A role for *Saccharomyces cerevisiae* Tpa1 protein in direct alkylation repair*

Gururaj Shivange†, Naveena Kodipelli†, Mohan Monisha‡, and Roy Anindya§

From the †Department of Biotechnology, Indian Institute of Technology Hyderabad, Ordnance Factory Estate, Yeddumailaram-502205, Hyderabad, India

‡Both authors made equal contributions to the manuscript.

§Running title: *Repair of DNA alkylation damage*

**ABSTRACT**

Alkylating agents induce cytotoxic DNA base-adducts. In this report we provide evidence to suggest, for the first time, that *S. cerevisiae* Tpa1 protein is involved in DNA alkylation repair. Little is known about Tpa1 as a repair protein beyond the initial observation from a high throughput analysis indicating that deletion of *TPA1* causes methylmethanesulfonate (MMS) sensitivity in *Saccharomyces cerevisiae*. Using purified Tpa1 we demonstrate that Tpa1 repairs both single and double-stranded methylated DNA. Tpa1 is a member of Fe(II) and 2-oxoglutarate-dependent dioxygenase family and we show that mutation of the amino acid residues involved in cofactor binding abolishes Tpa1 DNA repair activity. Deletion of *TPA1* along with base excision repair (BER) pathway DNA glycosylase MAG1 renders the *tpa1Δmag1Δ* double mutant highly susceptible to methylation-induced toxicity. We further demonstrate that trans-lesion synthesis DNA polymerases Polζ (REV3) plays key role in tolerating DNA methyl-base lesions and *tpa1Δmag1rev3Δ* triple mutant is extremely susceptible to methylation-induced toxicity. Our results indicate synergism between the BER pathway and direct alkylation repair by Tpa1 in *Saccharomyces cerevisiae*. We conclude that Tpa1 is hitherto unidentified DNA repair protein in yeast and plays a crucial role in reverting alkylated DNA base lesions and cytotoxicity.

The genomes of every cell are always exposed to DNA damaging alkylating agents that occur in the environment and also generated endogenously as byproduct of cellular oxidative metabolism. Some alkylating agents are also used in cancer chemotherapy (1). Simple methylating agents, such MMS, methylates double-stranded DNA and generate 7-methylguanine (7meG) and 3-methyladenine (3meA) (2). DNA synthesis is blocked by 3meA and it is considered a lethal lesion. Methylation of single-stranded DNA by MMS generates 1-methyladenine (1meA) and 3-methylcytosine (3meC) (3). In double-stranded DNA, these sites are protected by base pairing, but they can be transiently exposed during replication, transcription or recombination. Therefore, the major genotoxicity of MMS is caused by three main lesions, namely, 3meA,
1meA and 3meC (3, 4). As replicative polymerases are stalled at these lesions, specialized damage-tolerant DNA polymerases, can able to execute trans-lesion synthesis (TLS) (5). In S. cerevisiae, TLS is carried out by the Polη (Rad30), Polζ (Rev3, Rev7), and Rev1 polymerases, all of which have human homologs (6). Yeast Polη is dedicated to the repair of UV-induced cyclobutane-pyrimidine dimers in an error-free manner (7). In contrast, Polζ, in cooperation with Rev1, participates in error-prone TLS across lesions produced by variety of DNA-damaging agents, including MMS (8). In yeast, DNA glycosylase Mag1 specifically removes 3meA. Following Mag1-mediated removal of damaged base, Apn1 apurinic/apyrimidinic endonuclease cleaves the DNA strand at the abasic site for subsequent repair of the single strand break by base excision repair (BER) pathway. The enzyme that directly repairs 1meA and 3meC is alkylated repair protein-B (AlkB) in E. coli (9, 10). AlkB deficient E. coli cells accumulate alkylated lesions and are hypersensitive to alkylating agents (11). AlkB catalyzed demethylation reaction is coupled to the oxidative decarboxylation of 2OG to succinate and CO2 resulting removal of methyl group from 1meA and 3meC. The methyl group is hydroxylated and spontaneously released as formaldehyde (12, 13). AlkB is a member of the large Fe(II) and 2OG-dependent dioxygenases family and show similar conserved features like conserved HxDxH that coordinate the 2OG and iron (Fe(II)) and catalytic core consisting of double strand β-helix (DSBH) fold (14, 15).

Homologs of AlkB were identified across species ranging from bacteria to human (16, 17) except Saccharomyces cerevisiae (18). It was reported earlier that two genes of S. cerevisiae namely YFW1 and YFW12 could complement deficiency of AlkB in E.coli (19). However, YFW1 is an endoplasmic reticulum membrane protein and YFW12 is secreted-sterol binding protein, and they share no sequence homology with AlkB or any other Fe(II) and 2OG-dependent dioxygenases (20, 21) and, therefore, could not be considered AlkB homolog. No genetic interactions were reported although functional homolog of AlkB remained unknown in S. cerevisiae, search for the dioxygenase domain containing proteins in budding yeast revealed that an uncharacterized ORF named YER049W had the characteristic dioxygenase domain (22). Later the gene product of YER049W was renamed as ‘termination and polyadenylation protein’ (Tpa1) as it was found to be associated with eRF1, eRF3 as well as polyA binding protein (PABP) within the mRNA ribonucleoprotein complex (23). TPA1 deletion in yeast resulted decrease of translation termination efficacy and an increase in mRNAs stability (24). Structural analysis of Tpa1 revealed the presence of two domains; the N-terminal domain (NTD) and the C-terminal domain (CTD) (24, 25). Although the conserved DSBH fold was found in both domains, only NTD was found to have bound iron (23). A recent study demonstrated that Tpa1 probably function as prolylhydroxylase responsible for hydroxylation 40S ribosomal subunit protein (26). However, none of these studies provided any direct evidence for prolylhydroxylase enzymatic activity using purified Tpa1 (24–26).

The study reported here was initiated in response to the findings that Tpa1 is the only S. cerevisiae protein that belongs to Fe(II) and 2OG-dependent dioxygenase superfamily of proteins which also includes AlkB (22). Further, genetic screen in yeast deletion mutants revealed that TPA1 deletion caused mild MMS sensitivity (27), making it even more pressing to know the importance, if any, of this protein in repair of DNA alkylation damage. Herein we provide evidence that purified recombinant Tpa1 catalyzes the oxidative demethylation of methylated DNA and promote survival of MMS sensitive E. coli alkB mutant cells. Furthermore, we demonstrate genetic interaction between Tpa1, DNA glycosylase Mag1 and TLS polymerases Polζ (Rev3) in S. cerevisiae. We also show that Mag1 appears to have a synergistic relationship with Tpa1, because tpa1Δmag1Δ double mutant showed exacerbated phenotype. Most notably, we have uncovered a remarkable synergism among Mag1, Tpa1, and TLS polymerases Polζ (Rev3) in protecting against methylation damage, as indicated by the inability of the tpa1Δmag1Δrev3Δ triple mutant cells to recover from extremely low level of MMS-induced methylation damage.
EXPERIMENTAL PROCEDURES:

**Plasmid constructs-** For expression of recombinant proteins, all the plasmids were constructed in the expression vector pGex6p1 vector (GE Healthcare), in-frame with an N-terminal GST (Glutathione transferase). TPA1 gene was PCR amplified from an S. cerevisiae genomic DNA using the appropriate primers. Similarly, E. coli AlkB gene was PCR amplified from E. coli genomic DNA. Tpa1 N-terminal domain (NTD), which lacks amino acid (aa) 269-644, and C-terminal domain (CTD), which lacks aa 1-276, was also PCR amplified using specific primers. The PCR products of Tpa1, NTD, CTD and AlkB were cloned into the BamHI and SalI sites of pGex6p1 vector to yield pGex-Tpa1, pGex-Tpa1NTD, pGex-Tpa1CTD and pGex-AlkB, respectively.

In order to generate mutant Tpa1, PYMOL (http://www.pymol.org) was used to make the substitution mutations in silico using PyMol Mutagenesis Wizard. Molecular docking analysis was performed to confirm whether co-factor binding is indeed abolished using published structures of Tpa1 (24, 25). Initially, in order to assess the reliability of the docking method, 2OG was removed from the holoenzyme atomic structure (PDB ID: 3KT7) and then coordinates of 2OG were docked back into the rigid binding site. Based on the Tpa1 structure and molecular docking analysis we determined the amino acid residues involved in coordinating the iron in the active site. Accordingly we introduced site-specific mutations into the recombinant Tpa1 active site using PROVEAN algorithm (28). H159C, D161N, H227C, H237C and R238A were introduced to generate pGex-Tpa1mut. FoldX algorithm was used to make sure that the mutations did not affect the overall stability of protein (29).

**Functional complementation of alkB mutant E. coli-** Functional complementation of E. coli HK82 (alkB) cells transformed with either pGex-Tpa1, pGex-Tpa1NTD, pGex-Tpa1CTD, or pGex-AlkB were grown in LB medium at 37°C. Cultures were treated with 0.05%, 0.1%, and 0.15% (v/v) MMS for 2h. Cultures were treated with 0.05%, 0.1%, and 0.15% (v/v) MMS for 2h, diluted, and spread on LB agar plates. Colonies were counted 20h later, and survival was expressed as a percentage of colonies where no MMS was present.

**Purification of recombinant proteins-** Plasmids were transformed into the E. coli strain BL21-CodonPlus(DE3)-RIL (Stratagene), and protein expression was induced by the addition of 1mM isopropyl β-D-thiogalactopyranoside (IPTG). Cells were disrupted by sonication and proteins were purified using affinity purification using glutathione-Sepharose 4B medium (GE Healthcare) (32). Proteins were analyzed by 12% SDS-PAGE and subsequently by Coomassie Brilliant Blue staining and concentrations were determined by Bradford assays (Bio-Rad).

**UV/visible Spectroscopy-** UV/visible spectra of Tpa1, Tpa1 mutant, NTD and CTD were determined as described before (33). Briefly, recombinant proteins were purified as described before (32, 34) and concentrated to 0.04 μM. Spectra were recorded in the presence of buffer containing 25 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.5 mM 2OG, 4.0 mM Sodium Ascorbate, 880 μM FeSO4, by using a HITACHI UV-3900 model spectrophotometer.

**Preparation of methylated DNA-** Desalted oligonucleotides were purchased from Imperial Lifescience. Single stranded DNA was purchased from Sigma (D8899). Methylation adducts were generated by treating the oligonucleotide or single stranded DNA with MMS (Sigma, 129925).
as described before (35). Briefly, 40 µg ssDNA oligonucleotides were incubated with 5% (v/v) (0.59 M) MMS, 200 mM K$_2$HPO$_4$ in 500 µl of total reaction for 170 min at room temperature. Excess MMS was removed by dialysis against TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) using Spectra/Por dialysis membrane (MWCO: 3,500). Then the damaged substrate was precipitated by adding 0.3 M sodium acetate pH 5.5 and 2 volume of ice-cold ethanol. The precipitated ssDNA was washed with 70% ethanol and finally dissolved in water.

**Analysis of Tpa1 demethylation activity by direct detection of formaldehyde**- Formaldehyde reacts with ammonia and acetoacetanilide to form a fluorescent dihydropyridine product (36). The resulting fluorescent product could be analyzed using excitation wavelength of 365 nm and emission wavelength of 465 nm.

Direct detection of formaldehyde was carried out as follows. To a 50 µl sample containing formaldehyde, 40 µl Ammonium acetate (2M) and 10 µl of Acetoacetanilide (0.5 M) was added (0.05 M) to make the final volume 100 µl. Fluorescent compound was allowed to develop at 30°C for 30 minutes and fluorescence emission was measured at 465 nm using an excitation wavelength of 365 nm and a Spectramax multimode reader. For the quantification of formaldehyde release, standard curve was generated using pure formaldehyde.

DNA demethylase assays (50 µl) were performed in 96-well dark plates (Corning) in the presence of demethylase buffer (20 mM HEPES, pH 8.0, 200 μM 2OG, 2 mM L-Ascorbate, 20 μM Fe(NH$_4$)$_2$(SO$_4$)$_2$, 100 μg/ml bovine serum albumin), MMS-treated methylated DNA (100 ng/µl) and purified proteins, viz. Tpa1, Tpa-mutant, or AlkB (2 µM) at 30°C for 60 min.

**Analysis of Tpa1 demethylation activity by FDH-coupled assay**- Formaldehyde production was continuously monitored by a Formaldehyde dehydrogenase-coupled DNA repair assay. This assay was performed by incubating purified recombinant proteins, viz. Tpa1, mutant-TPA1, Tpa1-NTD, Tpa1-CTD (2 µM) with the reaction buffer (20 mM HEPES, pH 8.0, 200 μM 2OG, 2 mM L-Ascorbate, 20µM Fe(NH$_4$)$_2$(SO$_4$)$_2$, 100 µg/ml BSA) in the presence of damaged single-stranded DNA (10 ng/µl) in a total volume of 100µl. Reaction mixture also contained 0.01 U FDH and 1 mM NAD$^+$ and monitored continuously for the production of NADH (peak absorption at 340 nm) using a Spectramax multimode reader. Typically, three reactions were monitored concurrently for 30 min, each set containing one control (no DNA). The data was analyzed using the software (GraphPad Prism).

**Analysis of Tpa1 demethylation activity by methylation-sensitive restriction endonuclease-based assay**- For this assay the sequence of the oligonucleotide selected for methylation was 5'-GGA TGC CTT CGA CAC CTA GCT TTG TTA GGT CTG GATC-3' (MboI site is underlined). This 70 base oligonucleotide was designed such that upon annealing with the complementary oligonucleotide an MboI restriction site is formed at the middle.

Restriction digestion of this 70 bp oligonucleotide with MboI would result two 35 bp fragments. However, upon repair of this methylated substrate it becomes susceptible to MboI digestion. In other words, MboI digestion of methylated substrate would indicate successful DNA repair. DNA repair was carried out by incubating recombinant Tpa1 (0.8 µM) or Tpa1 mutant (0.8 µM) with 3.7µg of MMS-damaged single-stranded oligonucleotide for 2h at 30°C in the presence of repair buffer (20 mM HEPES, pH 8.0, 200 µM 2-oxoglutarate, 2 mM L-ascorbic acid, 20 µM Fe(NH$_4$)$_2$(SO$_4$)$_2$ and 100 µg/ml BSA) in a total reaction volume of 25 µl. Following the repair reaction, the complementary strand was allowed to anneal with the repaired strand for 30 min at 30°C. The resulting double-stranded DNA was cleaved with 2.5 U of methylation sensitive restriction enzyme MboI (New England Biolabs, R0147) at 37°C for 1 h. Tpa1 mediated removal of methyl adducts were determined by appearance of 35bp band due to cleavage of 70 bp oligonucleotide by MboI by agarose gel electrophoresis as described before (35).

**Yeasts Strains and procedures**- All the Saccharomyces cerevisiae strains used were isogenic with strain W303 (MATa; ura3-1 ade2-1
his3-11,5 trpl-1 leu2-3,112 can1-100) (37). The cells were grown and manipulated using standard techniques (38). The TPA1 gene was replaced with the HIS3 gene by a one-step replacement. The HIS3 gene was amplified from plasmid pRS303 (39) by using the specific primers, so that the PCR product contained the entire HIS3 gene with flanking homology to TPA1. The MAG1 and REV3 genes were similarly replaced by LEU2 and TRP1 gene obtained from pRS305 and pRS304, respectively. Strains were grown at 30°C in yeast extract-peptone-dextrose (YPD) or synthetic media. All primer sequences are available on request. Detailed list of the genotypes of the strains used in this study are available on request. Yeast strains were grown to early log phase (10^7 cells/ml) in appropriate medium. For transformations with plasmids or PCR products, we used the standard Lithium acetate-polyethylene glycol method.

Analysis of MMS sensitivity- Sensitivity studies for S.2 alkylating agents, was assayed with MMS. For MMS sensitivity experiment by spot test, cells were grown in liquid YPD, washed and suspended in water at a density of 1.5x10^7 cells/ml. Aliquots (7 µl) of the cell suspensions of different ten-fold dilution were spotted onto solid YPD media containing various concentrations of MMS and grown at 30°C for 3-5 days. For survival analysis and quantification, cells were spread onto YPD plates with 0.5-1.5 µg/ml MMS and incubated at 30°C and colonies were counted after 3-5 days.

Analysis of sensitivity to protein synthesis inhibitors- To study the effect of translational inhibitors, YPD plates supplemented with anisomycin (Sigma-A9789), cycloheximide (Sigma-180179), genetin (Himedia-TC026), Paromomycin (Sigma-P5057) were used. 6-Aza-uracil (Sigma-A1757)-100 µg/ml is used to assay the effect of transcriptional inhibitor. Anisomycin was dissolved in DMSO. Gentamicin and Paromomycin stock solutions and YPD plates were prepared in 0.1 M KPO4 buffer (pH 7.5). Ten-fold dilution spot assays to monitor sensitivity to anisomycin (1.0 µg/ml), paromomycin (0.5 mg/ml), genetin (80 µg/ml), 6-aza-uracil (100 µg/ml) and cycloheximide (0.03 µg/ml) were carried out as described previously (40).

RESULTS

Tpa1 functionally complements E.coli AlkB-Bacterial cells lacking AlkB are hypersensitive to exposure to S.2 alkylating agents such as MMS (41). Further, exponentially proliferating AlkB deficient cells were reported to be more sensitive to MMS than wild-type cells growing at a similar rate (42). We hypothesized that if S.cerevisiae Tpa1 were functionally similar to AlkB, then expression of Tpa1 would be able to suppress the MMS sensitive phenotype of E. coli alkB deficient strain (HK82). In order to express Tpa1 construct in E. coli HK82 strains, we cloned Tpa1 gene in pGex vector (GE Healthcare) directly downstream to lac promoter and operator region so that the recombinant protein can be expressed without the requirement of T7 polymerase. Before complementation experiments were carried out, comparable expression levels in HK82 strain from the different constructs were confirmed. We expressed S. cerevisiae Tpa1 and E. coli AlkB in HK82 cells and treated with 0.01% (v/v) MMS for 2h. Surprisingly, only the cells expressing Tpa1 and AlkB complemented the MMS sensitive phenotype (Fig. 1A). Interestingly, survival of Tpa1 expressing cells was similar to the cells expressing AlkB. However, cells expressing empty vector, as might be expected, completely lacked any complementation effect. To verify specificity of Tpa1-mediated complementation, two more E.coli dioxygenases, namely, mhpB gene, encoding catechol dioxygenase (43) and tauD, encoding taurine hydroxylase (44), were cloned into the same vector and expressed in HK82 cells. As expected, these dioxygenases could not rescue MMS sensitivity of alkB mutant E.coli (Fig 1A). Tpa1 structure reveals presence two domains, the N-terminal domain (NTD, amino acid 1-268) and C-terminal domain (CTD, amino acid 277-644). Although both of these domains are structurally similar, only NTD has conserved HxDxnH motif and binds iron and 2OG as in other Fe(II)/2OG-dependent dioxygenases (25). It would, therefore, be expected that only NTD might retain the catalytic function and complement AlkB function. When we examined the ability of NTD and CTD in suppressing MMS sensitive phenotype of E. coli alkB strain HK82, we observed that NTD successfully complemented...
AlkB function but CTD could not, suggesting that enzymatic activity of Tpa1 may be essential for complementing AlkB. We also analyzed complementation of E. coli alkB by Tpa1 at different concentrations of MMS (Fig 1C). HK82 cells expressing different Tpa1 constructs were exposed to 0.05%, 0.1%, and 0.15% (v/v) MMS for 2h and cell survival was determined. The MMS sensitivity of alkB cells was significantly reduced when transformed with Tpa1 or NTD, whereas cells expressing CTD had only a small difference in sensitivity compared to the control vector (Fig. 1C). These observations strongly indicate that only the first 298 amino acids are necessary and sufficient for full complementation of the alkB mutant of E. coli. This indicates that the DNA repair activity is contained within this domain. The CTD may participate in unrelated functions, or interactions with other proteins, but is not necessary for the DNA repair activity itself. The protein was claimed to function as a poly-A binding protein and the CTD may contribute to such other unrelated activities, interactions with other repair proteins, or both (24, 25).

**Design of Tpa1 mutation that abolishes cofactor binding** - The Tpa1 residues involved in binding to iron are H159, D161, H227 in the ternary structure of the Tpa1–Fe(III)–2OG complex (24, 25). Iron also coordinates with 2OG which in turn interacts with and I171, Y173, V229, R238 and S240 (25). In order to generate a Tpa1 mutant, molecular docking analysis was performed to confirm whether co-factor binding is indeed abolished using published structures of Tpa1 (24,25) (Fig. 2A). Based on the Tpa1 structure and molecular docking analysis we first introduced site-specific mutations of conserved residues H159, D161, H227 to Alanine and analyzed cofactor binding of this triple mutant by molecular docking. However, the mutant had little effect on iron and 2OG binding. Consequently, we decided to use PROVEAN algorithm to predict amino acid substitution that would result significant functional difference (28). According to our molecular docking analysis Tpa1 with H159C, D161N, H227C, H237C and R238A mutations combined together resulted altered 2OG binding and concomitantly diminished iron binding (Fig. 2B). Next, we wanted to experimentally confirm the whether the Tpa1 mutation indeed resulted loss iron binding. We expressed recombinant Tpa1, Tpa1 mutant, NTD and CTD in E. coli and the purified by affinity chromatography to ~90% purity as visualized via SDS-PAGE and Coomassie brilliant blue staining (Fig. 2C). Members of the Fe(II)/2OG-dependent dioxygenases forms chromophore associated with metal-to-ligand charge transfer transition. When we monitored purified Tpa1 by UV-visible spectroscopy under optimum condition mentioned before (45), it generated the characteristic absorption peak at 530 nm when both metal and cofactor were present (Fig 2D). This is in good agreement with the characteristic absorption peak of chromophore reported by others (33, 45, 46). However, Tpa1-mutant, which lacks active site coordination of iron and 2OG, did not produced any spectrum associated with chromophore formation (Fig. 2D). This result is consistent with our observations from the molecular docking studies. Absorption peak at 530 nm was also observed when NTD was analyzed (Fig. 2E). Interestingly, CTD absorption spectra in presence of metal ion and cofactor revealed no characteristic absorption peak at 530 nm (Fig. 2E). On the basis of these observations and previous structural studies, it is clear that Tpa1-mutant and CTD lacked 2OG-bound iron in the catalytic site.

**Tpa1 catalyses methyl-base lesion repair** - Since nothing is known about the DNA repair activity of Tpa1 protein, we used a simple *in vitro* repair assay using a synthetic 70 base oligonucleotide containing methylation sensitive restriction site MboI in the middle (35). Methylation sensitive restriction enzyme MboI does not cleave methylated DNA substrate. MMS-damaged oligonucleotide was directly incubated with Tpa1 for repair to take place, annealed with the complementary DNA to generate the double stranded DNA and analyzed on 3% agarose gel using 10mM Sodium Borate as electrophoresis buffer and stained with ethidium bromide (Fig. 3A). We observed that Tpa1 could repair methylated DNA, as evident from the appearance of 35 bp band (Fig. 3B, lanes 2 and 5). This result is consistent with our observation that Tpa1 forms characteristic chromophore in presence of metal ion and cofactor (Fig. 2E). Previously we also observed that Tpa1 mutant failed to form any
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complex with iron and 2OG (Fig. 2E). To investigate whether direct repair MMS-damaged DNA is abrogated in the mutant lacking cofactor binding, we analyzed Tpa1 mutant treated sample with MboI digestion. Interestingly, no MboI cleavable band was formed in the presence of Tpa1-mutant with either single or double-stranded DNA (Fig. 3B, lanes 3 and 6), suggesting that there was no DNA repair activity present in the Tpa1 mutant. These results establish that Tpa1 is catalytically active and depend on Fe(II) and 2OG binding for its activity.

To confirm specificity of this reaction, we analyzed effect of increasing concentration of Tpa1 (0.6-2.4 µM). As shown in Fig. 3C, lanes 2-5, we observed a gradual increase of 35bp band, both in the case of double and single-stranded DNA. These data, in agreement with earlier observations and showed that products with less number of methylated base-lesion can be obtained using high enzyme concentrations. Longer incubation with Tpa1 also resulted increased amount of product DNA free of methylated base-lesion as detected by MboI cleavage both in the case of double and single-stranded DNA (Fig. 3D, lanes 2-6). However, our assay detects repair by restoration of MboI restriction site and depends on the specificity of the restriction enzyme unlike the conventional HPLC-based assay that directly measures demethylation by retention of methylated nucleotides in DNA. So this approach could not confirm which lesions are recognized and repaired by Tpa1 protein as the nature of the MboI digestion inhibiting lesions was unknown. Nonetheless, this simple assay is sufficient to demonstrate that Tpa1 is catalytically active and able to carry out repair of methylated nucleotide that can restore DNA into a form that can be cut by MboI.

**Tpa1 repairs MMS-damaged DNA by oxidative demethylation** - The hallmark of AlkB-mediated oxidative demethylation is that the oxidized methyl group is removed as formaldehyde. Hydroxylation of the methyl group is followed by spontaneous release of the resulting hydroxymethyl moiety as formaldehyde (13, 47). Therefore, it would be expected that Tpa1 would catalyze conversion of methyl adducts which is spontaneously released as formaldehyde, regenerating the normal bases. To test whether formaldehyde is released during the Tpa1-mediated DNA repair reaction we used two different assay methods.

First, we used direct fluorescence-based formaldehyde detection using acetoacetanilide. Acetoacetanilide reacts with formaldehyde and forms a fluorescent dihydropyridine derivative with peak emission at 465 nm. We generated a standard plot using a series of dilutions (0.1-50 µM) of formaldehyde (Fig. 4A). Various concentrations of formaldehyde (50 µl) were added to ammonium acetate (40 µl) and acetoacetanilide (10 µl) in a 100 µl reaction. Based on emission peak at 465 nm (Fig. 4A), linear relationship was observed between concentration of formaldehyde and fluorescence of the reaction product (Fig. 4B). We found that detection limit of this assay is 0.5µM. After confirming that acetoacetanilide and formaldehyde reaction product could be detected by fluorescent spectrometry, we wanted to know whether demethylation repair activity of Tpa1 could also be detected by the presence of formaldehyde. To carry out in vitro DNA repair reaction, purified recombinant proteins were used. *E.coli* AlkB was used as positive control. Following the repair reaction, acetoacetanilide and ammonia were added directly to the reaction mix. We noticed distinct emission spectra with peak emission of 465 nm when *E.coli* AlkB or *S. cerevisiae* Tpa1 was present (Fig. 4C). Since we had already established that formaldehyde reacts with ammonia and acetoacetanilide to form fluorescent compound with peak emission of 465 nm, this result proved formation of formaldehyde as result of removal of methyl-adducts (Fig. 4C). Notably, 2 µM of AlkB and Tpa1 resulted similar amount of formaldehyde release as detected by formation of fluorescent dihydropyridine product, suggesting equal repair efficiency. Next, we decided to test whether Tpa1 mutation that affect iron and 2OG binding to the active site has reduced DNA repair activity. As expected, mutation of the residues involved in iron and 2OG binding resulted diminished DNA repair activity (Fig. 4C). However, the mutant Tpa1 still retained some marginal activity. We speculate that this could be due to low-level background activity contributed by the Tpa1 CTD that contains conserved DSBH fold found in all the
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AlkB homologs.

Prior studies have shown that Fe(II)/2OG-dependent oxygenases are sensitive to inhibition by divalent transition metal ions (48). To test whether Tpa1 is also inhibited similarly, we analyzed the effect of Nickel ion by incubating Tpa1 with 1 mM nickel ions. We found that nickel ion strongly inhibited demethylase activity of Tpa1 (Fig. 4D). Fe(II)/2OG-dependent oxygenases are also known competitively inhibited by compounds structurally similar to 2-oxoglutarate, e.g., succinate (49). As shown in Fig. 4D, 5 mM succinate strongly inhibited demethylase activity of Tpa1. These results clearly indicate that mechanism of inhibition of Tpa1 activity is very similar to other Fe(II)/2OG-dependent oxygenases.

To test if the repair associated formaldehyde release is affected in Tpa1 mutant, methylated DNA substrate was increased from 40-200 ng/µl, keeping the amount of Tpa1 (2 µM) or Tpa1-mutant constant. We observed a proportional increase in the formaldehyde production in Tpa1 mediated reaction (Fig. 4E). However, repair associated formaldehyde release was diminished in Tpa1 mutant. This result confirmed that mutation of the cofactor binding residues affected Tpa1 activity (Fig. 4E). Together, these data suggest that Tpa1 has DNA repair activity and specifically removes methyl-adducts as formaldehyde.

To further confirm that formaldehyde was indeed generated in Tpa1-mediated demethylation reaction, we used an alternate indirect assay method. Formaldehyde dehydrogenase (FDH) converts formaldehyde to formate using NAD⁺ as the electron acceptor whose reduction to NADH can be spectrophotometrically measured from absorbance at 340 nm (Fig. 5A). Such indirect assay is routinely used to monitor enzymatic reactions involving formaldehyde release, viz. JmjC histone demethylase catalyzed reaction (50). In FDH-coupled demethylation assay, we determined the DNA repair activity of Tpa1 by measuring the production of NADH. A standard curve that was first generated using FPLC purified 0.01 U FDH, 1 mM NAD⁺, and different amounts of formaldehyde. Within the range of 5-50 µM formaldehyde, a linear relationship was found between the production of NADH and the amount of formaldehyde used. Subsequently, the FDH-coupled demethylation assays were carried out within this linear range. The reactions were initiated by adding the MMS-damaged single stranded DNA as substrates. As the demethylation proceeded, measuring absorbance at 340 nm at different time points revealed production of formaldehyde. As shown in Fig. 5B, within the first 2 min of the reaction a robust increase of absorbance at 340 nm was observed, indicating that substantial amounts of formaldehyde were produced in the Tpa1-catalyzed demethylation reaction. The fact that formaldehyde was generated in the demethylation of damaged DNA reaction strongly suggests that the reaction had occurred as proposed in Fig. 4A.

As positive control we used purified recombinant E. coli AlkB and we detected formaldehyde release comparable to Tpa1, suggesting that Tpa1 and AlkB has similar ability to demethylate MMS-modified single-stranded DNA. Furthermore, only when MMS-damaged DNA was incubated with the Tpa1, but not GST, we detected a robust increase in the absorbance at 340 nm, indicating the production of formaldehyde is specific for Tpa1 and result of successful demethylation (Fig. 5B). Next, we wanted to confirm effect of Tpa1 mutation that affect iron and 2OG binding to the active site. As expected, mutant Tpa1 had diminished DNA repair activity (Fig. 5B). FDH-coupled Tpa1-mediated repair assay also proved that 1mM nickel ion and 5mM succinate strongly inhibited demethylase activity of Tpa1 (Fig. 5C). We turned next to using the FDH-coupled AlkB assay to investigate the specificity of Tpa1 reaction. By keeping the amount of Tpa1 (1 µM) constant, when methylated DNA was increased from 2-20 ng/µl, a proportional increase in the formaldehyde production was observed (Fig. 5D). Formaldehyde release was observed only when MMS-damaged DNA was used. In the presence of undamaged DNA, formaldehyde release was not detected. Together, all these data from the indirect FDH-coupled repair assay supported our observations from the direct repair assay.

Collectively, these results from direct and indirect formaldehyde detection assay strongly indicate that budding yeast Tpa1, like bacterial AlkB, could directly convert methyl adducts to
susceptibility to MMS-induced toxicity. Increased sensitivity to alkylation agent MMS was also reported to arise when deficiency of proteins involved in BER pathway is combined with the context of DNA repair pathways have evolved to provide cell survival.

**MMS sensitivity mag1Δtpa1Δ double mutant strain is not due to defective protein translation** - Increased sensitivity to alkylation agent MMS was also reported to arise when deficiency of proteins involved in BER pathway is combined with the context of DNA repair pathways have evolved to provide cell survival.

**Genetic interaction of tpa1 with DNA glycosylase mutant mag1** - To better understand the role of Tpa1 in vivo with the context of DNA damage response, we analyzed the sensitivity of the tpa1Δ mutant to the DNA alkylating agent MMS by spot test and survival assays. Survival or recovery assays unveils nature of genetic interactions by scoring whether double mutants show epistatic, additive or synergistic effects for survival or recovery. We observed that the tpa1Δ mutant was very little sensitive to chronic exposure to MMS at lower concentration and only modestly MMS-sensitive at higher concentration (Fig. 6A). Interestingly, tpa1Δ strain was indeed reported as mild MMS-sensitive in a genetic screen (27).

These results prompted us to examine if there is any redundancy in repair of methylated bases by 3meA specific DNA glycosylase Mag1. As shown in Fig. 6A, deletion of MAG1 results modest MMS-sensitivity at higher concentration of MMS and this is in consistence with earlier report (51). However, when tpa1Δ was combined with the deletion of the DNA glycosylase Mag1, the double mutant tpa1Δmag1Δ was significantly more sensitive to MMS than the individual single mutant (Fig. 6B). These results suggest that there is an overlap of function (synergism) between Mag1 initiated BER and direct repair mediated by Tpa1 for the repair of alkylation damage. In order to quantify MMS sensitivity of the single and double mutants, survival was monitored by scoring colony growth on media containing different concentration of MMS.

When exposed to methylation damage, cells lacking either MAG1 or TPA1 expression showed susceptibility to methylation-induced toxicity. However, susceptibility to MMS-induced methylation damage was increased significantly in double mutant cells (Fig. 6C). Loss of Tpa1 and Mag1 alone affected survival but to a lesser extent than mag1Δtpa1Δ double mutation, as no survivors are recovered on 1μg/ml MMS in the double mutants (Fig. 6C). In *S. cerevisiae*, Mag1 is known to remove 3meA. Our observation that mag1Δtpa1Δ double mutants are synergistically more susceptible to methylation damage hints at overlapping substrate specificity of Mag1 glycosylase and Tpa1 dioxygenase. Given the remarkable ability of 3meA to stall replication it would not be surprising if the two different repair pathways have evolved to provide cell survival.

**Role of Tpa1 in vivo** - Tpa1 for the repair of alkylation damage. In order to quantify MMS sensitivity we analyzed the sensitivity of the double mutants, survival was monitored by scoring whether double mutants show epistatic, additive or synergistic effects for survival or recovery. We observed that the tpa1Δ mutant was very little sensitive to chronic exposure to MMS at lower concentration and only modestly MMS-sensitive at higher concentration (Fig. 6A). Interestingly, tpa1Δ strain was indeed reported as mild MMS-sensitive in a genetic screen (27).

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mag1Δtpa1Δ double mutant were also not sensitive to translation inhibitor cycloheximide or transcription inhibitor 6AU (Fig. 7), suggesting that these mutants do not have any general protein synthesis defect. From these findings it is clear that the MMS sensitivity of mag1Δtpa1Δ double mutant could possibly due to incomplete DNA repair and not due to lack of translational accuracy.

Genetic interaction of tpa1 with error-prone DNA polymerase mutants- We observed that although mag1Δtpa1Δ double mutant was sensitive to MMS, it could tolerate lower doses of MMS (Fig. 6D). To investigate how single mutant displayed increased susceptibility to MMS than ζ (Rev3) is probably the TLS polymerase that Pol these results provide strong evidence to suggest that Polζ (Rev3) is probably the TLS polymerase that contributes to cell survival in the presence of MMS damaged DNA. Interestingly, both mag1Δrev3Δ and tpa1Δrev3Δ had functional Tpa1 and Mag1, respectively. Therefore, this genetic interaction apparently indicates that the damage bypass polymerase Polζ may possibly be functionally secondary and less preferred by cells than Mag1 and Tpa1 in dealing with MMS damaged DNA.

Further, we sought to determine whether removing the Polζ function in mag1Δtpa1Δ double mutant strain would completely abolish the entire cellular defense mechanism against DNA alkylation damage. As shown in Fig. 6D and 6E, survival of tpa1Δmag1Δrev3Δ triple mutant was dramatically reduced compared to tpa1Δmag1Δ double mutants and display sensitivity to lower concentration of MMS. These genetic interactions establish a connection between Tpa1 activity, alkyl base DNA glycosylase Mag1 and error-prone DNA polymerase Polζ, and confirm that, indeed, Tpa1 has an important contribution in overall cellular defense against DNA alkylation damage.

DISCUSSION
This work comprises the first report of existence of direct alkylation damage reversal pathway apart from Mgt1 in Saccharomyces cerevisiae and identification of Tpa1 as the functional homolog of E. coli AlkB. Since the discovery AlkB decades ago, the budding yeast homolog has remained elusive. To our knowledge, no DNA repair function has been attributed to the Tpa1 protein so far. We provide evidence not only to establish S. cerevisiae Tpa1 as a Fe(II)/2OG-dependent dioxygenase, but also unravel that Tpa1 synergistically act with BER pathway against the S,2-type alkylating agents. We also establish that the synergistic MMS sensitivity phenotype is not due to defective protein synthesis.

Rescue of the alkylation-sensitive phenotype of E. coli alkB mutant- Since DNA alkylation repair pathways are highly conserved between prokaryotes and eukaryotes, we speculated that AlkB homolog(s) might also exist in S. cerevisiae. The only protein with significant amino acid sequence homology and structural similarity to the Fe(II)/2OG-dependent oxidative demethylases including E. coli AlkB protein is Tpa1 (22). Tpa1 was considered to be a...
prolylhydroxylase (24, 26). To test this idea that Tpa1 could be the functional homolog of AlkB more rigorously, we sought to examine if expression of Tpa1 in alkB mutant E. coli strain could suppress the MMS sensitivity. Remarkably, full-length Tpa1 as well as NTD rescued MMS-hypersensitivity of alkB mutant (HK82) cells (Fig. 1). Only two human homologs of AlkB with DNA repair activity, hALKBH2 and hALKBH3, also showed similar suppression of MMS-hypersensitivity of alkB mutant (HK82) cells (14, 55). Importantly, CTD, which lacked conserved catalytic residues, failed to provide protection against MMS (Fig. 1 and 2), indicating that between the two domains, Tpa1 NTD may be functionally active. Taken together, these observations made a strong case for Tpa1 being functional homolog of E. coli AlkB.

*In vitro oxidative demethylation*- Fe(II)/2OG-dependent dioxygenases display spectral properties associated with metal-to-ligand charge transfer transition in presence of iron. Although Tpa1 was characterized before (24–26), this spectroscopic characteristic was not known in Tpa1. We added excess Fe(II) and 2OG to recombinant Tpa1 and observed UV-vis band with peak absorption at 530 nm for the native protein as described previously (33, 45, 46). This is the first evidence of with metal-to-ligand charge transfer transition in Tpa1, clearly indicating a catalytic function.

Among the human AlkB homolog, hALKBH3 and hALKBH2 conclusively showed DNA repair activity (56). Using highly purified recombinant protein, we demonstrated that Tpa1 could demethylate single and double-stranded DNA (Fig. 3). This enzymatic activity imply that Tpa1 may have role in maintenance of global genomic DNA as well as nuclear single-stranded DNA, found during cellular events like transcription. Human genome codes for at least nine AlkB homologs and it seems rather likely that these homologs functionally complement each other; Therefore it was tempting to speculate Tpa1 may possess some of the functions of these homologs. Using single stranded DNA as substrate, Tpa1 activity was found to be very similar to AlkB (Fig 5B). Iron and 2OG binding to the active site of Tpa1 was found to be crucial for activity, as mutant Tpa1 which lacked iron-coordinating residues failed to repair DNA (Figure 3, 4 and 5).

Finally, Fe(II)/2OG-dependent dioxygenases including AlkB were known to be strongly inhibited by divalent transition metal cations and competitively inhibited by succinate (48, 49); all these phenomena were also observed here with Tpa1 (Fig. 4 and 5). Overall, these *in vitro* assays unambiguously establish Tpa1 as a Fe(II)/2OG-dependent oxidative demethylase specific to restoring MMS-damaged DNA. Our analysis of the mutant Tpa1 revealed. Often DNA repair-related proteins are associated with chromatin. Indeed, previous studies have shown that Tpa1 is associated with yeast chromatin and co-purified with the histone acetyltransferase complex NuA3 (57, 58). Future studies of Tpa1 should focus on recruitment and activity of Tpa1 in the context of chromatin and how this might be regulated.

*Further insights into collaboration between DNA repair pathways obtained from genetic interactions of Tpa1*- The genetic interactions between alkylation-specific demethylase and glycosylase in *S. cerevisiae* was revealed for the first time in this study. This not only offered a qualitative assessment of repair dynamics but also allowed the analysis of pathway-dependent effects in eukaryotes. Although previously observed in *E. coli* (59), interdependence of alkylation specific demethylase and glycosylase in protecting the cells from MMS-induced damage that we unveil here was hitherto unknown in eukaryotes. Significant overlap of function between Mag1 and Tpa1 also cause lack of susceptibility to sub-lethal dose of MMS in *tpa1Δ* cells (Fig. 6A). This also explains earlier observation why *mag1Δ* cells are also not particularly susceptibility to sub-lethal dose of MMS (60, 61). It is possible that it is due to this reason Tpa1 was never identified before as an obvious candidate gene associated with DNA repair in genetic screens (27, 62). Our analysis also revealed that in the absence of Rev3 (Polζ), Tpa1 and Mag1 equally contribute to DNA alkylation repair. Surprisingly, Polζ could compensate absence of either Tpa1 or Mag1 when other one is present. However, when both Mag1 and Tpa1 were deleted and only Polζ was present, it could not substitute the role of both Tpa1 and Mag1.

It is very difficult to imagine whether mammalian Polζ would be able to compensate the loss of both 3meA-specific DNA
glycosylases and oxidative demethylase. For example, when mammalian alkA homolog Aag was mutated along with two functional alkB homologs (hALKBH2 and hALKBH3), such Aag<sup>−/−</sup>Abh2<sup>−/−</sup>Abh3<sup>−/−</sup> triple knockout mice did not exhibit significantly increased susceptibility to alkylating agent MMS compared Aag<sup>−/−</sup> single knockout mice (63). However, this does not necessarily mean cells were actually protected from MMS-induced damage by mammalian Polζ. Since mammalian genome codes for nine AlkB homologs, this could as well be due to presence of other active AlkB homologs. For example, weak AlkB-like repair activities have also been shown for hALKBH1 and FTO in vitro (17, 30). Nonetheless, Polζ, and perhaps Polη and Polκ, may also have important role in promoting replication through the 3meA adduct (5). It is also difficult to address the role of either DNA glycosylases or oxidative demethylases in the absence of mammalian TLS polymerase Polζ. This is because Rev3<sup>−/−</sup> knockouts was reported as embryonic lethal and Rev3<sup>−/−</sup> cell lines could not be established (64). By contrast, we clearly show that in S. cerevisiae, in the absence of either Mag1 or AlkB homolog Tpa1, error-prone repair by Polζ support cell survival (Fig. 5D and E). Therefore, this rather simple yeast system may help to decipher complex network of alkylation damage repair pathways in other eukaryotes.

Relevance of the genetic interaction between alkyl base glycosylase and oxidative DNA demethylase to cancer therapy- The study of the genetic interaction among 3meA-specific DNA glycosylases, oxidative demethylases and trans-lesion DNA polymerases is particularly important in view of its potential role in determining the efficacy and specificity of cancer therapy. If the same DNA repair deficiencies that sensitize tumor cells to chemotherapy or radiotherapy also allow them to continue replicating regardless of damage and simultaneously reduce their efficiency of DNA repair, then any DNA-damaging therapy could carry a risk of allowing further deleterious mutations to accumulate in any tumor cells that survive the treatment. Therefore it is important to consider the actual DNA repair pathway operating in response to particular forms of damage in particular tumors. For example, it has long been recognized that solid tumors frequently contain hypoxic regions (65). Lack of oxygen leads to inhibition of Hypoxia inducible factor (HIF) prolyl-hydroxylase, a Fe(II) and 2OG-dependent dioxygenase (66). Hypoxic solid tumors also accumulate fumarate and/or succinate, which act as 2-oxoglutarate analogues and inhibit HIF prolylhydroxylase (67, 68). In such tumors alkylation specific Fe(II)/2OG-dependent demethylases may also be inhibited just like HIF prolylhydroxylase and such tumors may actually be dependent on alkylation-specific DNA glycosylases for damage removal and BER for single-strand break repair. Our genetic analysis indicates that, when only TLS polymerase Polζ was present, it failed to substitute the role of both oxidative demethylase Tpa1 and alkyl base glycosylase Mag1 in S. cerevisiae. We speculate that, in such situation alkylation by MMS would cause multiple fork-blocking lesions on DNA during the S phase, giving alkylating drugs a much greater impact. In checkpoint-deficient tumor cells and checkpoint-mutant yeast strains (69, 70), such fork stalling would be irreversible and the stretches of DNA between collapsed forks would remain unrepaired at the end of the S phase. When any such cells will complete mitosis, it would result catastrophic chromosome breakage and rearrangement. In this regard, future work will include the study of repair pathways to clinically relevant chemotherapeutic drugs including alkylating agents.

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Acknowledgement: This work was funded by "Innovative Young Biotechnologist Award (IYBA)" fellowship (2008) to A.R. by the Department of Biotechnology (DBT), Govt. of India. *E. coli* HK82 (*alkB*) cells were kindly provided by Dr Hans Krokan, Norwegian University of Science and Technology.

FOOTNOTES

†Both authors made complementary and equal contributions to the manuscript.

*This work was supported by DBT, Govt. of India.

§To whom correspondence should be addressed: †Roy Anindya, Department of Biotechnology, Indian Institute of Technology Hyderabad, Ordnance Factory Estate, Yeddumailaram-502205, Hyderabad, India. Tel.: +91-40-23016083, Fax: +91-40-23016032, Email: anindya@iith.ac.in (R. Anindya)

Abbreviations used are: 1meA, 1-methyl adenine; 2OG, 2-Oxoglutarate; 3meC, 3-Methyl cytosine; 6-AU, 6-Azauracil; CTD, C-terminal Domain; DSBH, Double strand β-helix; eRF, eukaryotic release factor; ESCRT, Endosomal sorting complexes required for transport; FDH, Formaldehyde dehydrogenase; hALKBH, Human AlkB homolog; HIF, Hypoxia inducible factor; MMS, Methyl methane sulphonate; NTD, N-terminal Domain; NuA3, Nucelosomal acetyl transferases of H3; PROVEAN, Protein variation effect analyzer; Tpa, Termination and poly-adenylation associated; TLS, Trans-lesion synthesis.
FIGURE LEGENDS

FIGURE 1.

Functional complementation of MMS sensitivity of *E. coli* alkB strain HK82 by *S. cerevisiae* Tpa1. (A) MMS sensitivity of *E. coli* alkB deficient strain (HK82) expressing Tpa1, TauD, AlkB, MphB and empty vector. (B) MMS sensitivity of *E. coli* alkB deficient strain (HK82) expressing Tpa1 NTD and CTD. Cells were treated with 0.1% (v/v) MMS for 3h and plated on LB-Agar and incubated at 37°C for 20h. Survival was compared to cells expressing *E. coli* AlkB (positive control) and empty vector (pGex6p1; negative control). All strains were grown in duplicates. (C) Survival analysis of *E. coli* alkB strain HK82 expressing *S. cerevisiae* Tpa1 constructs. Survival curve of Tpa1-rescued *E. coli* alkB strain. *E. coli* strain HK82 (alkB) was transformed with different Tpa1 constructs and grown in cultures containing 0.05%, 0.1%, and 0.15% (v/v) of methylating agent MMS for 2h; Survival was determined by plating cells on LB-Agar at 37°C for 20h and colony counting. The viability of cells not treated with MMS was 100%. Error bars indicate the standard error of the mean. All the strains are the isogenic derivatives of W303.

FIGURE 2.

Interaction of 2OG and iron with the active site residues of wild type and mutant Tpa1. (A) Diagrams showing the crystal structures of the 2OG-bound Tpa1 (PDB: 3KT7). (B) Diagrams showing altered orientation and diminished atomic interactions of a representative position of docked 2OG bound to mutant Tpa1 (H159C, D161N, H227C, H237C and R238A, shown in red). Ribbon representations show the position of 2OG and iron with respect to the alpha helices, beta strands of Tpa1. Stick representations depict the amino acids and atomic bonds are represented with dotted lines. (C) Recombinant GST-AlkB, -Tpa1, -CTD and -NTD were purified by glutathione sepharose and analyzed by 12% SDS-PAGE. (D) UV-Visible spectroscopic analysis showing evidence for binding of Fe(II) and 2OG by Tpa1 (solid line) and Tpa1 mutant (broken line). (E) UV-vis spectra of Fe(II)-CTD (broken line) and NTD (solid line). Peak absorption was recorded at 530 nm. Proteins were mixed with buffer containing 25 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.5 mM 2OG, 4.0 mM Sodium ascorbate, 880 µM FeSO4 and spectra were recorded.

FIGURE 3.

Tpa1 is catalytically active and removes methyl adducts. (A) Schematic representation of restriction enzyme-based repair assay. 70bp oligonucleotide comprised of recognition site for the methylation sensitive restriction enzyme MboI (5'-GATC-3') in the middle was treated with MMS. MMS-damaged oligonucleotides were incubated with Tpa1 and repaired DNA was annealed to complimentary undamaged oligonucleotide and cleaved with MboI restriction enzyme. (B) Tpa1 catalyzed repair was detected by MboI digestion followed by agarose gel electrophoresis and ethedium bromide staining. Tpa1, mutant Tpa1 and GST were incubated with methylatedoligonucleotide. Following Tpa1-mediated repair, MboI digestion produced 35bp band, whereas MboI did not cleave mock-repaired (GST) and mutant Tpa1-repaired substrate and 70bp substrate remained intact. The arrows indicate position of 70 and 35 base pair band. Single and double stranded DNA is represented as ssDNA and dsDNA, respectively. Lane 4 represents molecular size markers. (C) Increasing amounts of purified Tpa1 was added to methylated DNA substrate for 2h at 30°C followed by MboI restriction analysis. Reactions were analyzed by agarose gel analysis as mentioned before. (D) Time-course of Tpa1 repair. Tpa1 was added to methylated DNA substrate for 0.5h, 1h, 2h and 3h at 30°C followed by MboI restriction analysis. Reactions were analyzed by agarose gel analysis as mentioned before.
FIGURE 4.

Tpa1 is involved in direct repair of methylated DNA. (A) Emission spectra of the product of reaction between ammonia and acetoacetanilide and different concentration of formaldehyde. (B) Standard plot of absorbance versus different concentration of formaldehyde. (C) Emission spectra of the product of reaction between ammonia, acetoacetanilide and sample containing either AlkB, or Tpa1 or mutant-Tpa1-repaired methylated DNA. Repair reaction was carried out with fixed amount (2 µM) of bacterially expressed and purified recombinant protein (GST-Tpa1, GST-Tpa1-mutant and GST-AlkB) and MMS-damaged single-stranded DNA substrate (100 ng/µl) at 30°C for 1h. A sample containing 2 µM GST and MMS-damaged single-stranded DNA (100 ng/µl) was used as background fluorescence correction. (D) Emission spectra of the product of reaction between ammonia, acetoacetanilide and sample containing Tpa1 in the presence of divalent Nickel ion and competitive inhibitor Succinate. Reaction components were same assays as in C except 1mM Ni(II) or 5mM Succinate was present. (E) Bacterially expressed and purified Tpa1 and Tpa1-mutant proteins were incubated with MMS-damaged single-stranded DNA substrate of different concentrations. DNA repair activity was monitored by quantifying formaldehyde release as mentioned in C and plotted. Graphs represent averages of triplicate experiments. Curve fitting was carried out using Graphpad Prism software.

FIGURE 5.

Formaldehyde dehydrogenase (FDH)-coupled assay to detect formaldehyde produced during Tpa1-mediated repair. (A) Chemical reaction for formaldehyde production and its detection by formaldehyde dehydrogenase (FDH)-coupled assay. (B) Demethylation reaction using purified GST-fused recombinant proteins (2 µM), viz. Tpa1, Tpa1-NTD, Tpa1-CTD, AlkB and MMS-damaged single-stranded DNA substrate (10 ng/µl). The repair reaction was coupled to FDH-reaction by adding 0.01 U FDH and 1 mM NAD⁺. The reaction was monitored continuously for the production of NADH (absorption at 340 nm). (C) Direct reversal of methylated DNA as reveled by formaldehyde release by wild type Tpa1 but not with Tpa1 mutant that lacks cofactor binding. Reactions in the presence of divalent Nickel ion and competitive inhibitor Succinate were same assays as in (B) but contained 1 mM Ni(II) or 5 mM Succinate. (D) FDH-coupled demethylation assays with fixed amount (2 µM) of Tpa1 and varying amounts (2-20 ng/µl) of the MMS-damaged single-stranded DNA substrate. Undamaged single-stranded DNA was used as negative control.

FIGURE 6.

Effect of the deletion of Tpa1 and BER pathway DNA glycosylase Mag1 on cell viability during MMS treatment. (A) Mild MMS sensitive phenotypes of tpa1Δ and mag1Δ mutant. 10-fold dilutions of log-phase cultures of wild-type strain (W303), mag1Δ, and tpa1Δ strains were spotted on YPD supplemented with increasing concentrations (0.005% and 0.015%(v/v) of MMS. (B) Hyper-sensitivity of tpa1Δ mag1Δ double mutant strain to MMS-induced DNA damage as compared with tpa1Δ and mag1Δ. Colonies of each strains were streaked on an YPD plate containing either no MMS or 0.005%(v/v) MMS. As a control wild-type strain (W303) was streaked. Plates were incubated at 30°C for 3 days and then photographed. (C) The effect of MMS on the viable cell population of Saccharomyces cerevisiae wild-type strain (W303), mag1Δ, tpa1Δ and mag1Δtpa1Δ double mutant. Cells were spread onto MMS-containing YPD plates and incubated at 30°C and colonies were counted after 3–5 days. Graphs represent averages of four experiments. (D) MMS sensitive phenotypes of tpa1Δmag1Δ double mutant. 10-fold dilutions of log-phase cultures of tpa1Δ, mag1Δ and tpa1Δmag1Δ strains were exposed to increasing concentrations (0.002%, 0.003% and 0.005%(v/v)) of MMS on YPD plates and incubated at 30°C for 3 days. Representative images of repeat experiments are shown.
FIGURE 7.

Effect of deletion of Tpa1 and Mag1 on cell viability during treatment with translational inhibitors. Tenfold dilutions of cells harboring the indicated mutations were spotted on to YPD medium containing indicated concentration of cycloheximide, anisomycin, paromomycin, geneticin and 6-azauracil. Cells were incubated for 3 days at 30°C, and growth was monitored as compared to growth on plates in the absence of drug. Each strain and drug was assayed at least thrice.

FIGURE 8.

Effect of the deletion of Tpa1, Mag1 and TLS DNA polymerases Polζ (Rev3) on cell viability during MMS treatment. (A) MMS sensitive phenotypes of tpa1Δmag1Δrev3Δ triple mutant. 10-fold dilutions of log-phase cultures of mag1Δrev3Δ, tpa1Δrev3Δ and tpa1Δmag1Δrev3Δ strains were exposed to increasing concentrations (0.002%, 0.003% and 0.005%(v/v)) of MMS on YPD plates and incubated at 30°C for 3 days. Representative images of repeat experiments are shown. (B) MMS sensitivity of tpa1Δ mag1Δ rev3Δ triple mutant strain compared with tpa1Δmag1Δ, tpa1Δrev3Δ and mag1Δrev3Δ double mutant strains. Colonies of each strains were streaked on an YPD plate containing either no MMS or 0.005% (v/v) MMS. Strains were grown as before. (C) MMS sensitivity of strains lacking TLS polymerases Polη (Rad30) and Rev1. 10-fold dilutions of log-phase cultures of rev1Δ, rad30Δ, mag1Δrad30Δ, tpa1Δrad30Δ, mag1Δrev1Δ, tpa1Δrev1Δ strains were exposed to increasing concentrations (0.005%, 0.01% and 0.02%(v/v)) of MMS on YPD plates and incubated at 30°C for 3 days. Representative images of repeat experiments are shown.
Figure 1

A

No MMS  0.1%(v/v) MMS

B

No MMS  0.1%(v/v) MMS

C

Survival (%)

MMS Concentration (%v/v)

- AlkB
- Tpa1
- NTD
- CTD
- Vector

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Figure 2
**Figure 3**

A. MboI digestion pattern with GATC sites.

B. Gel electrophoresis with bands at 70bp and 35bp.

C. Concentration of Tpa1 (μM) with bands at 70bp and 35bp.

D. Time (h) with bands at 70bp and 35bp.

- + + methylated DNA
- + - Wildtype Tpa1
- - + Tpa1-mutant
+ - - GST

Wildtype Tpa1 methylated DNA
Tpa1-mutant
 GST
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
A role for **Saccharomyces cerevisiae** Tpa1 protein in direct alkylation repair
Gururaj Shivange, Naveena Kodipelli, Mohan Monisha and Roy Anindya

*J. Biol. Chem.* **published online November 7, 2014**

Access the most updated version of this article at doi: [10.1074/jbc.M114.590216](https://doi.org/10.1074/jbc.M114.590216)

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