## Supporting Information

# Repair of Alkylation Damage in Eukaryotic Chromatin Depends on Searching Ability of Alkyladenine DNA Glycosylase

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## Contents of Supporting Information:

## SUPPORTING METHODS

**Proteins.** Full-length human AAG was expressed as an N-terminal $His_6$-Smt3 fusion protein using a previously described vector.[1] Mutations in AAG were generated using site-directed mutagenesis from the wild-type AAG plasmid, and their sequences were confirmed by DNA sequencing. AAG was expressed in *E. coli* BL21 Star (DE3) pRare2 cells using autoinduction media.[2] Typically, cells were grown at 37 °C until they reached an optical density of 0.5–1.0 at 600 nm. Cultures were then transferred to a 16 °C shaker, and proteins were expressed for 24 hours with final $OD_{600}$ values at 15–25 before cells were harvested and frozen at –80 °C. Cell pellets were suspended in lysis buffer (25 mM potassium phosphate, pH 7.0, 250 mM NaCl, 10% (v/v) glycerol, 0.1% (v/v) NP-40) and disrupted by a high pressure homogenizer (EmulsiFlex, Avestin). Cell lysates were slowly precipitated with polyethylenimine to remove nucleic acids, and AAG proteins were purified by metal affinity chromatography. Cleavage with ULP1 removed the $His_6$-SUMO tag to produce full-length AAG with an N-terminal serine in place of the initiator methionine of the native protein. Subsequent ion exchange chromatography (HiTrap SP, GE Healthcare), followed by gel filtration (Superdex 200, GE Healthcare) or dialysis (MWCO 12 000–14 000, Fisherbrand), yielded homogeneous protein as judged by SDS-PAGE with Coomassie staining. The active concentration of AAG was determined by burst analysis as described previously, and the reported concentrations of protein were corrected for the fraction active.[3]

**Annealing of DNA Oligonucleotides.** DNA duplexes were annealed in 10 mM NaMES pH 6.5, 50 mM NaCl by heating samples to 95 $^{o}$C and slowly cooling to 4 $^{o}$C. DNA annealing reactions had a 2-fold excess of unlabeled over labeled strand, except for the substrates used in the $k_{cat}/K_M$ assays for which DNA was annealed at a 1:1 ratio of the two strands.

**Single-turnover Kinetics.** The single-turnover assay was performed with 25mer DNA substrates containing εA (25E•T) or Hx (25I•T) at 50 mM $Na^+$, as previously described.[4-6] The concentration of AAG was varied in excess over the DNA substrates (25–100 nM) to ensure single-turnover conditions. The enzyme concentrations ranged from 250 to 500 nM for the 25E•T DNA substrate, and from 200 nM to 10 μM for the 25I•T DNA substrate. Due to the very fast reaction of AAG on the 25I•T DNA substrate, these assays were performed at 25 °C. In all

cases the reactions followed single exponential kinetics according to eq 1, in which F is the fraction product, A is the reaction amplitude, and k is the observed single-turnover rate constant. At saturating concentration of enzyme, the single-turnover rate constant is independent of the concentration of enzyme and designated $k_{max}$ (the maximal single-turnover rate constant).

$$F = A(1-\exp(-kt)) \quad (1)$$

**Multiple-turnover Kinetics to Measure $k_{cat}/K_M$.** Catalytic efficiency ($k_{cat}/K_M$) for excision of εA (25E•T) and Hx (25I•T) was determined from the substrate concentration dependence under sub-saturating conditions ([DNA]<<$K_M$). We used high concentrations of salt in order to eliminate the contribution of facilitated diffusion to the second order rate constant $k_{cat}/K_M$ (1 M and 515 mM $Na^+$ were used for 25E•T and 25I•T, respectively). Under these reaction conditions, the $K_M$ values are in the μM range for the wild-type enzyme. For the 25E•T DNA substrate, reactions were performed using 2 or 3 nM AAG variants and 25–300 nM substrate DNA. The only exception is R197S, which reacts more slowly than other mutants, and 40 nM enzyme and 1–4 μM DNA were used. For the 25I•T DNA substrate, reactions were performed using 2 nM AAG variants and 100–300 nM substrate DNA, with the exception of R197S, which was assayed using 40 nM enzyme and 1–4 μM DNA. The $k_{cat}/K_M$ values were calculated from the slopes of the linear fits (eq 2).

$$V_{init}/[E] = (k_{cat}/K_M)[DNA] \quad (2)$$

**Multiple-turnover Processivity Assay.** Multiple-turnover assays were performed on a 47mer processivity substrate containing two εA lesions separated by 25 bp (47E2F2; Supporting Figure S1) at 100 and 150 mM $Na^+$, with 100-fold excess of substrate (200 nM) over enzyme (2 nM).[3,7] The initial rates for formation of intermediate ($V_{int}$) and product ($V_{prod}$) were determined from linear fits of the first 10% of substrate consumption. In all cases, the two products and two intermediates were formed at identical rates, indicating that AAG encounters either lesion with equal probability. The fraction processive values were calculated by eq 3, and corrected for the small amount of ring-opened εA by eq 4 as described previously.[7,8]

$$F_{P,obs} = (V_{prod} - V_{int})/(V_{prod} + V_{int}) \quad (3)$$

$$F_P = (F_{P,obs} - 0.05)/(0.92 - 0.05) \quad (4)$$

**Pulse-chase Assays to Measure the Efficiency of Excision.** The complex of AAG bound to

Fam-labeled εA-DNA was formed by incubating 100 nM of AAG variants with 50 nM εA-DNA (25E•T) for 20 s, followed by a chase consisting of 1 μM unlabeled pyrrolidine-containing DNA inhibitor (25Y•T) at 150 mM $Na^+$. Control reactions in which AAG was preincubated with chase DNA prior to addition of εA-DNA confirmed that this amount of chase was sufficient for each of the AAG variants (Supporting Figure S4). AAG-catalyzed base excision yields labeled product, whereas dissociation of AAG releases unreacted labeled substrate. The data were converted to fraction product and fit by a single exponential followed by a steady-state phase ($V_{ss}$*t) that reflects rebinding of AAG to εA-DNA (eq 5).

During the 20 s pre-incubation of AAG and the substrate DNA, substrate is converted to product at a rate constant of approximately 0.22 $min^{-1}$ for the wild-type enzyme (from the single-turnover reaction), and this corresponds to formation of 0.07 fraction of product prior to the addition of the chase. Therefore, we took time points at 20 s in the single-turnover assay for each variant and used the measured results as the baseline level of product formation when the chase species was added ($A_{20s}$). This term was incorporated into curve fitting for the pulse-chase assay so that the pre-existing product was not included in the burst phase (eq 5). After obtaining the burst amplitude ($A_{PC}$) values from the data fitting, the efficiency of excision can be determined with eq 6, in which $A_{max}$ is the maximal amplitude in the single-turnover reaction (without chase).

$$F = A_{20s} + A_{PC}(1-\exp(-kt)) + V_{ss}*t \quad (5)$$

$$\text{Efficiency of Excision } (E_{ex}) = A_{PC}/(A_{max} - A_{20s}) \quad (6)$$

**Competitive Inhibition by Nonspecific DNA.** AAG variants (2–10 nM) were incubated with excess nonspecific inhibitor DNA at 150 mM $Na^+$. Ratios of the inhibitor DNA (25A•T) to substrate (25I•T) were varied from 0 to 79, while the total DNA concentration was kept constant at 4 μM. The observed rates from linear fits of the initial 15% of the reactions were normalized to the reaction rate without the inhibitor. A competitive inhibition model (eq 7) was used to fit the [I]/[S] dependence, yielding the ratio of $K_i$ for the inhibitor ($K_d$ for the nonspecific DNA, $K_{d,ns}$) to the $K_M$ for substrate.[9] The $V_{obs}$ is the initial velocity at a given ratio of inhibitor to substrate and the $V_{max}$ is the initial velocity in the absence of inhibitor.

$$V_{obs}/V_{max} = 1/(([I]/[S])/(K_i/K_M)+1) \quad (7)$$

**Yeast Strains and Growth Conditions.** The pYES2-N169S AAG plasmid was generously provided by M. Wyatt (University of South Carolina).[10] Wild-type and mutant forms of AAG were constructed by site-directed mutagenesis and the sequences were confirmed by sequencing both strands. The BY4741-Mag1Δ yeast strain (*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 mag1::KanMX*), which is highly sensitive to alkylating agent treatment, was a kind gift from D. Engelke (University of Michigan, Ann Arbor).[11,12] Single colonies from re-streaked plates following plasmid transformation were inoculated into 5 mL SD-URA drop-out broth containing glucose or raffinose as the only carbon source for 3 days until yeast growth reached saturation. The saturated cell cultures were then inoculated into 25–100 mL fresh SD-URA drop-out broth containing glucose (from the glucose culture) or galactose (from the raffinose culture) with a starting $OD_{600}$ of 0.05. Cells were grown for ~12 hours and reached an $OD_{600}$ of ~1 before they were collected for the MMS DNA damage assay, the yeast survival assay and the measurement of the AAG expression level as detailed in the sections below.

**Analysis of DNA Damage by MMS.** Yeast cells expressing WT AAG and the empty vector were incubated at room temperature on bench with slow rotation (LabQuake, Thermo Scientific) with or without 0.3% (v/v) MMS for 1 h, and they were immediately harvested for yeast genomic DNA isolation. Approximately $10^9$ cells were collected by centrifugation, washed twice in water, and resuspended in 0.3 mL of cell disruption buffer (2% (v/v) Triton X-100, 1% (w/v) SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8, 1 mM EDTA). 0.3 mL of phenol:chloroform: isoamylalcohol was then added into the cell suspension, and supplemented with acid-washed glass beads (425–600 μm, Sigma-Aldrich) to break open cells by vortexing (3 times for 1 min, with 2 min gaps). After centrifugation, the top aqueous layer was transferred to a new tube, and nucleic acids were ethanol precipitated, resuspended in 0.4 mL TE and incubated with 3 μL RNase A (10 mg/mL) at 37 °C for 15 min to remove RNA. DNA was ethanol precipitated in the presence of 10 μL 3 M sodium acetate (pH 5.2) and washed once with 70% (v/v) ethanol. The DNA pellet was dissolved in TE buffer and used for alkaline agarose gel electrophoresis as described previously.[13] Briefly, the purified DNA samples (2–10 μg) were incubated with excess WT AAG and APE1 endonuclease (20 μM each) in the reaction buffer (50 mM NaHEPES, pH 7.5, 1 mM DTT, 1 mM EDTA, 5% (v/v) glycerol) at 37 °C for 90 min, and the resulting strand breaks were resolved on a 1% (w/v) alkaline agarose gel and stained with SYBR gold.

Quantification and calculation of number averaged DNA length used ImageQuant software (Molecular Dynamics) as described previously.[14]

**Yeast Survival Assay.** Yeast cells hosting different AAG mutants and the pYES2 vector were incubated at room temperature on bench with slow rotation with or without 0.3% (v/v) MMS for 1 h, and they were then immediately diluted and plated on YPD rich media (Figure 6A). Plates were incubated at 30 $^{o}$C for 2–3 days before colonies were scored. Efforts were made to have 20–300 colonies per plate for accurate counting. Survival rates were calculated by dividing the number of colonies after MMS treatment by the total number of colonies in the control samples without MMS treatment.

**Determination of AAG Expression Level in Yeast Cells**. AAG expression level was measured using an activity assay in whole cell extracts that were prepared from the same cultures that were used in the yeast survival assays. Cells were split at $OD_{600}$ of ~1 after overnight cultivation, and 1 mL of the cell cultures was used for the yeast survival assay and the rest were harvested by centrifugation and stored at –80 $^{o}$C. Cell pellets were resuspended with 50 μL buffer (25 mM NaHEPES, pH 7.5, 250 mM NaCl, 10% (v/v) glycerol, 1 mM DTT, 1 mM EDTA) for the glucose cultures and 150 μL buffer for the galactose cultures. The cell suspensions were mixed with equal volumes of ice-cold glass beads (acid washed 425–600 μm, Sigma-Aldrich) and disrupted at 6.5 m/s using a MP FastPrep®-24 benchtop homogenizer (MP Bio) for 1 min to make yeast whole cell extracts. Cell lysates were centrifuged at 13.2k rpm for 60 min to remove cell debris and pellets, and the supernatants were snap frozen in liquid nitrogen and kept at –80 $^{o}$C for the glycosylase assays.

The prepared yeast whole cell extracts were assayed for AAG glycosylase activity using the 25I-bulge DNA as the substrate at 250 mM $Na^{+}$ under the standard glycosylase activity assay condition, except that 3 mM EDTA was used to eliminate unwanted nonspecific DNA degradation. As an internal quality control of the cell extract preparation protocol, all the extracts were also assayed for endogenous uracil DNA glycosylase (UNG) activity on the 25U•G DNA substrate under the same reaction conditions. Concentrations of the AAG variants in the yeast cells were calculated using the measured activity divided by the measured rate constants using the recombinant AAG variants under the same reaction conditions (Supporting Table S2).

**25I•T**
5′-Fam-CGATAGCATCCT**I**CCTTCTCTCCAT
3′-GCTATCGTAGGA**T**GGAAGAGAGGTA

**25E•T**
5′-Fam-CGATAGCATCCT**E**CCTTCTCTCCAT
3′-GCTATCGTAGGA**T**GGAAGAGAGGTA

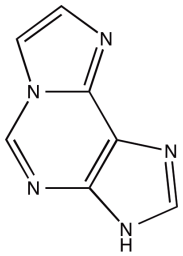
**25Y•T**
5′-CGATAGCATCCT**Y**CCTTCTCTCCAT
3′-GCTATCGTAGGA**T**GGAAGAGAGGTA

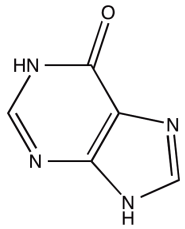
**25A•T**
5′-CGATAGCATCCT**A**CCTTCTCTCCAT
3′-GCTATCGTAGGA**T**GGAAGAGAGGTA

**25I-bulge**
5′-Fam-CGATAGCATCCT(**I**)CCTTCTCTCCAT
3′-GCTATCGTAGGA-GGAAGAGAGGTA

**25U•G**
5′-Fam-CGATAGCATCCT**U**CCTTCTCTCCAT
3′-GCTATCGTAGGA**G**GGAAGAGAGGTA

**47E2F2**
5′-Fam-TAGCATCCT**E**CCTCGTGTAGGTATTAGATCCGACT**E**CCTTGTGTCCT-Fam-3′
3′-ATCGTAGGA**T**GGAGCACATCCATAATCTAGGCTGA**T**GGAACACAGGA-5′


εA; E denotes 2'-deoxy-1,$N^6$-ethenoadenosine


Hx; I denotes 2'-deoxyinosine

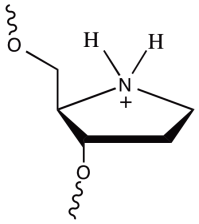

Pyrrolidine (Y)

**Figure S1. Sequences of oligonucleotides**. The lesion-containing strands were labeled with fluorescein (6-aminohexyl linker; Fam). 25I•T was used in the active site titration measurement for active concentration determination of the purified AAG variants. 25I•T and 25E•T were used in single-turnover and multiple-turnover assays for $k_{max}$ and $k_{cat}/K_M$ measurements. 25E•T and 25Y•T were used in the pulse-chase assay for the efficiency of excision measurement. 25I•T and 25A•T were used in the competitive inhibition assay for the measurement of $K_i/K_M$ and $K_d$ on nonspecific DNA. 25I-bulge was used to determine the active concentrations of the plasmid expressed AAG variants in yeast cell extracts. 25U•G was used to compare the relative concentrations of the yeast UNG enzyme in different yeast cell extracts. 47E2F2 was used in the processivity assay to determine the searching efficiency of AAG variants.

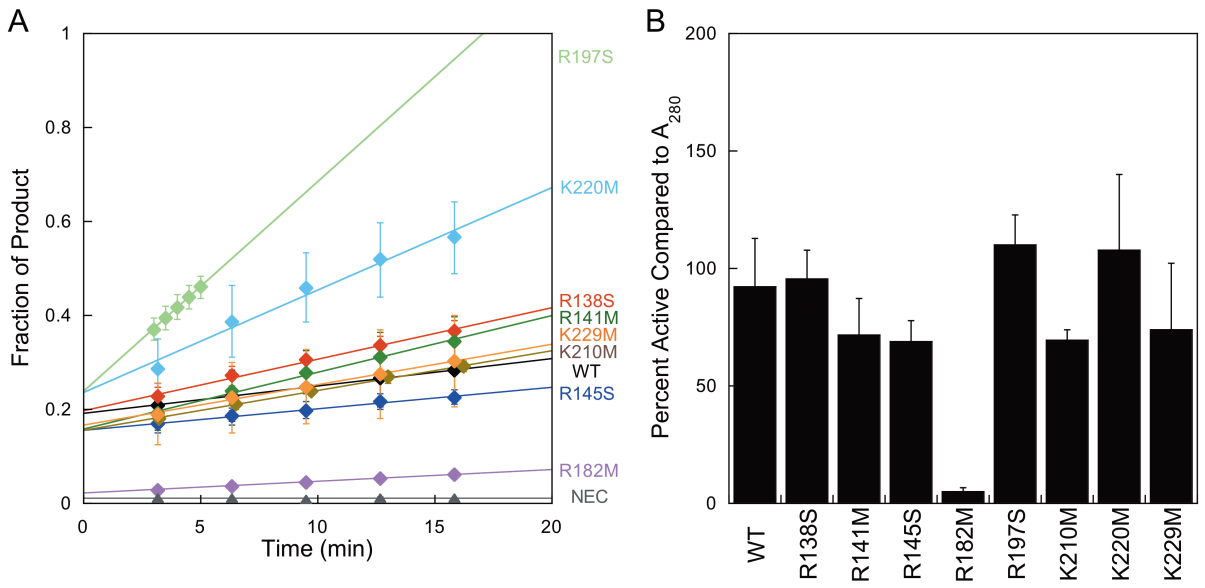



**Figure S2. Active site titration of the AAG variants. (**A) Excision of Hx base from the 25I•T substrate DNA shows burst kinetics under conditions of low salt concentration, and therefore the amount of active enzyme can be obtained from extrapolation of the steady-state phase back to the *y* axis. The results for the different AAG variants are shown. The concentration of the DNA substrate was 1 μM and the concentration of the AAG variants were 200 nM as determined from absorbance at 280 nm. Reactions were performed in triplicate, and the average and standard deviation are shown (mean ± S.D., n = 3). As the R197S mutant exhibits a faster steady-state reaction, it was necessary to use a shorter time course. Reactions were also performed using 100 nM AAG variants and gave approximately half the burst amplitudes (data not shown). NEC denotes the no enzyme control reaction, which shows the nonenzymatic baseline level of product formation at approximately 1% of the substrate. The active enzyme concentrations were corrected for this baseline level of product formation. (B) Summary of the active percentage of the purified AAG variants (mean ± S.D., n ≥ 3). Most of the variants have active enzyme concentrations close to those determined by $A_{280}$, except for R182M. The corrected active concentrations of the variants were used throughout.

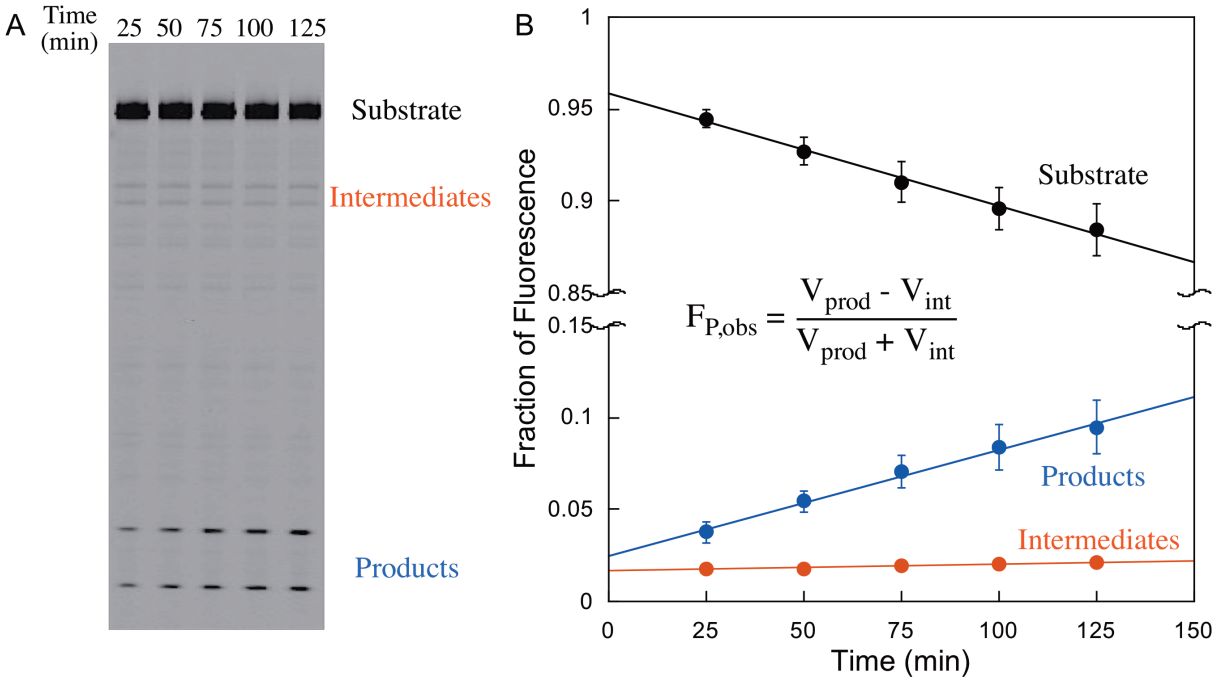



**Figure S3. Multiple-turnover processivity assay. (**A) Representative gel showing a time course for the turnover of the processivity substrate (47E2F2) by wild-type AAG at 150 mM $Na^+$. (B) Reaction process curves for consumption of substrate (black), and formation of intermediates (red) and products (blue). The averaged values of three independent measurements are plotted, and the error bars indicate the standard deviation. The fraction processive was calculated from the slopes of linear fits of products and intermediates within the first 10% of reaction.

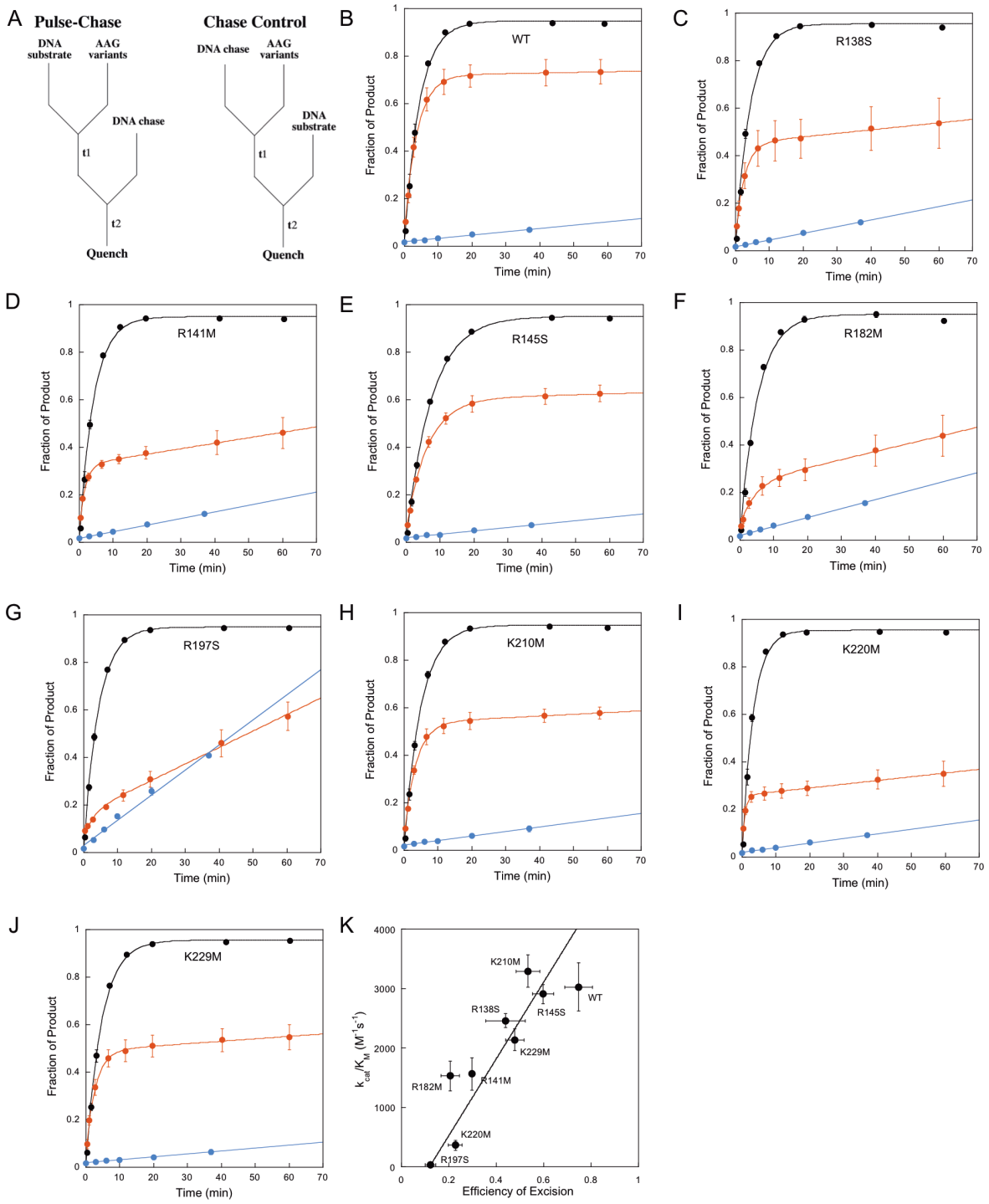



**Figure S4. Determination of the efficiency of excision.** (A) Experimental design of the pulse-chase and chase control assays. (B) – (J) Determination of the efficiency of excision of AAG variants on 25E•T DNA. No chase (black symbols), with chase (red symbols) and chase control (blue symbols) are shown for each AAG variant. Efficiency of excision was calculated according to eq 6. The absence of a burst phase in the chase control reactions indicates that sufficient chase was used. Chase control assays were performed in duplicate, and the rest were in triplicate. (K) Efficiency of excision correlates with $k_{cat}/K_M$ with a $R^2$ value of 0.95 (weighted by *y* values).

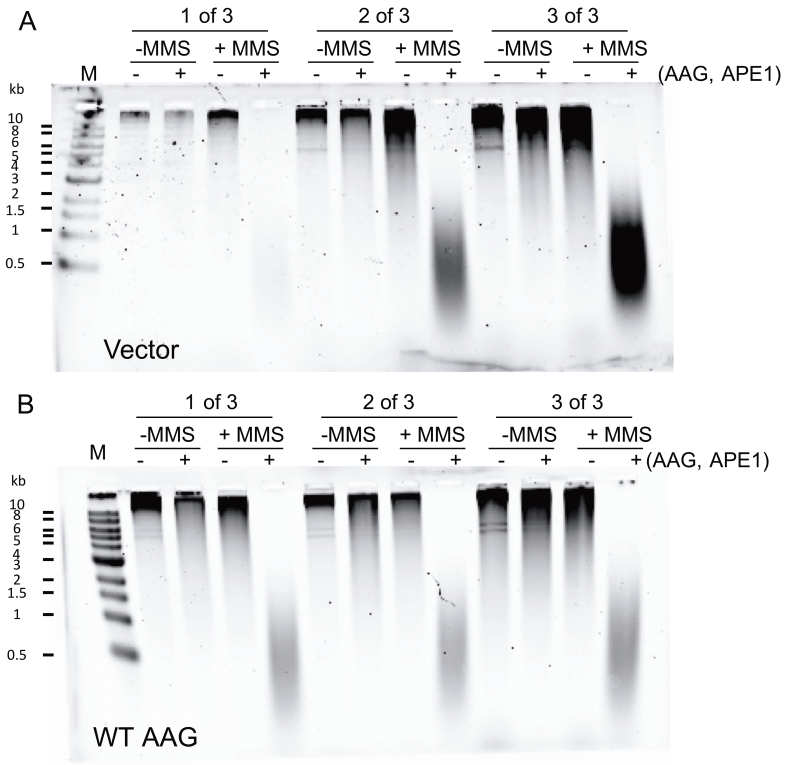



**Figure S5. Analysis of MMS-induced DNA damage in yeast cells.** Genomic DNA from yeast containing empty vector (A) or wild-type AAG (B) were analyzed on 1% (w/v) alkaline agarose gels stained with SYBR gold. Genomic DNA was isolated from cells treated with or without 0.3% (v/v) MMS for one hour at room temperature, and aliquots were incubated with (+) or without (-) AAG and APE1. Three independent genomic DNA preparations from each strain were used for the assay. DNA size marker (M) is labeled on the left of the gels, and used for the calculation of ensemble average size of DNA. After AAG/APE1 reaction, which cleaves at methylated purines, the MMS-treated samples give an ensemble average size of DNA at ~0.32 kb, which corresponds to approximately 3 alkylated purine lesions per kb DNA. The calculation assumes a Poisson distribution of DNA fragments as described previously.[14,15] This calculated damage density is consistent with a previous study, which showed that treatment with 0.035% (v/v) MMS produced 0.3 to 0.5 alkylated purine lesions per kb DNA.[13]

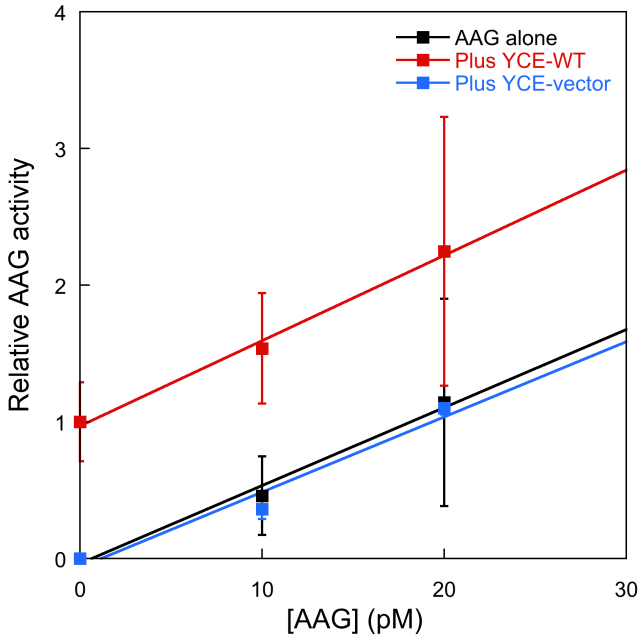



**Figure S6. Representative data for glycosylase assays in cell extracts.** To validate that the glycosylase assay with the 25I-bulge substrate DNA can be used to determine the concentration of AAG in yeast whole cell extracts, purified recombinant AAG was added to yeast extract from cells lacking AAG (YCE-vector; blue line) or expressing wild-type AAG (YCE-WT; red line). The activity of AAG in cell extracts is within error of reactions carried out in buffer (AAG alone; black line), suggesting that no yeast components interfere with the assay, and the AAG activity assay accurately reports on the amount of active AAG in the yeast extract. The values are the mean ± SD (n≥4).

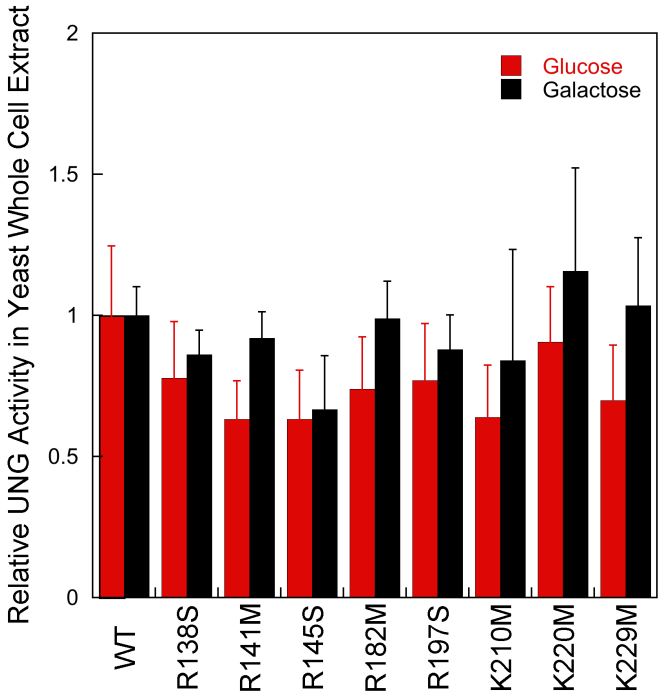



**Figure S7. Determination of UNG activity in cell extracts.** The yeast UNG activity was measured from the initial rates of uracil excision from the 25 U•G substrate as a control to validate the preparation of the individual cell extracts. Values are the mean ± SD (n=4 for the glucose conditions and n=2 for the galactose conditions).

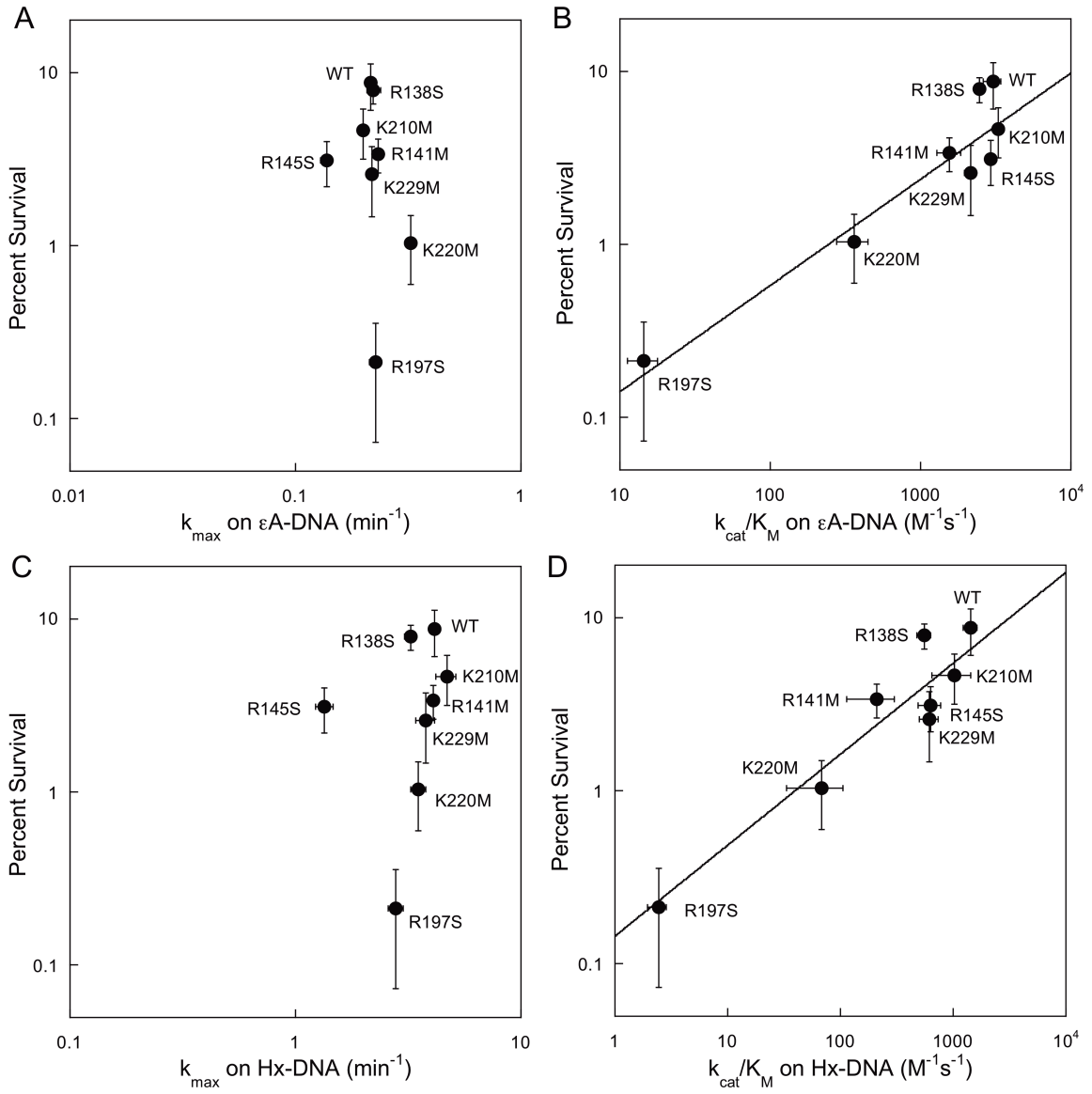



**Figure S8. Correlations of in vivo and in vitro parameters.** The survival rate of yeast cells cultured in glucose is positively correlated with $k_{cat}/K_M$ on 25E•T (B) and 25I•T DNA (D) with $R^2$ values of 0.82 and 0.79, respectively, but not with $k_{max}$ on 25E•T (A) and 25I•T DNA (C). The lines are from weighted linear regression analyses using the errors in percent survival.

Table S1. In vitro and in vivo parameters for AAG variants [a]

| AAG Variant | Excision of εA | | | | | Excision of Hx | | Nonspecific DNA | | In Vivo Yeast Survival [j] | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | $k_{max}$ [b] ($min^{-1}$) | $k_{cat}/K_M$ [c] ($M^{-1}s^{-1}$) | $F_P$ [d] 100 mM $Na^+$ | $F_P$ [d] 150 mM $Na^+$ | $E_{ex}$ [e] 150 mM $Na^+$ | $k_{max}$ [f] ($min^{-1}$) | $k_{cat}/K_M$ [g] ($M^{-1}s^{-1}$) | $K_i/K_M$ [h] | $K_{d,ns}$ (mM) [i] | Glucose | Galactose |
| WT | 0.22 ± 0.01 | 3000 ± 400 | 0.97 ± 0.06 | 0.93 ± 0.04 | 0.75 ± 0.06 | 4.1 ± 0.2 | 1400 ± 200 | 13 ± 3 | 0.64 ± 0.17 | 8.6 ± 2.6 | 11.3 ± 2.7 |
| R138S | 0.22 ± 0.02 | 2500 ± 120 | 0.96 ± 0.09 | 0.54 ± 0.05 | 0.44 ± 0.08 | 3.2 ± 0.2 | 550 ± 70 | 10 ± 1 | 0.99 ± 0.15 | 7.8 ± 1.3 | 11.7 ± 4.5 |
| R141M | 0.23 ± 0.01 | 1600 ± 300 | 0.95 ± 0.12 | 0.45 ± 0.01 | 0.30 ± 0.01 | 4.1 ± 0.2 | 210 ± 90 | 6 ± 1 | 1.9 ± 0.94 | 3.4 ± 0.7 | 15.7 ± 2.7 |
| R145S | 0.14 ± 0.01 | 2900 ± 200 | 0.88 ± 0.05 | 0.69 ± 0.04 | 0.60 ± 0.04 | 1.3 ± 0.1 | 630 ± 140 | 12 ± 5 | 0.43 ± 0.19 | 3.1 ± 0.9 | 14.8 ± 0.4 |
| R182M | 0.17 ± 0.01 | 1500 ± 300 | 0.65 ± 0.02 | 0.37 ± 0.04 | 0.21 ± 0.04 | 1.8 ± 0.2 | 170 ± 30 | 12 ± 4 | 2.2 ± 0.86 | 0.08 ± 0.02 | 0.6 ± 0.4 |
| R197S | 0.23 ± 0.01 | 15 ± 2 | 0.60 ± 0.09 | 0.10 ± 0.03 | 0.12 ± 0.02 | 2.8 ± 0.2 | 3 ± 1 | 6 ± 1 | 29 ± 13 | 0.2 ± 0.1 | 18.6 ± 3.8 |
| K210M | 0.20 ± 0.01 | 3300 ± 300 | 0.96 ± 0.04 | 0.57 ± 0.02 | 0.53 ± 0.05 | 4.7 ± 0.5 | 1000 ± 400 | 9 ± 2 | 0.65 ± 0.29 | 4.6 ± 1.5 | 14.1 ± 4.3 |
| K220M | 0.32 ± 0.01 | 360 ± 80 | 0.92 ± 0.07 | 0.42 ± 0.05 | 0.23 ± 0.03 | 3.5 ± 0.3 | 70 ± 40 | 8 ± 3 | 6.9 ± 4.5 | 1.0 ± 0.4 | 17.8 ± 2.2 |
| K229M | 0.22 ± 0.01 | 2100 ± 200 | 0.94 ± 0.03 | 0.70 ± 0.06 | 0.48 ± 0.04 | 3.8 ± 0.4 | 610 ± 110 | 7 ± 1 | 0.74 ± 0.18 | 2.6 ± 1.1 | 19.0 ± 1.2 |
| E125Q | | | | | | | | | | 0.06 ± 0.05 | 0.04 ± 0.03 |
| vector | | | | | | | | | | 0.03 ± 0.01 | 0.06 ± 0.03 |

[a] In vitro enzymatic assays were performed at 37 °C in 50 mM NaMES, pH 6.1, 1 mM DTT, 1 mM EDTA, 0.1 mg/mL BSA, 10% (v/v) glycerol unless otherwise stated. Values are the mean ± SD of ≥3 independent determinations.

[b] Maximal single-turnover rate constant ($k_{max}$) values were measured with 25E•T at 50 mM $Na^+$ (Figure 2C in the main text).

[c] $k_{cat}/K_M$ values were measured with 25E•T at low DNA concentrations under multiple-turnover conditions at 1 M $Na^+$ (Figure 2E in the main text).

[d] The fraction processive values were measured for the 47E2F2 processivity substrate under multiple-turnover conditions at 100 and 150 mM $Na^+$ (Figure 3B in the main text).

[e] Efficiency of excision ($E_{ex}$) values were measured with 25E•T using the pulse-chase assay at 150 mM $Na^+$ (Supporting Figure S4 and Figure 3E in the main text)

[f] Maximal single-turnover rate constant ($k_{max}$) values were measured with 25I•T at 50 mM $Na^+$ and 25 °C (Figure 4B in the main text).

[g] $k_{cat}/K_M$ values were measured with 25I•T at low DNA concentrations under multiple-turnover conditions at 515 mM $Na^+$ (Figure 4D in the main text).

[h] The ratios of $K_i/K_M$ were measured by competitive inhibition of Hx excision by excess nonspecific 25A•T DNA under multiple-turnover conditions at 150 mM $Na^+$.

[i] Values of the macroscopic dissociation constant for binding to the 25A•T nonspecific DNA ($K_{d,ns}$) were calculated using the measured parameters $k_{max}$ and $k_{cat}/K_M$ for 25I•T and $K_i/K_M$: $K_{d,ns} = k_{max}*(K_i/K_M)/(k_{cat}/K_M)$, in which the single-turnover rate constant $k_{max}$ was used to approximate the multiple-turnover rate constant $k_{cat}$ (Figure 5C in the main text).

[j] Yeast survival frequency in glucose and galactose culture were measured by treating exponential growth phase cells in 0.3% (v/v) MMS at room temperature for one hour (survival frequency is given by the number of colonies surviving MMS treatment, dividing by the number of colonies in the control without MMS, and multiplying by 100; Figure 6B in the main text).

Table S2. Determination of AAG expression level in yeast cells

| AAG Variant | $V_{init}/[E]$ ($min^{-1}$) [a] | Glucose Condition | | | | Galactose Condition | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $V_{init}$ (nM/Hr) [b] | [AAG] (nM) [c] | Molecules/cell [d] | $[AAG]_{rel}$ [e] | $V_{init}$ (nM/min) [b] | [AAG] (μM) [c] | Molecules/cell [d] | $[AAG]_{rel}$ [e] |
| WT | 2.8 ± 0.5 | 21 ± 5 | 5.8 ± 1.7 | 10 ± 3 | (1) | 138 ± 8 | 6.9 ± 1.4 | 12500 ± 2500 | (1) |
| R138S | 1.1 ± 0.1 | 10 ± 2 | 6.9 ± 1.5 | 12 ± 3 | 1.19 | 55 ± 8 | 6.7 ± 1.0 | 12200 ± 1900 | 0.98 |
| R141M | 0.4 ± 0.1 | 2.2 ± 1.2 | 4.5 ± 2.5 | 8 ± 4 | 0.77 | 12 ± 1 | 4.6 ± 0.8 | 8200 ± 1500 | 0.66 |
| R145S | 1.6 ± 0.2 | 13 ± 8 | 6.1 ± 3.9 | 11 ± 7 | 1.05 | 52 ± 22 | 4.6 ± 2.0 | 8200 ± 3600 | 0.66 |
| R182M | 0.5 ± 0.1 | ND [f] | – | – | – | < 0.2 | < 0.07 | <125 | < 0.01 |
| R197S | 0.05 ± 0.00 | 0.4 ± 0.4 | 6.0 ± 5.9 | 11 ± 11 | 1.03 | 0.85 ± 0.04 | 4.6 ± 0.3 | 8300 ± 500 | 0.67 |
| K210M | 2.0 ± 0.3 | 9.9 ± 1.6 | 3.9 ± 0.8 | 7 ± 1 | 0.67 | 58 ± 5 | 4.1 ± 0.6 | 7400 ± 1100 | 0.59 |
| K220M | 0.2 ± 0.1 | 1.2 ± 0.5 | 3.7 ± 1.8 | 7 ± 3 | 0.64 | 8 ± 1 | 4.8 ± 1.3 | 8600 ± 2400 | 0.69 |
| K229M | 1.1 ± 0.2 | 6.9 ± 1.3 | 4.8 ± 1.2 | 9 ± 2 | 0.83 | 34 ± 11 | 4.3 ± 1.6 | 7700 ± 2800 | 0.62 |

[a] Specific activity ($V_{init}/[E]$) was determined using known concentrations of the recombinant AAG variants and 200 nM 25I-bulge DNA. This DNA was convenient to use in the cell extracts, because AAG exhibits fast multiple-turnover excision without product inhibition, and the Mag1 deletion strain does not show any detectable activity with this substrate (Supporting Figure S6).

[b] $V_{init}$ values were measured by using yeast cell extracts prepared from cells grown under the glucose or galactose growth conditions (see Supporting Methods for cell extract preparation). The reported values shown in the table reflect the final results after taking into consideration of sample dilution in the glycosylase reaction system. Different units are used for the $V_{init}$ values under the two different growth conditions out of convenience.

[c] The concentrations of enzyme in yeast cells were calculated using $V_{init}$ values of the yeast cell extracts and $V_{init}/[E]$ values of the recombinant proteins. Different units are used for the two growth conditions out of convenience. The cellular AAG variant concentrations were calculated by using a cell density of $2 \times 10^7$ cells/$OD_{600}$/mL, an optical density of 1 upon harvest, a total culture volume of 20 mL, a disruption efficiency of 90%, a yeast cell extract volume of 50 μL for the glucose condition and 150 μL for the galactose condition (Supporting Methods), and a yeast nucleus volume of 3 $\mu m^3$.

[d] The number of AAG molecules per yeast cell were calculated by $[AAG] \times V_{yeast\ nucleus} \times$ Avogadro constant.

[e] The relative AAG concentrations were normalized based on the expression level of the WT AAG under their respective growth condition.

[f] ND, not detected.

## Supporting References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