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4 **Biochemical characterization and mutational studies of the thermostable uracil**
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6 **DNA glycosylase from the hyperthermophilic euryarchaeon *Thermococcus***
7 ***barophilus* Ch5**
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63 **Abstract**
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65 Uracil DNA glycosylases (UDGs) play an important role in removing uracil from
66 DNA to initiate DNA base excision repair. Here, we first characterized biochemically
67 a thermostable UDG from the hyperthermophilic euryarchaeon *Thermococcus*
68 *barophilus* Ch5 (Tba UDG), and probed its mechanism by mutational analysis.
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70 The recombinant Tba UDG cleaves specifically uracil-containing ssDNA and dsDNA
71 at 65°C. The enzyme displays an optimal cleavage activity at 55–75°C. Tba UDG
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73 cleaves DNA over a wide pH spectrum ranging from 4.0 to 9.0 with an optimal pH of
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75 5.0–8.0. In addition, the Tba UDG activity is independent on a divalent metal ion;
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77 however, both Zn²⁺ and Cu²⁺ completely inhibits the enzyme activity. Furthermore,
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79 the Tba UDG activity is also inhibited by high NaCl concentration. Tba UDG
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81 removes uracil from DNA by the order: U≈U/G>U/T≈U/C>U/A. The mutational
82
83 studies showed that both the E118A and N159A mutants completely abolish the
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85 cleavage activity and retain the compromised binding activity, suggesting that
86
87 residues E118 and N159 in Tba UDG are important for uracil recognition and removal.
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89 Our work provides a basis for determining the role of Tba UDG in the base excision
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91 repair pathway for uracil repair in *Thermococcus*.
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103 **Keywords:** *Thermococcus barophilus*; Uracil DNA glycosylase; Base excision
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105 repair
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1. Introduction

Uracil bases in DNA are created by deamination of cytosine or by dUMP incorporation catalyzed by a DNA polymerase. It is estimated that the deamination of cytosine leads to up to 500 uracil residues in a single human cell each day [1-2]. The rate of deamination of cytosine is greatly enhanced at elevated temperatures [3], and thus hyperthermophilic organisms that live at temperatures above 80°C are facing a serious threat caused by cytosine deamination. Since uracil has a strong ability to form mismatch with adenine (A), a G-C base pair would be subsequently converted to an A-T base pair if DNA replication occurs before the uracil is repaired [4], potentially leading to mutations in the genome. However, the estimated spontaneous mutation rates in hyperthermophilic bacteria and archaea are similar to those observed in *Escherichia coli* [5-6], suggesting that hyperthermophilic microorganisms are more efficient in repairing hydrolytic and oxidative damage to DNA bases [7]. Increased GC to AT mutations by replicating uracil that originates from deamination of cytosine are detrimental to the cells, which may cause genome instability or even cancer occurrence. In response, cells have evolved a base excision repair (BER) pathway to counteract potential mutations generated by uracil replication. Uracil DNA glycosylase (UDG) is the first BER enzyme to remove uracil from DNA, which leads to apurinic/apyrimidinic (AP) site. The generated AP site is subsequently repaired by different enzymes: AP endonuclease, DNA deoxyribosephosphodiesterase, DNA polymerase and DNA ligase [8].

UDGs are ubiquitous in bacteria, archaea, eukarya and viruses, and can remove

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181 uracil from DNA through hydrolyzing their glycosyl bonds. Based on sequence
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183 similarity, UDGs have currently been classified into six families [9]. Family 1 UDGs
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185 have been well studied in *Escherichia coli* and human [10-15], and can remove uracil
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187 base efficiently from ssDNA and from ds DNA. Mismatch-specific DNA glycosylases
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189 and single-stranded specific monofunctional UDGs form families 2 and 3,
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191 respectively [16-17]. Family 4 UDGs are exclusively observed in the
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193 hyperthermophilic microorganisms and possess a 4Fe - 4S cluster [18-20]. Family 5
194
195 UDGs have broad substrate specificity, but lack a polar residue at the active-site motif
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197 [20, 21-22]. Last, family 6 UDGs can cleave hypoxanthine instead of uracil, and
198
199 thus belong to hypoxanthine DNA glycosylase family [23].
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205 The *Archaeoglobus fulgidus* UDG is the first enzyme to be identified and
206
207 characterized from archaea [24]. Currently, another eight UDG homologues from
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209 hyperthermophilic archaea have been reported from *Pyrobaculum aerophilum* [25],
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211 *Pyrococcus furiosus* [26-27], *Methanococcus jannaschii* [28], *Aeropyrum pernix* [29],
212
213 *Sulfolobus solfataricus* [30], *Sulfolobus tokodaii* [31-32], *Sulfolobus acidocaldarius*
214
215 [33], and *Thermoplasma acidophilum* [34]. The *A. fulgidus* UDG is the most
216
217 thermostable, retaining activity even after 1.5 hr of heating at 95°C [24]. It is able to
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219 remove uracil from dsDNA containing a U/A or U/G base pair, as well as from
220
221 ssDNA. This enzyme is inhibited by apurinic sites. *M. jannaschii* UDG possesses the
222
223 helix-hairpin-helix and [4Fe-4S]-binding cluster to recognize and bind uracil, and can
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225 efficiently cleave uracil in ssDNA and dsDNA and 8-oxoG in DNA [28]. The crystal
226
227 structure of *S. tokodaii* UDG shows that residues Leu169, Tyr170 and Asn171 in the
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240 leucine-intercalation loop of the enzyme play important roles in uracil-binding [32]. *T.*
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242 *acidophilum* UDG is involved in mediating the transfer from long patch repair to short
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244 patch repair [34]. Several studies on archaeal UDGs have shown that they can interact
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246 with PCNA (proliferating cell nuclear antigen) [26, 30, 35], which is an important
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248 component in DNA replication and repair in and eukarya and archaea [36].
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250 Furthermore, due to their thermostability, they have been shown to have potential
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252 applications to enhance PCR yield or to help in jump starting the reactions [29].
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257 *Thermococcus* is an important branch of euryarchaea comprising more than 40
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259 described species, which mostly thrive in the hottest deep-sea hydrothermal vent
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261 systems. Thus, similar to other hyperthermophilic archaea and bacteria,
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263 *Thermococcus* is also facing severe challenges due to increased deamination of
264
265 cytosine dependent on high-temperature [37]. It is expected that the
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267 hyperthermophilic *Thermococcus* would have evolved a repair pathway to counteract
268
269 the mutational effects of cytosine deamination in order to maintain their genome
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271 stability. *Thermococcus barophilus* is one of the most extreme member of the
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273 *Thermococcus* genus, being hyperthermophilic, piezophilic and capable of
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275 auxotrophic growth on carbon monoxide. Strain Ch5 was isolated from a deep-sea
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277 hydrothermal field of the Mid-Atlantic Ridge (Logachev field chimney, 3,020 m
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279 depth) [38]. *T. barophilus* Ch5 has a pressure optimum of 40 MPa and a temperature
280
281 optimum of 85°C [39]. The completed genome sequence of *T. barophilus* Ch5 shows
282
283 that this strain possesses two uracil DNA glycosylases (Tba UDGs) [40]. In this study,
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285 we cloned one of the Tba UDGs gene, purified and characterized its product. In
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299 addition, we probed the mechanism of cleaving uracil by Tba UDG through
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301 mutational analysis. We report here that Tba UDG is a thermostable enzyme cleaving
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303 specifically uracil-containing DNA, and its residues E118 and N159 are important for
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305 its catalysis. To the best of our knowledge, this is the first report of the biochemical
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307 characterization and mutational studies of a thermostable UDG from *Thermococcus*
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309 species.
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313 **2. Materials and methods**

314 *2.1. Cloning, expression and purification of Tba UDG*

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316 The Tba UDG in this work is encoded by gene TBCH5v1_0629 (GenBank
317
318 accession number: CP013050.1). The genomic DNA of *T. barophilus* Ch5 was
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320 extracted as described by Oger et al. [40] and then used as a template to amplify the
321
322 gene TBCH5v1_0629 using the Phusion DNA polymerase (Thermo Scientific,
323
324 Waltham, MA, USA) and the two primers (Tba UDG F and Tba UDG R, Table 1).
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326 The amplified DNA product was inserted into the vector pET-30a (+) (Novagen,
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328 Merck, Darmstadt, Germany). The recombinant plasmid harboring a sequence
329
330 encoded a 6 × His-tag at the C-terminus of Tba UDG was sequenced to verify the
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332 accuracy of the sequence of the enzyme gene and transformed into *E. coli* BL21
333
334 (DE3) cells (Transgene, Beijing, China) for protein expression.
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342 The expression strain *E. coli* harboring the recombinant plasmid was cultured
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344 into LB medium with 100 µg/mL kanamycin at 37°C until an OD₆₀₀ of 0.6, at which
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346 point protein expression was induced with isopropyl thiogalactoside (IPTG) at a final
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348 concentration of 0.1 mM. The culture was further shaken for 10 hr at room
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358 temperature until it reached an OD₆₀₀ of 1.1.
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360 The cells were harvested by centrifugation (5,000 × g for 20 min at room
361 temperature). The resultant pellet was resuspended with Ni column buffer A (20 mM
362 Tris-HCl pH 8.0, 1 mM dithiothreitol (DTT), 500 mM NaCl, 50 mM imidazole and
363 10% glycerol). The cells were disrupted by sonication into an ice bath. After
364 centrifugation (16,000 × g for 30 min at 4°C), the supernatant was collected into a 50
365 mL tube and heated at 70°C for 20 min. The non-thermostable *E. coli* proteins were
366 almost removed by centrifugation (16,000 × g for 30 min at 4°C). The resulting
367 supernatant was loaded to a HisTrap FF column (GE Healthcare, Uppsala, Sweden)
368 and purified with NCG™ Chromatography System (Bio-Rad, Hercules, CA, USA). A
369 linear gradient of 50–500 mM imidazole was used to elute the Tba UDG protein.
370 Fractions of Tba UDG protein were harvested and run by a 12% sodium dodecyl
371 sulfate-polyacrylamide gel electrophoresis. The Tba UDG protein was stained and
372 visualized by Coomassie-staining method. Finally, the purified Tba UDG protein was
373 dialyzed in a storage buffer containing 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM
374 DTT and 50% glycerol, and was stored at –80°C. The protein concentration was
375 determined using the Bradford Protein Assay Kit (Bio-Rad).
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398 399 *2.2. Construction, overexpression and purification of the Tba UDG mutants* 400

401 Using the wild-type plasmid harboring the Tba UDG gene as a template, the site-
402 directed mutagenesis was performed by a SDM Kit to construct E118A and N159A
403 mutants, according to its manual instruction. Note that residues E118 and N159 in Tba
404 UDG are located in the conserved Motif B and Motif D, respectively (Fig. 1). The
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414 sequences of mutagenic primers are listed in Table 1. The mutant plasmids were
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416
417 verified by sequencing. Similar to the wild-type protein, the Tba UDG mutant
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420 proteins were overexpressed, purified and quantitated.
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423 2.3. DNA substrate

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425 Normal and uracil-containing deoxyoligonucleotides were synthesized by
426
427 Sangon Biotech company, China. The sequences of these deoxyoligonucleotides are
428
429 shown in Table 2. The Cy3-labeled deoxyoligonucleotide duplexes shown in Table 3
430
431
432 were prepared by annealing the Cy3-labeled deoxyoligonucleotides to the
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434 complementary deoxyoligonucleotides in a buffer containing 20 mM Tris-Cl pH 8.0
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436 and 100 mM NaCl. The mixture was heated at 100°C for 5 min and cooled slowly at
437
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439 least 4 hours to room temperature.
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443 2.4. Glycosylase assays

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445 The standard assays of Tba UDG activity were carried out in the reactions (10
446
447 μL) which contained 20 mM Tris-HCl pH 8.0, 5 mM DTT, 50 mM NaCl, 1 mM
448
449 EDTA, 8% glycerol, 200 nM DNA, wild-type or mutant Tba UDG with varied
450
451 concentrations. The reactions were performed at 75°C for 10 min for ssDNA cleavage
452
453 and at 65°C for 10 min for dsDNA cleavage. 1 μL 500 mM NaOH and 9 μL
454
455 formamide-EDTA (98% formamide and 20 mM EDTA) were added to stop the
456
457 reactions. The reaction products were heated at 95°C for 5 min and chilled rapidly on
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459 ice for 5 min, and then loaded onto a denaturing 15% polyacrylamide gel with 8M
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461 urea. After electrophoresis, the gels were scanned and the Cy3-labeled DNA was
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464 visualized with a Molecular Image analyser (PharosFx System, Bio-Rad). The
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476 ImageQuant software was used for the quantitative analysis. All experiments of the
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478 glycosylase assays were replicated three times.
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481 *2.5. Biochemical characterization assays*

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483 The optimal temperature of Tba UDG to cleave DNA in the reactions (10 μ L)
484 contained 800 nM enzyme and 200 nM Cy3-labeled ssDNA with uracil as a target.
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486 The reactions were performed at 35, 45, 55, 65, 75, 85 and 95°C for uracil-containing
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488 ssDNA for 10 min.
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492 To examine the thermostability of the enzyme, Tba UDG was heated at 80, 85,
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494 90, 95 and 100°C for 30 min. The activity of the heated Tba UDG protein were
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496 investigated under the same conditions but using 1 μ M of the heated enzyme protein.
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498 Samples were treated as described above.
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502 The effect of pH on the Tba UDG activity was evaluated by examining DNA
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504 cleavage in similar 10 μ L reactions at 75°C under pHs ranging from 4.0 to 11.0 using
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506 1 μ M of the enzyme protein. The varied pHs were adjusted with five different buffers
507
508 (all at 20 mM concentrations): acetate-sodium acetate (pH 4.0 and pH 5.0), sodium
509
510 phosphate-NaOH (pH 6.0 and pH 7.0), Tris-HCl (pH 8.0), Gly-NaOH (pH 9.0), and
511
512 NaHCO₃-NaOH (pH 10.0 and pH 11.0).
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516
517 The effect of divalent metal ions on Tba UDG activity was investigated by
518
519 adding of 2 mM of Mg²⁺, Mn²⁺, Ca²⁺, Zn²⁺ or Cu²⁺ (analytical purity) to the reaction
520
521 mixture. Assays were performed at 75°C with 250 nM of the enzyme. Samples were
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523 treated as described above.
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526 To evaluate the effect of salinity on Tba UDG activity, glycosylase assays were
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535 performed in the presence of various NaCl concentrations ranging from 50 to 1,000
536 mM using 1 μ M of the enzyme. Samples were treated as described above.
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539 540 2.6. *Glycosylase single-turnover assays*

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542 The reaction mixtures containing 800 nM wild-type or mutant Tba UDG and 200
543 nM DNA substrates were incubated at 65°C for various times. Samples were treated
544 as described above. Data from the DNA cleavage experiments under single-turnover
545 conditions were fit to a single-exponential decay equation:
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$$551 \quad [\text{Product}] = A \exp(-k_{\text{endo}} t)$$

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553 where A and k_{endo} represent the reaction amplitude and observed DNA cleavage
554 rate, respectively.
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557 558 2.7. *Substrate specificity*

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560 To investigate the substrate specificity of the enzyme, we employed normal
561 ssDNA and dsDNA, ssDNA with uracil and dsDNA with U (U/T, U/C, U/G or U/A),
562 and dsDNA with a mismatch (G/T) as the substrates to perform the glycosylase assays
563 at 65°C for 10 min using 800 nM enzyme. Samples were treated as described above.
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571 2.8. *DNA-binding Assays*

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573 Electrophoresis mobility shift assays (EMSA) were performed by incubating the
574 wild-type or mutant Tba UDG with uracil-containing ssDNA and dsDNA in a DNA
575 binding buffer (10 μ L) containing 20 mM Tris-HCl pH 8.0, 5 mM DTT, 8% glycerol,
576 200 nM DNA and the wild-type or mutant Tba UDG with varied concentrations at
577 25°C for 10 min. The samples were electrophoresed on a 4% native polyacrylamide
578 gel in 0.1 \times TBE (Tris-borate-EDTA) buffer. After electrophoresis, the gels were
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591 scanned and Cy3-labeled DNA was visualized with a Molecular Image analyser (Bio-
592 Rad). ImageQuant software was used for the quantitative analysis.
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598 **3. Results**

600 *3.1. Tba UDG is a thermostable uracil DNA glycosylase*

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602 The alignment result of partial amino acid sequences of UDGs from
603 hyperthermophilic archaea and bacteria shows that Tba UDG possesses six conserved
604 motifs (A-F) that are characteristics of family 4 UDGs (Fig. 1A), suggesting that this
605 enzyme belongs to family 4 UDGs. On the other hand, Motif B and Motif F in Tba
606 UDG are conserved in all six family UDGs (Fig. 1B). Tba UDG displays 21%, 19%,
607 22%, 22%, 18%, 20%, 19%, and 21% similarities to those of from *P. furiosus*, *P.*
608 *horikoshii*, *A. fulgidus*, *P. aerophilum* UDGA, *A. pernix*, *S. solfataricus*, *S. tokodaii*
609 and *Thermotoga maritima*, respectively. The low similarity between Tba UDG and
610 other UDGs suggests that Tba UDG might be a novel glycosylase.
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625 The Tba UDG gene from the hyperthermophilic archaeon *T. barophilus* Ch5 was
626 cloned into the pET-30a (+) expression vector, and expressed in *E. coli* BL21(DE3).
627 The recombinant Tba UDG protein was successfully expressed as a His-tag fusion
628 protein (Fig. 1C). By means of sonication, heat treatment (70°C for 20 min) and
629 purification by affinity chromatography with a Ni column, we purified the Tba UDG
630 protein (~27 kDa) (Fig. 1C).
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639 We used the normal, uracil-containing ssDNA and dsDNA as the substrates to
640 investigate DNA cleavage by Tba UDG at 65°C. Using normal ssDNA and dsDNA as
641 the substrates, no product was formed by the enzyme, however, the cleaved product of
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653 the enzyme was observed in the presence of uracil-containing ssDNA and dsDNA
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655 (Fig. 1D). The results showed that Tba UDG is a thermostable glycosylase, capable of
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657 removing uracil from ssDNA and dsDNA at 65°C.
658

659
660 The heating treatment (70°C for 20 min) during the purification of Tba UDG
661
662 protein can denature most of *E. coli* proteins (Fig. 1); however, there was a slight
663
664 possibility of *E. coli* UDG contamination, which would interfere with our results. To
665
666 test this possibility, we used cell extracts made from cells expressing the empty pET-
667
668 30a (+) vector. We could detect no cleavage product when using this heated
669
670 supernatant produced from the empty vector (data not shown), thus ruling out the
671
672 possibility of an *E. coli* UDG contamination during purification of Tba UDG. Overall,
673
674 our results suggest that Tba UDG can cleave uracil-containing DNA at high
675
676 temperature.
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681 682 3.2. Biochemical characterization of Tba UDG 683

684 Since *T. barophilus* Ch5 thrives at high temperature (85°C) and we could show
685
686 that Tba UDG can cleave uracil-containing DNA at 75°C, we first investigated the
687
688 optimal temperature for the enzyme to cleave uracil-containing DNA by using the
689
690 uracil-containing ssDNA as the substrate. The cleavage percentage of Tba UDG
691
692 increased from 54% to 98% when increasing reaction temperature from 35 to 75°C
693
694 (Fig. 2A). Interestingly, even at the lowest tested temperatures, e.g. 35°C and 45°C,
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696 Tba UDG displayed a significant activity, with 54% and 92% cleavage efficiencies
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701 (Fig. 2A), respectively. At the temperatures higher than 75°C, the efficiency
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712 decreased to reach 24% at 95°C (Fig. 2A), suggesting that the optimal activity of the
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714 enzyme to cleave uracil-containing ssDNA is between 55°C and 75°C.
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716
717 To further investigate the thermostability of the enzyme, we heated Tba UDG at
718
719 various temperatures prior to activity assessments. When heated at 80°C for 30 min,
720
721 Tba UDG retained about 90% of cleavage activity (Fig. 2B). The enzyme activity
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723 rapidly decreased at higher temperatures to reach 23% for 85°C and no remaining
724
725 activity above 90°C (Fig. 2B). Overall, these observations suggest that Tba UDG is
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727 thermostable.
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730
731 We examined the impact of pH on the endonuclease activity of Tba UDG over a
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733 wide pH range from 4.0 to 11.0 in the standard DNA cleavage reactions. No activity
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735 could be detected at the highest pHs (pH=10 and pH=11, Fig. 2C). By contrast, we
736
737 could detect significant cleavage activity even at the lowest pH (pH=4, activity =
738
739 69%). The maximal activity was observed for pH ranging from 5 to 8, ranging from
740
741 96% to 91%, respectively (Fig. 2C). At pH 9.0, Tba UDG retained 43% cleavage
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743 efficiency. These results suggest that Tba UDG cannot effectively cleave uracil-
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745 containing DNA at high pHs (pH>10.0) and that the optimal pH for this enzyme to
746
747 cleave uracil-containing DNA was between 5.0 and 7.0.
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752
753 To evaluate the effects of various divalent metal ions (Mg^{2+} , Mn^{2+} , Ca^{2+} , Zn^{2+}
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755 and Cu^{2+}) on the DNA cleavage activity of Tba UDG, we reduced the concentration
756
757 of the enzyme in the reactions. In the absence of a divalent ion and in the presence of
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759 EDTA, Tba UDG displayed about 70% cleavage activity (Fig. 2D), suggesting that a
760
761 divalent metal ion is not required for the enzyme to cleave uracil-containing DNA.
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771 We observed no inhibition of Ca^{2+} or Mg^{2+} on the activity of Tba UDG, with cleavage
772 efficiencies ca. 77% in the presence of both ions (Fig. 2D). Two metals, e.g. Zn^{2+} or
773
774 Cu^{2+} , were found to totally inhibit the enzyme. Last, the activity of the enzyme was
775
776 partially inhibited in the presence of Mn^{2+} . Overall, our results suggest that a divalent
777
778 metal ion is not needed for Tba UDG to effectively cleave uracil-containing DNA.
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781
782 To uncover the effect of salinity on the Tba UDG activity, we added NaCl with
783 various concentrations in the DNA cleavage reactions. Under the standard conditions
784
785 in the absence of NaCl, Tba UDG cleaved almost completely DNA substrate with
786
787 97% of cleavage efficiency (Fig. 2E). No impact of salinity was observed below 200
788
789 mM, at which salinity Tba UDG retained 96% of cleavage efficiency, which is similar
790
791 to the control reactions (Fig. 2E). However, only 13% cleavage efficiency of Tba
792
793 UDG activity was observed in the presence of 400 mM NaCl (Fig. 2E). No cleaved
794
795 DNA product was observed at NaCl concentrations from 600 to 1000 mM (Fig. 2E).
796
797 These results show that the Tba UDG is a salt-tolerant enzyme, inhibited only by high
798
799 NaCl concentrations (>400 mM).
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802 3.3. *Substrate specificity of Tba UDG*

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805 To evaluate the substrate specificity of the enzyme, we used the mismatched
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807 DNA (G/T), four mismatched DNA with uracil, and ssDNA with uracil as the
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809 substrates to examine the enzyme activity. As shown in Fig. 3A, the cleavage
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811 efficiencies of Tba UDG were 95%, 88%, 30% and 85% when using the mismatched
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813 DNA with U/G, U/C, U/A and U/T as the substrates, respectively. These results
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815 suggest Tba UDG exhibit various cleavage efficiencies on uracil-containing dsDNA.
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830 Furthermore, no cleavage product was found when using mismatched dsDNA (G/T)
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832 (Fig. 3B). The cleavage efficiency of the enzyme was 93% when using uracil-
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834 containing ssDNA as the substrate, which is close to that of using uracil-containing
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836 dsDNA (U/G). Thus, Tba UDG has a preference for substrates with the order from
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838 high to low: $U \approx U/G > U/T \approx U/C > U/A$.
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840 841 842 *3.4. Kinetics of DNA cleavage by Tba UDG* 843

844 Here, we carried out time course of DNA cleavage activity of Tba UDG under
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846 the optimal reaction condition as described above. As the reaction time extended,
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848 DNA cleavage product of Tba UDG was gradually enhanced until the uracil-
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850 containing ssDNA (Fig. 4A) and dsDNA (Fig. 4B) were almost cleaved. When
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852 reaction time was 10 min, the percent of Tba UDG for cleaving uracil-containing
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854 ssDNA and dsDNA reached 97% and 93%, respectively. These observations suggest
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856 that the enzyme has a strong activity for cleaving uracil-containing DNA at high
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858 temperature.
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863 The molar amount of remaining DNA substrate in the DNA cleavage reactions
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865 catalyzed by Tba UDG was plotted as a function of reaction time (Fig. 4C), and the
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867 data were fit to the single-exponential decay equation to yield k_{exo} . The k_{exo} values
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869 are $0.25 \pm 0.03 \text{ min}^{-1}$ and $0.31 \pm 0.04 \text{ min}^{-1}$ for uracil-containing ssDNA and dsDNA,
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871 respectively. Therefore, Tba UDG displays similar rates for cleaving uracil-containing
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873 ssDNA and dsDNA.
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878 879 *3.5. Mutational analysis of Tba UDG* 880

881 As shown in Fig. 5A, the crystal structure of *S. tokodaii* UDG shows that
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889 residues Glu42, His164, Asn82, Phe55 and Glu48 might be key for uracil recognition
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891 [32]. Note that there is no a corresponding amino acid residue in Tba UDG for residue
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893 Glu48 in *S. tokodaii*, and residues Glu42, His164, Asn82 and Phe55 in *S. tokodaii*
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895 UDG correspond to residues Glu118, His216, Asn159 and Tyr127 in Tba UDG.
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897 Sequence comparison shows that these conserved residues Glu118, His216, Asn159
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899 and Tyr127 in Tba UDG are located in Motif B, C, D and F (Fig. 1A), respectively.
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901 To investigate the function of these residues of Tba UDG, we mutated two of these
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903 residues to alanine. The purification profiles of the Tba UDG E118A and N159A
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905 mutants are shown in Fig. 5B.
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911 In the control reaction with the wild-type Tba UDG, the enzyme can effectively
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913 cleave the uracil-containing ssDNA (Fig. 6A) and dsDNA (Fig. 6D). When using 500
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915 nM enzyme, the cleavage percent of Tba UDG reached approximate 90%. By
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917 contrast, the E118A and N159A mutants had no cleaving activity, no matter what the
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919 uracil-containing ssDNA or dsDNA was used (Figs. 6B, 6C, 6E and 6F). Therefore,
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921 our data suggest that both mutations enable Tba UDG to abolish its activity, and thus
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923 residues E118 and N159 in the enzyme play essential roles in cleaving uracil-
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925 containing DNA.
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929 930 *3.6. DNA-binding of the wild-type and mutant Tba UDGs*

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933 To assess the effect of these two mutations on the affinity of the enzyme to
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935 uracil-containing DNA, we investigated whether or not the wild-type and mutant Tba
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937 UDGs binds to uracil-containing ssDNA or dsDNA by employing EMSA. As shown
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939 in Figs. 7A and 7D, the free uracil-containing ssDNA and dsDNA were gradually
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945 bound as increasing the enzyme concentrations. At $\geq 1,100$ nM Tba UDG, the uracil-
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948 containing ssDNA and dsDNA was almost bound by the enzyme (Figs. 7A and 7D).
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952 In contrast, the maximal binding percents of the E118A mutant only reached
953 35% for uracil-containing ssDNA and 25% for uracil-containing dsDNA even in the
954 presence of high enzyme concentration (1,500 nM) (Figs. 7B and 7D), suggesting that
955 the E118A mutant retains the compromised ability to bind to uracil-containing ssDNA
956 and dsDNA.
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965 Compared with the wild-type protein, the N159A mutant displayed the clearly
966 reduced efficiencies for binding to uracil-containing ssDNA at lower concentration
967 ($< 1,100$ nM) (Fig. 7C). However, the binding percent of the N159A mutant was 92%
968 at 1,500 nM enzyme, which is similar to that of the wild-type protein. On the other
969 hand, the N159A mutant had the lower efficiency for binding to uracil-containing
970 dsDNA than the wild-type protein (Fig. 7F). Furthermore, the binding efficiencies of
971 the N159A mutant were higher than those of the E118A mutant. Thus, the residue
972 N159 in the Tba UDG is essential for catalysis, and also are involved in binding to
973 uracil.
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988 **4. Discussion**

989 In this work, we characterized biochemically for the first time the thermostable
990 UDG from the hyperthermophilic archaeon *T. barophilus* Ch5, and revealed that Tba
991 UDG can specifically cleave uracil-containing DNA at temperatures ranging from 35
992 to 95°C. Similar to that of the closest homologue, *Pyrococcus furiosus* UDG, the
993 optimal temperature of the enzyme activity is 55–75°C, which is lower than that of
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1007 the optimal temperature of *T. barophilus* Ch5 [40]. Compared with *A. fulgidus* UDG
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1009 (80°C) [42], Tba UDG has an slightly lower optimal activity temperature. However,
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1011 the optimal temperature of Tba UDG activity is clearly higher than that of *S.*
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1013 *solfataricus* UDG [30], and similar to that of *A. pernix* UDG [29], *P. aerophilum*
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1015 UDG [41] and *T. maritima* UDG [43]. Thus, the optimal temperatures of archaeal
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1017 UDGs vary with hyperthermophilic organisms, which might be due to distinct living
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1019 environments. Furthermore, Tba UDG still retains the pronounced endonuclease
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1021 activity even when heated at 85°C for 30 min, suggesting that Tba UDG is a
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1023 thermostable endonuclease. Since the rate of deamination of cytosine increases with
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1025 temperature, significant amount of uracil might be generated at 85°C, which is the
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1027 optimal growth temperature of *T. barophilus* Ch5 [40]. Thus, the activity of Tba UDG
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1029 might be essential for mutation prevention in this organism in response to the known
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1031 mutagenic potential of uracil.
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1038 DNA cleavage efficiencies by UDGs vary with pH. Tba UDG exhibits maximal
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1040 activity over a broad pH range from 5.0 to 7.0, which is close to that of the purified
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1042 recombinant *A. fulgidus* UDG that has a optimal pH 4.8 [42]. Interestingly, the native
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1044 *A. fulgidus* UDG displays maximal activity around pH 6.2 [42]. The difference pHs
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1046 for optimal activity of *A. fulgidus* UDG from native cells and expression cells might
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1048 be related to covalent modifications or accessory factors, or a different folding when
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1050 expressed in the native host. By contrast, the optimal pH for the *A. pernix* UDG
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1052 activity is estimated to be 8.0 to 10.5, with the highest removal of uracil from ssDNA
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1054 at pH 9.0 [29]. In addition, the optimal pH for Tba UDG strongly differs from that of
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1066 its closest homologue, that of *P. furiosus*, which is ca. pH 9 [27]. The rationale for this
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1068 strong divergences in optimal pHs is quite surprising since most of these Archaea
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1070 have near neutral intracellular pHs.
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1073 The reported UDGs are independent on a divalent metal ion [27, 29], which is
1074
1075 also the case for Tba UDG. Similar to *A. pernix* UDG [29], Tba UDG is almost
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1077 inactive to cleave DNA in the presence of Zn²⁺ or Cu²⁺. However, both Mg²⁺ and
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1079 Mn²⁺ have no detectable effect on DNA cleavage of Tba UDG. However, Mn²⁺ shows
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1081 some inhibition of the activity of *A. pernix* UDG [29].
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1085 Tba UDG displays substrate specificity for cleaving DNA in the order:
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1087 U≈U/G>U/T≈U/C>U/A. By contrast, the *P. furiosus* UDG, as its closest homologue
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1089 of Tba UDG, removes uracil from various DNA substrates with the following order:
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1091 U/T≈U/C>U/G≈U/AP≈U/->U/U≈U/I≈U/A [27]. On the other hand, the *A. fulgidus*
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1093 UDG exhibits opposite base-dependent excision of uracil by the following order:
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1095 U>U/T>U/C=U/G=U/A [44]. Furthermore, the uracil-releasing activity of *M.*
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1097 *jannaschii* UDG is observed by the following order U/T>U/C>U/G>U/A [28]. In
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1099 addition, *A. pernix* UDG exhibits the uracil removal as follows:
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1101 U/C=U/G>U/T=U/AP=U/->U/U=U/I>U/A [29]. Moreover, *P. aerophilum* UDG
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1103 shows the substrate specificity by the order: G/U>A/U>ssU [25]. Overall, the
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1105 substrate specificities of archaeal UDGs vary with these organisms.
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1112 On the other hand, Tba UDG has no detected activity on G/T mismatched DNA,
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1114 similar to *S. solfataricus* UDG [30]. By contrast, the *P. aerophilum* UDG h can cleave
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1116 normal mismatched DNA (G/T) and U/G [41]. Furthermore, the preferred substrates
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1125 of *S. solfataricus* UDG and Tba UDG appear to be the G:U-containing double-
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1127 stranded oligonucleotide. In addition, both Tba UDG and *S. solfataricus* UDG can
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1129 cleave single-stranded DNA containing uracil; however, Tba UDG displays higher
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1131 efficiencies for this cleavage than *S. solfataricus* UDG.
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1134 The uracil recognition mechanisms of several UDGs have been reported,
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1136 however, an complete understanding on how archaeal UDGs recognize and cleave
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1138 uracil-containing DNA remains elusive. The crystal structure of *S. tokodaii* UDG
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1140 suggest that this UDG has a special structure of the leucine-intercalation loop [32],
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1142 which is distinct from other UDGs, Further mutational analysis on the loop indicates
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1144 that Tyr170 in *S. tokodaii* UDG is critical for substrate DNA recognition and the
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1146 catalysis [32]. Mutational studies on the iron sulfur cluster loop motif in the *A.*
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1148 *fulgidus* uracil-DNA glycosylase suggest that the R86A, C85A and C101A mutants
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1150 exhibit reduced activity for uracil removal only within double-stranded DNA, while
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1152 the K100A mutant exhibits enhanced uracil excision activity [45]. In this work, we
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1154 did the mutational studies based on the *S. tokodaii* UDG structure by mutating
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1156 residues E118 and N159 in Tba UDG to alanine, which are the corresponding residues
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1158 E42 and N82 in *S. tokodaii* UDG. Our data show that residues E188 and N159 are key
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1160 for uracil recognition and removal, suggesting that the conserved motif B and Motif
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1162 D are important for uracil recognition and removal. Thus, our observations provide
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1164 new insight into understanding mechanism and function of archaeal UDGs.
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1173 **5. Conclusion**

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1175 In summary, we present the biochemical characteristics and mechanism of the
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1184 thermostable UDG from *T. barophilus* Ch5 in this work, which is first report on UDG
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1186 from *Thermococcus* species. The recombinant Tba UDG displays specifically uracil-
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1188 containing DNA cleavage activity with the highest efficiency at 55–75°C and with an
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1190 optimal pH of 5.0–7.0. A divalent metal ion is not required for the enzyme to cleave
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1192 uracil-containing DNA. Furthermore, the enzyme activity is inhibited by Zn²⁺ or Cu²⁺,
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1194 and high NaCl concentration. The enzyme exhibits the substrate specificity by the
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1196 order: U≈U/G>U/T≈U/C>U/G>U/A. Mutational studies suggest that residues E118
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1198 and N159 in Tba UDG are essential for uracil recognition and removal. Our work
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1200 provides a basis for determining the role of Tba UDG in the base excision repair
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1202 pathway for repairing potentially elevated uracils in *Thermococcus*.
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1230 **Author contributions**

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1243 LZ, ZY and PO designed experiments; HS, QG, HH, HC, YX and LM performed
1244
1245 experiments; LZ and PO analyzed data; LZ, ZY and PO wrote and revised the paper.
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Figure legends

Fig. 1. Tba UDG can cleave uracil-containing ssDNA and dsDNA at high temperature. A. Partial amino acid alignment of UDGs from hyperthermophilic crenarchaea, euryarchaea and bacteria. Tba: *Thermococcus barophilus* (WP_056934618.1); Pfu: *Pyrococcus furiosus* (WP_011012532.1); Pho: *Pyrococcus horikoshii* (WP_048053599.1); Afu: *Archaeoglobus fulgidus* (GenBank: AIG99287.1); Pae: *Pyrobaculum aerophilum* (GenBank: AAL62921.1); Ape: *Aeropyrum pernix* (GenBank: BAA79385.2); Sso: *Sulfolobus solfataricus* (GenBank: AKA78326.1); Sto: *Sulfolobus tokodaii* (PDB: 4ZBY); Tma: *Thermotoga maritima* (PDB: 1L9G_A). B. The conserved Motif B and Motif F in six families of UDG. Family 1, Eco (*E. coli*) UDG (EMBL:J03725); Family 2, Human TDG (EMBL: U51166); Family 3, Human SUMG1 (EMBL: AF125182); Family 4, Tba UDG247 (NCBI reference sequence: WP_056934618.1); Family 5, *P. aerophilum* (Pae) UDGb (NP_559226); Family 6, Mba (*Methanosarcina barkeri*) HDG (YP_304295.1). C. Overexpression and purification of Tba UDG. M: Protein marker. D. DNA cleavage assays of Tba UDG. DNA cleavage reactions were performed by Tba UDG in the presence of normal and uracil-containing ssDNA and dsDNA at 65°C. CK: the reaction without the enzyme.

Fig. 2. Biochemical characterization of Tba UDG. A. The optimal temperature of the enzyme. B. The thermostability of the enzyme. C. The pH adaptation of the enzyme. D. Effects of divalent metal ions on the enzyme activity. E. Effect of NaCl on the enzyme activity. Reaction products were detected by electrophoresis through running

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1715 a 15% denaturing PAGE. CK: the reaction without the enzyme; CK1 in the panel B:
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1717 the reaction without the enzyme; CK2 in the panle B: the reaction with the unheated
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1719 enzyme.
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Fig. 3. Substrate specificity of Tba UDG. DNA cleavage reactions of Tba UDG were
1723 performed using the uracil-containing ssDNA and dsDNA, and mismatched DNA
1724 (G/T) as the substrates. Reaction products were analyzed by electrophoresis through
1725 running a 15% denaturing PAGE. A. The substrates were ssDNA with U, and
1726 mismatched dsDNA with U/T, U/C, U/G, or U/A. B. The substrates were mismatched
1727 DNA (G/T). CK: the reaction without the enzyme.
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Fig. 4. Kinetic analysis of DNA cleavage of Tba UDG. DNA cleavage reactions by
1739 Tba UDG were performed under the optimal reaction condition at various time (10
1740 sec – 30 min). Reaction products were analyzed by electrophoresis through running a
1741 12% denaturing PAGE. A. Uracil-containing ss DNA cleavage; B. Uracil-containing
1742 ds DNA cleavage. CK: the reaction without the enzyme; C. Rate of DNA cleavage
1743 catalyzed by Tba UDG. By using the single-exponential decay equation, the amount
1744 of remaining substrate was plotted as a function of time to yeild the best fit (the solid
1745 lines). Tba UDG cleaved the uracil-containing ssDNA (\circ) and dsDNA (\square) at the rates
1746 of $0.25 \pm 0.03 \text{ min}^{-1}$ and $0.31 \pm 0.04 \text{ min}^{-1}$, respectively.
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Fig. 5. Possible uracil recognition mechanism of Tba UDG. A. Interactions between
1761 amino acid residues and uracil of Tba UDG. The residues E42, N82, H164 and F55 in
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1763 *S. tokodaii* UDG that correspond to the residues E118, N159, H216 and Y127 in Tba
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UDG are depicted in blue, red, cyan and yellow sticks, respectively. The figure was adapted from the *S. tokodaii* UDG structure (PDB: 4zby) by Pymol [32]. Tba UDG residues are indicated in parentheses. The uracil is shown with dots. B. Purification of the wild-type, E118A and N159A Tba UDG mutant proteins. M: Protein marker.

Fig. 6. DNA cleavage assays of the wild-type and mutant Tba UDGs. DNA cleavage reactions of Tba UDG were performed using uracil-containing ssDNA and dsDNA as the substrates at 65°C for 10 min, respectively. Reaction products were analyzed by electrophoresis through running a 15% denaturing PAGE. A. Cleaving uracil-containing ssDNA by the wild-type; B. Cleaving uracil-containing ssDNA by the E118A mutant; C. Cleaving uracil-containing ssDNA by the N159A mutant; D. Cleaving uracil-containing dsDNA by the wild type; E. Cleaving uracil-containing dsDNA by the E118A mutant; F. Cleaving uracil-containing dsDNA by the N159A mutant. CK: the reaction without the enzyme.

Fig. 7. The binding assays of the wild-type and mutant Tba UDGs. The uracil-containing ssDNA and dsDNA (U:G) were employed as the substrates to examine the DNA-binding of the wild-type and mutant Tba UDGs. The wild-type and mutant Tba UDGs and DNA were incubated at 25°C for 10 min, and were run by electrophoresis on a 4% native polyacrylamide gel. A. Binding to uracil-containing ssDNA by the wild-type protein; B. Binding to uracil-containing ssDNA by the E118A mutant; C. Binding to uracil-containing ssDNA by the N159A mutant; D. Binding to uracil-containing dsDNA by the wild-type protein; E. Binding to uracil-containing dsDNA

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by the E118A mutant; F. Binding to uracil-containing dsDNA by the N159A mutant.

CK: the binding assay without the enzyme.

Table 1 Sequences of the oligonucleotides used to clone the Tba UDG gene and construct its mutants

Name	Sequence (5'-3')
Tba UDG F	GGAATTCC <i>ATATG</i> CTGCTGGAGTTTGAACGCC
Tba UDG R	CCGCTCGAGTTT <i>AGTAATATTTA</i> AGCTTTTCC
E118A F	AAAGGTTGTTTTGGTCGGGG <u>GCGG</u> CTCCAGGAAGGAAAGGCT
E118A R	AGCCTTTCCTTCCTGGAGCC <u>GCC</u> CCCGACCAAACAACCTTT
N159A F	TTTTGTGTATATCACAGCTGTTGTAAAATGCAATC
N159A R	<u>AGCT</u> GTGATATACACAAAATCGGGGTTAATTCCGA

The italic nucleotides represent restriction sites.

The substitution bases are underlined.

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Table 2 Sequences of the oligonucleotides used in this work

Number	Sequence (5'-3')
1	CGAACTGCCTGGAATCCTGACGAC <u>U</u> TGTAGCGAACGATCACCTCA
2	CGAACTGCCTGGAATCCTGACGAC <u>C</u> TGTAGCGAACGATCACCTCA
3	CGAACTGCCTGGAATCCTGACGAC <u>G</u> TGTAGCGAACGATCACCTCA
4	TGAGGTGATCGTTCGCTACAG <u>G</u> TCGTCAGGATTCCAGGCAGTTTCG
5	TGAGGTGATCGTTCGCTACAC <u>G</u> TCGTCAGGATTCCAGGCAGTTTCG
6	TGAGGTGATCGTTCGCTACA <u>A</u> GTCGTCAGGATTCCAGGCAGTTTCG
7	TGAGGTGATCGTTCGCTACA <u>I</u> GTCGTCAGGATTCCAGGCAGTTTCG

The underlined base is used to prepare normal and uracil-containing dsDNA.

Table 3 DNA substrates prepared with the oligonucleotides in Table 2

Strand	labeling	Combination	Base pair
ssDNA	Cy3	1*	U/-
ssDNA	Cy3	2*	C/-
dsDNA	Cy3	1*+4	U/G
dsDNA	Cy3	1*+5	U/C
dsDNA	Cy3	1*+6	U/A
dsDNA	Cy3	1*+7	U/T
dsDNA	Cy3	2*+4	C/G
dsDNA	Cy3	3*+6	G/T

The symbol “*” indicates the labeled strand.

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		<u>Motif A</u>	<u>Motif B</u>	<u>Motif C</u>		<u>Motif D</u>
Tba	101	GLPFANGVWGSKVVLVGEAP	G---RKGCYTG	ICFYRDASGMLLRKALFSL	-GINPD	FVYITNVVKCNPPENKLGVD--KREL
Pfu	29	PVP-GYGNDAKIMFVGEAP	GYWEDQKGLPFVG	-----KAGKVLDELDDGI	-GLTRE	DVYITNVVKCRPPNRRDPTTEEIKACS
Pho	23	PVP-GDGSYDTRKIMFVGEAP	GYWEDQMGLPFVG	-----KAGKVLDELKLI	-GLKRS	EVYITNIVKCRPPNRRDPTTEEIKACA
Afu	26	YVP-GVGNKAEIVFVGEAP	GRDEDLKGEPFVG	-----AAGKLLTEMLASI	-GLRRE	DVYITNVVKCRPPNRRDPTPEEVEKCG
Pae	25	AVP-GEGEMKLGVMIVGEAP	GASEDEAGRPFVG	-----AAGQLLTEALSRL	-GVRRG	DVYITNVVKCRPPNRRTPNREEVEACL
Ape	28	AVP-GEGPGEAGVMVIVGEAP	GRMEDRLGRPFVG	-----PAGKLLDLSLELA	-GLSRG	EVYITNVVKCRPPGNRRDPREEIEACL
Sso	32	PVP-PNGQIDAEIVIVGLAPAGNGGNRTGRMFTGD	-----ESSNNLANALYAV	-GLSNQPFVSKDDGLKLF	NVYITSAVKAPPONK-PNKDEIINCS	
Sto	30	GYP-----KAEIMFVGEAP	GENEDKGRPFVG	-----AAGKLLTQMIKEIL	GLERD	QVYITNVVKCRPPNRRDPEEDEITACS
Tma	30	VVV-GEGNLDTRIVFVGEAP	GEEEDKTGRPFVG	-----RAGMLLTELRES	-GIRRE	DVYICNVVKCRPPNRRDPTPEEQACG

Motif E

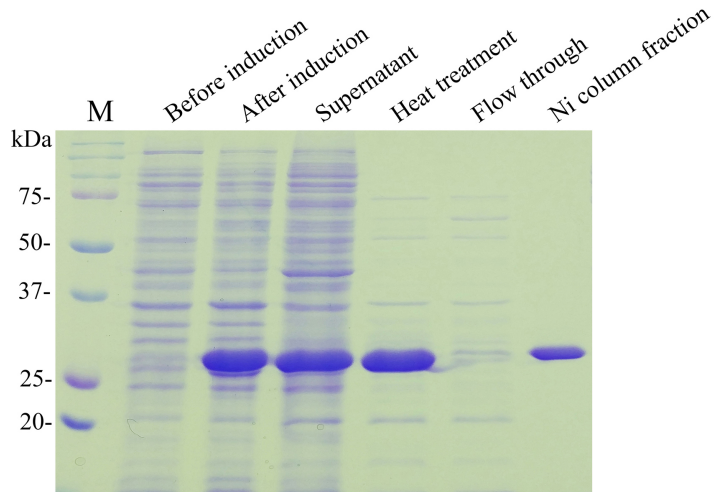
Motif F

Tba	179	SLLKKELEIFR	-PKAIFALGR	----TAEKALKRVGFDA	-----VYLR*	HPAWYVRRGLREPNDMLEEY
Pfu	106	PYLDQQIDIIK	-PKVIVTLGR	---HSTNYILKKFGFDLEPISKIH	GKVFKA	KTLFGLTY-IFPTYHPAVALYRP--QLKEELKQDF
Pho	100	PYLDQQIDIIK	-PKVIVTLGR	---FSTAYIMKKYGFNVEPISKIH	GRVFEARTLFGKIY-IVPMYHPAVALYRP--QLRRELEEDF	
Afu	103	NYLVRQLEAIR	-PNVIVCLGR	---FAAQFIENLFDLEFTTISR	VKGKVEVERWGGKVK-VIAIYHPAAVLYRP--QLREEYESDF	
Pae	102	PYLTIQQIGILK	-PRRIIALGL	---ISAKALMELMGRRAEKLGDVVK	GKCYQGR	IAGVQVE-LCITYHPAAVLRKP--ALRGEFQKDL
Ape	105	PYLVEQISLIR	-PRLVIAVGR	---HAGRTLFRLAGLRWPLARARG	GRVWRGRIGGVELL-IAVTYHPAAALYNP--GLRGELEDF	
Sso	123	VFLVEEVRIKNTKVI	IALGKI	AWDSLIIYVFKKIGYNVNPVRFYH	GALVKVVKPDM	SIWLVGSYHPSPRNMKTG-RLTINMLIEI
Sto	104	PYLDQQIDIIIM	-PKIIVTLGR	---HSTKYIFSKMGENFSSITKVR	GKSYVWKYKEKE-IIVFPTYHPAAALYNPN--LRKILEEDF	
Tma	107	HFLLAQIEIIN	-PDVIVALGA	---TALSFFV---DGKKV	SITKVRGNPID--WLGKKV-I-PTFHPSYLLRNRSNELRRIVLEDI	

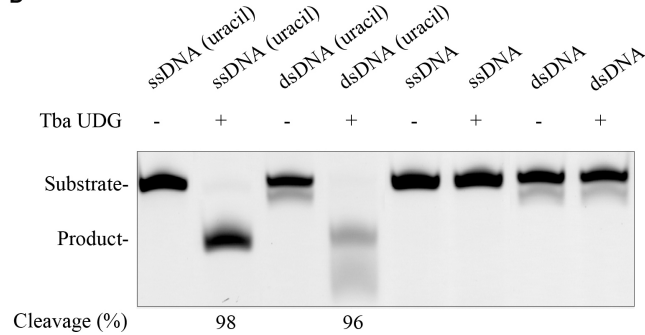
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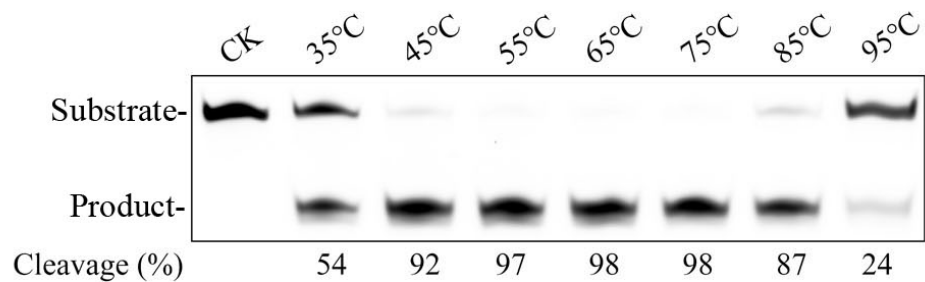
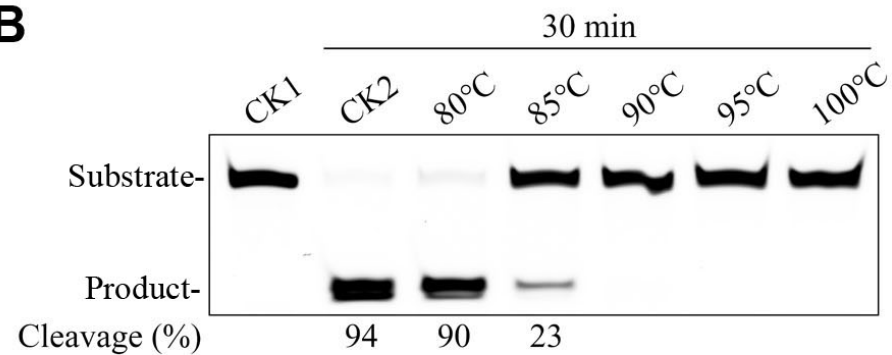
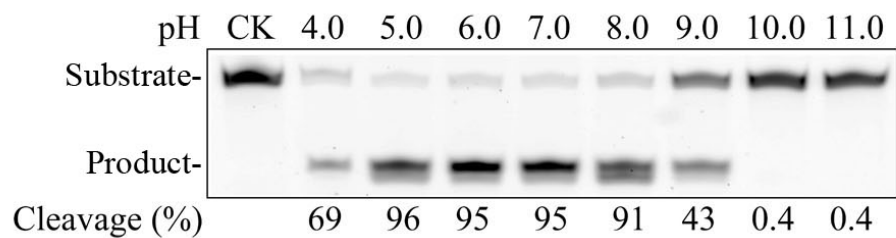
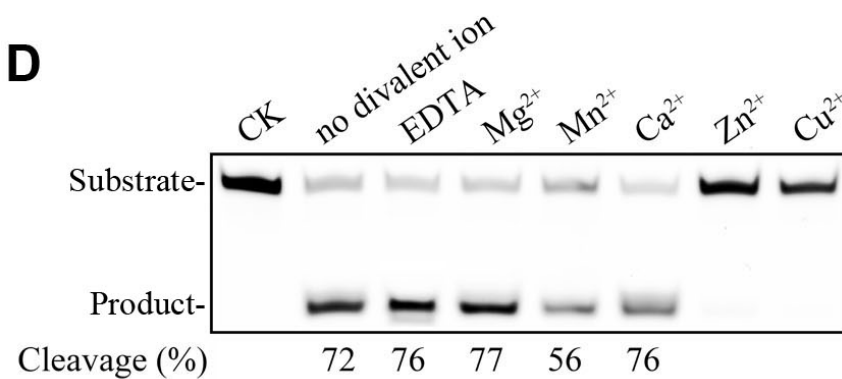
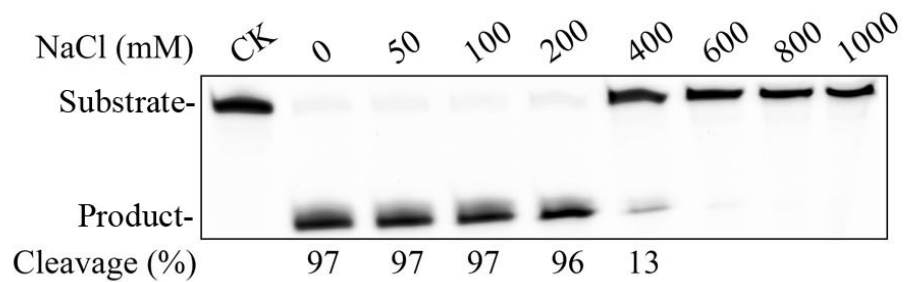
		<u>Motif B</u>	<u>Motif F</u>
Family 4	Tba UDG	113 V V L V G E A P G	216 H P A W Y V R
Family 1	Eco UDG	58 V V I L G Q D P Y	187 H P S P L S A
Family 2	Human TDG	134 I V I I G I N P G	269 M P S S S A R
Family 3	Human SMUG1	79 V L F L G M N P G	239 H P S P R N P
Family 5	Pae UDGb	62 V M V V G L A P G	196 H P S P L N V
Family 6	Mba HDG	42 W R L L G S I I G	136 S S S G A N R

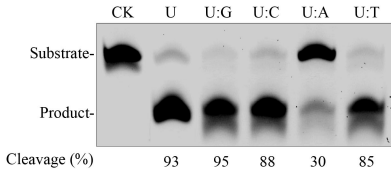
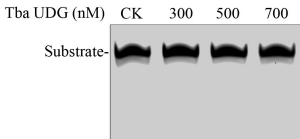
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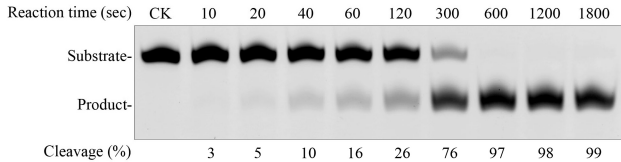
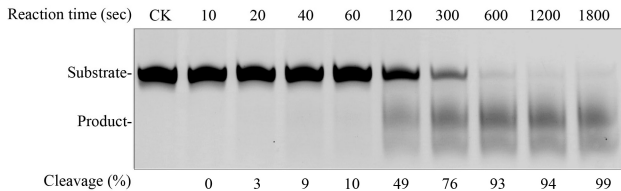
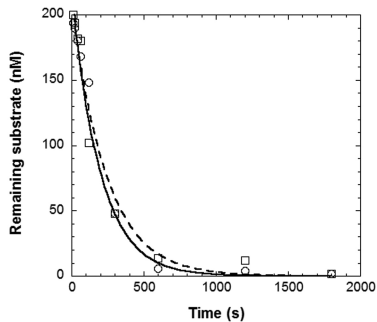


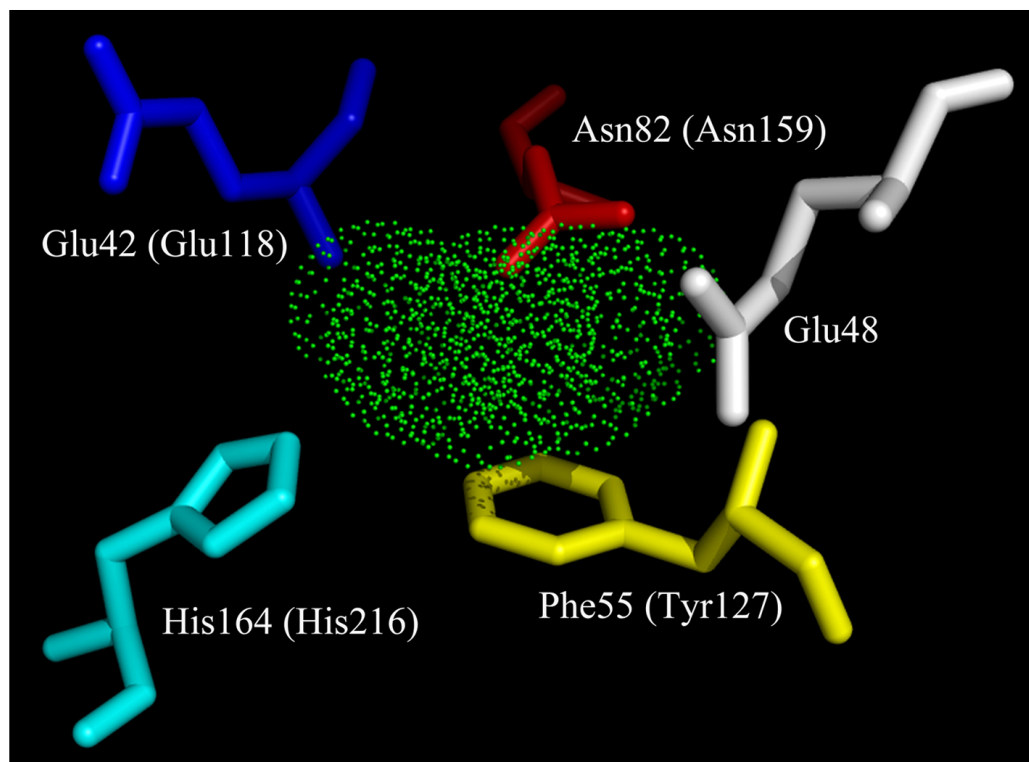
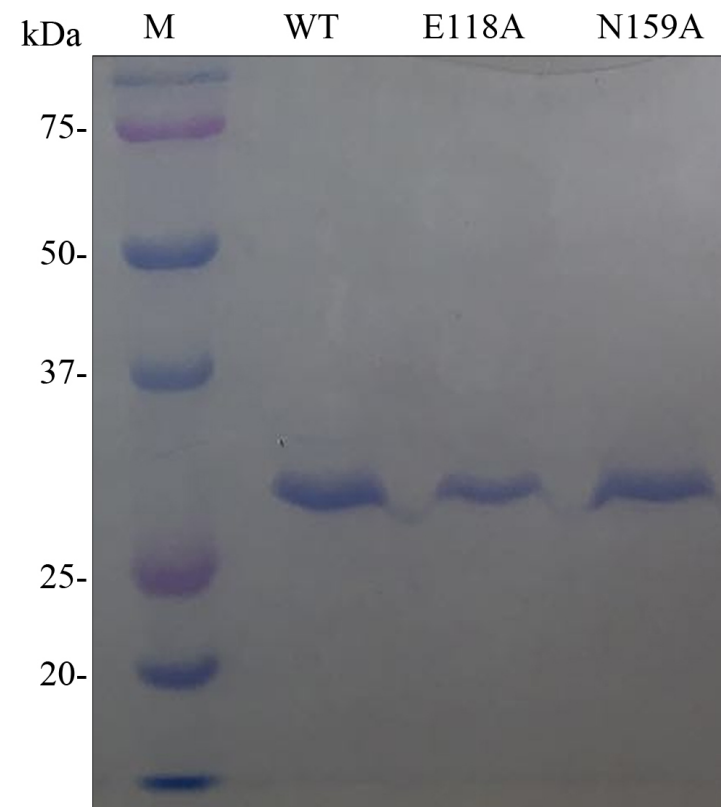
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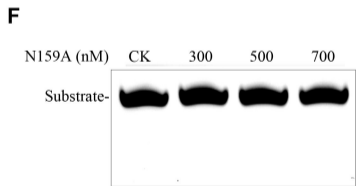
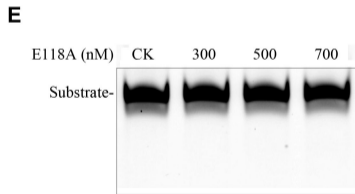
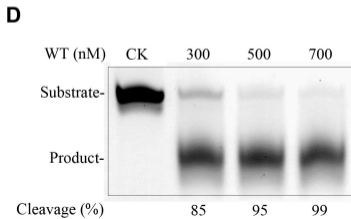
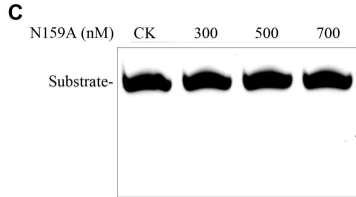
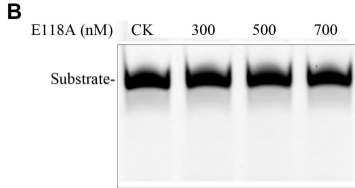
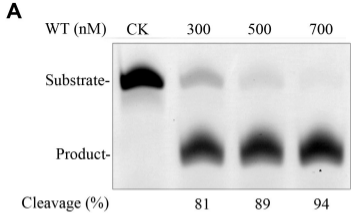


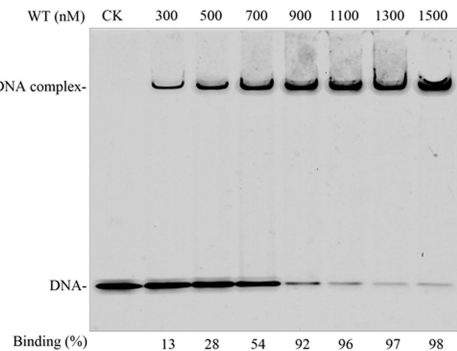
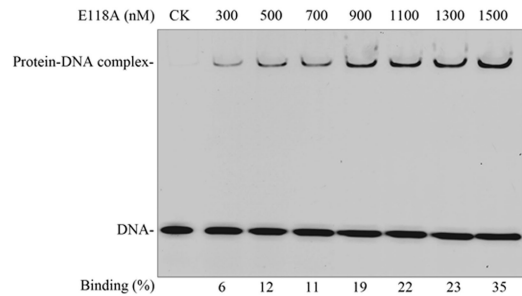
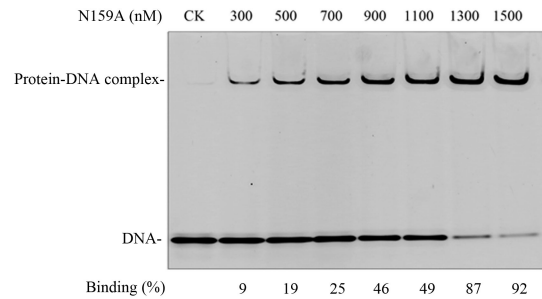
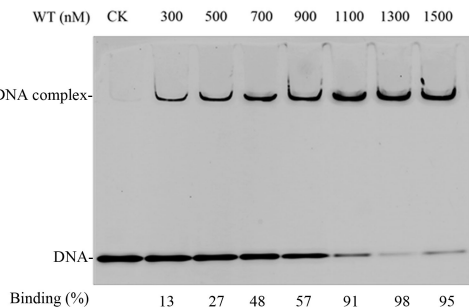
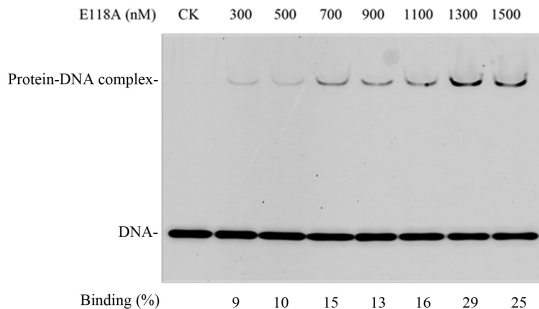
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