and thus it is likely that the V165A mutation disrupts the protein fold to affect the shape of

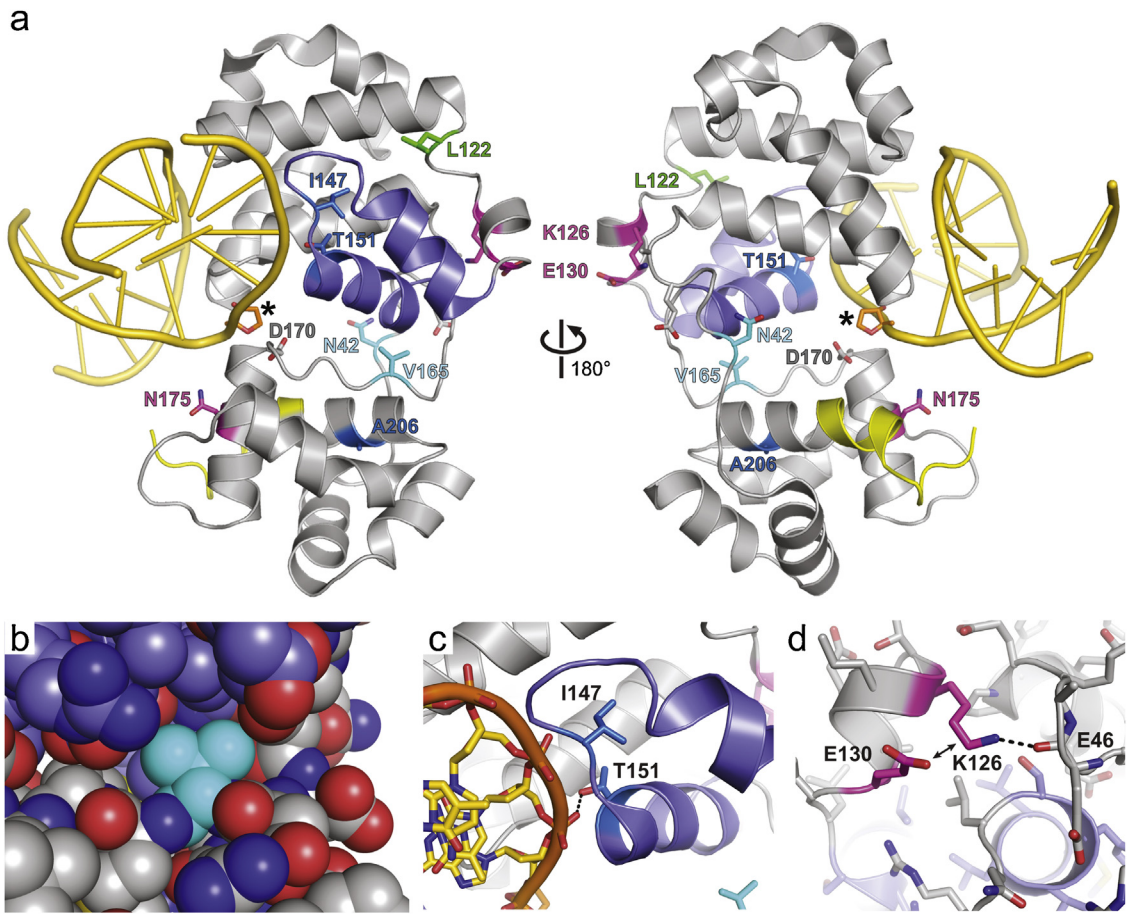

Fig. 8. Structural analysis of *sp*Mag1 mutants. (a) Locations of residues associated with enhanced viability and/or likely to affect enzyme activity are mapped onto the crystal structure of *sp*Mag1 (PDB ID 3S6I). Side chains are shown as sticks and colored according to mutation (magenta, K126N E130G N175D; blue, I147V T151A A206S; cyan, N42Y V165A; green, L122M; silver, D170N). The helix–hairpin–helix motif is colored blue. DNA is gold, and the location of the flipped abasic site is marked by an asterisk. (b) Space-filling model of the local environment around V165 (cyan). Residues are colored as in panel a. (c) Proximity of I147 and T151 to the DNA. (d) Close-up of K126 and E130. Hydrogen bonds are depicted as dashed lines. The double-headed arrow represents the short distance (3.0 Å) between side chains, which likely form an ionic bond.

the active site. N42 and V165 both reside on the strands located at the hinge region between the two domains of the protein. Thus, the N42Y mutation may compensate for the deleterious effects of V165A. Similarly, in the I147V T151A A206S mutant, both T151A and A206S mutations had highly significant SIFT scores. T151 is located within the helix–hairpin–helix DNA binding domain and forms a hydrogen bond with the DNA backbone (Fig. 8c). A206 is located in a buried cluster of helices near the C-terminus. This position appears to be critical for folding a variety of glycosylases, since *E. coli* TAG coordinates a Zn atom, *H. pylori* MagIII contains a carbamylated lysine, and several glycosylases contain iron-sulfur clusters at this position [31,40]. The presence of these two highly deleterious mutations in a mutant that shows decreased but still detectable 3MeA glycosylase activity suggests a positive epistatic interaction.

The K126N E130G N175D mutant of Mag1, by contrast, likely represents an example of negative epistasis since, based on SIFT analysis, each individual mutation is unlikely to affect activity substantially (score >0.2 in all three cases, Table 2) even though this triple mutant showed a clear decrease in repair of MNNG-generated lesions. Both N175D and the K126N E130G doublet contributed to the increased cell survival observed for the triple mutant, with K126N E130G making the largest contribution (Suppl. Fig. S1). The N175D mutation resides close to the DNA backbone immediately adjacent to the active site, and possibly interferes with a protein-DNA interaction (Fig. 8a). Interestingly, E130 and K126 are located on the opposite side of the protein, and form an ionic interaction on the same face of a short solvent-exposed helix immediately behind the helix–hairpin–helix DNA binding motif. In addition, K126 forms a hydrogen bond with the carbonyl oxygen of the E46 backbone on an adjacent segment of the protein. Thus, the E130G and K126N mutations may contribute to reduction in glycosylase activity by disrupting secondary and tertiary contacts that impact how the helix–hairpin–helix motif engages the DNA.

5. Conclusion

3MeA glycosylases can be divided into two categories: those that are constitutively expressed and those that are inducible. These two categories differ in their catalytic efficiency (low for constitutive, high for inducible) and in their substrate specificity (narrow for constitutive and broad for inducible). This divide across categories is particularly striking given that there is no apparent genetic barrier for constitutive glycosylases to evolve broader substrate specificity. Our complementation and directed evolution experiments demonstrate a tradeoff between the deleterious effects of constitutive 3MeA glycosylase expression and the protection that a 3MeA glycosylase provides against exposure to exogenous alkylating agents. This work suggests that constitutive 3MeA glycosylase activity must be limited to the minimum necessary to protect cells from endogenous methylation, leaving cells vulnerable to increased exposure to exogenous alkylating agents. These findings therefore suggest that inducible 3MeA glycosylases likely arose as a way to increase glycosylase activity during exposure to

exogenous alkylation while minimizing negative effects on organismal fitness in the absence of exogenous alkylating agents.

Conflict of interest

There are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mrfmmm.2014.03.007.

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