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IDENTIFYING GENETIC DETERMINANTS OF ALKBH2 EVOLVABILITY

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ABSTRACT

ALKBH2 is an Fe(II), alpha-ketoglutarate dependent dioxygenase that repairs methyl and etheno lesions. Recently, ALKBH2 has been described as a tumor suppressor, and somatic ALKBH2 mutations have been reported in tumors. To understand selective pressures driving ALKBH2 evolution, we evolved an ALKBH2 mutant library under MNNG selection, which generates methyl adducts that are not known ALKBH2 substrates. Mutants from our selection overlap with tumor mutations. We used *E. coli* complementation systems to study how these mutations alter protection against both known methyl and etheno substrates, and against non-canonical cytotoxic lesions. We find that: 1) ALKBH2 mutant activity against known substrates is decreased but not abolished 2) Activity against methyl adducts can be differentially modulated relative to etheno adducts. 3) Some of our MNNG-selected mutations exhibit protection to additional cytotoxic lesions. These findings open the door for genetic modulation of ALKBH2 substrate specificity, which has implications for alkylating agent therapy.

DEDICATION

I dedicate this thesis to my wonderful family and friends. I need to thank my siblings Joe, Alicia, and Diana for being there for me over the years. I especially need to thank my parents, who have always supported me and pushed me to do great things. Without their support I could have never accomplished this, and I will always be grateful for the things they have taught me.

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Chapter 1:

Introduction to DNA Damage and Repair Mechanisms

1. DNA Damage

1a. Alkylation damage leads to cytotoxic methyl lesions

DNA is constantly exposed to a variety of endogenous and exogenous agents that are responsible for damaging DNA. Alkylating agents are one such class of DNA damaging agents that are responsible for introducing methyl or ethyl groups on oxygen or nitrogen atoms on DNA bases (Nieminuszczy 2007). These agents are widespread in the environment as pollutants (such as tobacco smoke), as well as produced endogenously through the action of methyl donors, such as S-adenosylmethionine for example (Fu, 2012).

Alkylating agents can introduce methyl groups through one of two mechanisms, S_N1 or S_N2 nucleophilic substitution. Chemical agents that use the S_N1 mono-molecular mechanism of attack, such as N-methyl-N-nitrosourea (MNU) or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), introduce alkyl adducts at both N and O atoms. S_N2 agents, such as methyl methanesulfonate (MMS) and naturally occurring methyl halides, use a bimolecular mechanism introducing lesions on the N-position of DNA bases (Nieminuszczy 2007, Sedgwick 2004). Sites of S_N2 alkylation damage are protected by Watson-Crick base pairing in dsDNA, and therefore these agents only induce lesions in ssDNA (Mishina 2006, Figure 1). These lesions protrude into the minor groove, blocking polymerases and result in primarily cytotoxic effects causing replication arrest and transcription interruption (Sedgwick, 2007, Nieminuszczy 2007). Due to the cytotoxic nature of these lesions, they are detrimental to human health. However, this cytotoxicity is often harnessed in many chemotherapeutic

agents, which take advantage of disregulation in DNA homeostasis in tumor cells to selectively kill them.

1b. Mutagenic Etheno adduct formation

Etheno adducts are a class of bulky adducts that form on DNA bases. Etheno adducts are an exocyclic-ring DNA adduct that is formed when bifunctional electrophilic compounds attack an exocyclic-nitrogen and subsequently attack a nitrogen at the other end, closing the ring (Hang, 2004). The etheno ring on DNA bases are a noninstructive lesion that interferes with the formation of hydrogen bonds between complementary base pairs, blocking progression of the DNA polymerase and causing mutagenesis (Palajwala 1993, Hang, 2004, Basu 1993).

Etheno adducts arise from both exogenous and endogenous sources. An increase in etheno adduct formation has been found to correlate with both cigarette smoke exposure (Chen 2007) as well as a diet high in ω-6 fatty acids (Hagenlocher 2001). Endogenously, etheno adducts arise from lipid peroxidation (LPO). Epoxy derivatives from LPO such as the α,β-unsaturated aldehydes HNE, 8-HETE and 12-HETE, are more reactive toward DNA and are the primary metabolites responsible for generating exocyclic etheno adducts (Berquist 2012, Dosanjh 1994). Evidence of LPO resulting in etheno adduct build-up was shown by the measure of increased etheno adducts (εA and εC) when rats were exposed to excess iron and treated with carbon tetrachloride (Nair 1999). Carbon tetrachloride is metabolized by cytochrome p450 in the liver and has been proven to initiate radical-mediated oxidation and is a common model for oxidative damage and lipid peroxidation *in vivo* (Yoshida 2005).

There is some evidence indicating that etheno adducts are a potential driver of carcinogenesis. When compared to asymptomatic tissues, a build-up of etheno adducts has been observed in the tissues of patients with chronic hepatitis, liver cirrhosis, HCC, CP, and cancer-prone colon tissues (Bartsch 2005). However, the strongest evidence comes from a study on vinyl chloride (VC) exposure. VC is metabolized by cytochrome p450 isoenzyme CYP2E1 in hepatocytes (Dogliotti 2006) and forms the reactive metabolites chloroethylene oxide (CEO) and chloroacetaldehyde (CAA) (Barbin 1981). Due to rearrangement of CEO to CAA, and testing of the mutagenic potential of CAA in supF shuttle vectors, CAA has been determined to be the ultimate carcinogenic form of vinyl chloride (Matsuda 1995). These metabolites were shown to have the same reaction products with nucleic acids, etheno adducts, and both display a similar mutational pattern (Malaveille 1975). A study done on 1658 workers that were employed at a plant with high VC exposure confirmed that there is a causal relationship between VC exposure and liver angiosarcomas. Workers also exhibited an increased risk for hepatocellular carcinomas (HCC) and liver cirrhosis (Mastrangelo 2004). Since etheno adducts are the primary reaction product of VC metabolism and these lesions are primarily mutagenic, it can be concluded that the carcinogenicity of VC exposure is likely due to etheno adducts.

Similar to other types of DNA damage such as methyl adducts and double-strand breaks, etheno adducts can also differentially kill tumor cells. The anticancer drug Ifosfamide, which is part of a group of oxazaphosphorines that are widely used

to treat breast cancer, is mainly metabolized to CAA which mediates the therapeutic effects of Ifosfamide, (Knouzy 2009). Since etheno adducts are the main product of CAA (Malaveille 1975), this implies induced etheno adduct formation is a potential antitumor strategy and suggests the role of etheno-adduct formation in promoting tumor formation is more complex than initially thought, with both pro- carcinogenic (genetic instability) and antitumorigenic (cytotoxic) effects.

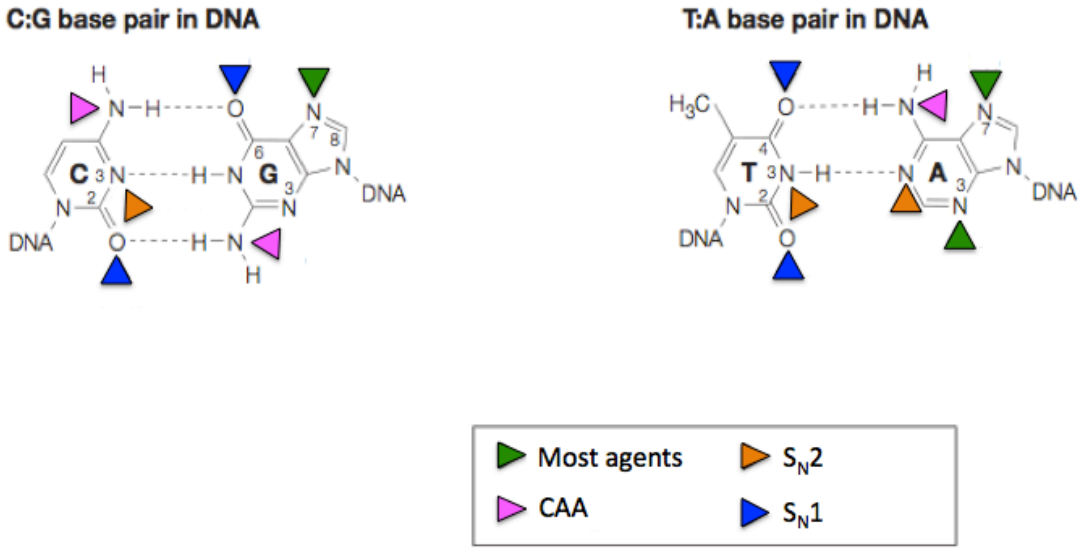

Fig 1: Sites of methyl and etheno adduct formation. Orange arrows indicate sites damaged by S_N2 alkylating agents. Damage at these sites can only be induced in ssDNA. Blue arrows indicate sites of alkylation damage by S_N1 agents. Green arrows indicate sites that are damage by most alkylating agents. Pink arrows indicate sites of etheno adduct formation by chloroacetaldehyde (CAA). (Modified from Sedgwick 2004).

2. Mechanisms of DNA Repair: Base Excision Repair (BER) and Direct Repair

2a. Base Excision Repair

Base excision repair (BER) is a conserved process between mammals and bacteria. The process is initiated by glycosylases, which remove alkylated bases from the phosphate backbone of DNA by cleaving the glycosylic bond (Memisoglu 2000). Endo/exonucleases then act to cleave the sugar phosphate backbone, allowing DNA polymerase to replace the damaged base and a DNA ligase to complete the repair process by ligating the DNA sugar phosphate backbone (Sedgwick 2004). Although BER is the principal mechanism by which adducts are removed, it involves a coordinated effort of several enzymes working in succession, and if any enzyme becomes limiting or overexpressed, the resulting repair intermediate can become even more toxic than the initial lesion itself (Glassner 1998).

2b. Direct Repair by the ALKBH family

There are two main direct repair enzymes in humans: O^6-methylguanine-DNA methyltransferase (MGMT) and the ALKBH family. MGMT repairs O^6-methylguanine, a miscoding base in DNA generated by S_N1 alkylating agents, by transferring the methyl group to a specific cysteine residue in the repair enzyme. The transfer of the methyl group is irreversible, and the methylated MGMT protein is then degraded, making MGMT a suicide enzyme (Sedgwick 2007). The focus of my thesis, however, is on the other direct repair mechanism: oxidative demethylation by the ALKBH family, specifically by the AlkB human homologue 2 (ALKBH2).

ALKBH2 is one of nine human homologues to the *E. coli* AlkB protein (Kurowski et. al 2003, Wei et. al 1996). In *E. coli*, the AlkB protein was found to be one of three proteins that take part in a coordinated response to reactive DNA alkylation known as the adaptive response pathway, which is restricted to prokaryotes (Sedgwick 2004). The AlkB protein was characterized as an Fe(II)/2-oxoglutarate-dependent dioxygenase that repairs 1-methyladenine and 3-methylcytosine lesions in ssDNA as well as ethyl, hydroxyethyl and propyl adducts (Falnes 2002, Trewick 2002, Duncan 2002). The AlkB protein was also found to have 9 human homologues (ALKBH family) whose sequence conserves key Fe(II)/2-oxoglutarate-dependent dioxygenase residues.

Although the focus of my thesis is on ALKBH2, other ALKBH homologues implicate oxidative demethylases as having additional roles in DNA damage protection. In addition to ALKBH2, two other ALKBH homologues, ALKBH1 and ALKBH3, have been found to have nucleic acid demethylation activity of 1-meA and 3-meC, although these two homologues repair single stranded DNA (Duncan 2002, Falnes 2004, Westbye 2008). However, alternative activities despite oxidative demethylation have been suggested for ALKBH homologues. ALKBH1 has been characterized as a mitochondrial protein that can demethylate 3-meC in both DNA and RNA, and in addition has also exhibited lyase activity at abasic sites (Muller 2009). ALKBH5 is a direct target of hypoxia inducible factor-1 (HIF-1), and has therefore been suggested to have a role in regulation of cellular response to hypoxia (Thalhammer, et. al 2011). ALKBH8 has been shown to have methyltransferase

activity with specific activity as a tRNA methyltransferase required for uridine wobble modifications implicated in translational decoding (Fu 2010, Songe-Moller 2010). These recent findings suggest that the ALKBH family is a complex group of oxidative demethylases that may play a large role in protecting against multiple types of DNA damage, which could implicate this protein in cancer progression.

ALKBH2 acts to remove methyl adducts, N1-methyladenine (1-meA) and N3-methylcytosine (3-meC) (Duncan 2002, Sedgwick 2004), or etheno adducts N6-ethenoadenine (εA), and ethenocytosine (εC) on DNA bases (Ringvoll 2008, Fu 2012). The methyl and etheno adducts are repaired by oxidizing the adduct making an unstable intermediate that is then released as formaldehyde or glyoxal, respectively. To repair methyl or etheno damaged bases, ALKBH2 uses a hairpin motif to probe for unstable base pairs, and then uses an arginine finger to flip the damaged base into the active site (Yi 2013). Once in the active site, the damage is oxidized and then released leaving the original base in tact. ALKBH2 preferentially repairs dsDNA, which is in contrast to the ssDNA preference of AlkB and other ALKBH homologues, due to the presence of a unique hydrophobic β-hairpin next to the active site that binds the complementary DNA strand (Chen 2010). The enzyme also has a Phe102 residue that is responsible for taking the position of the flipped out base, in order to maintain base-stacking interactions and stability (Yi 2013).

Alterations in ALKBH2 levels have been observed in human cancer cell lines. ALKBH2 down-regulation has been observed in 68% of primary gastric cancers, and a study by Gao et. al. (2011) has shown that ALKBH2 overexpression significantly

inhibits gastric cancer cell proliferation, while knockdown increased cell growth (Gao 2011). Overexpression of ALKBH2 can also have pro-oncogenic effects. In human bladder cancer cells increased expression of MUC1 (Fujii 2013), a glycoprotein that modulates signaling pathways responsible for affecting oncogenesis, metastisis and motility resulting in increased cell proliferation (Nitta 2000, Makiguchi 1996). Decreased ALKBH2 expression resulted in a decrease in MUC1 associated cell proliferation, indicating a potential role for ALKBH2 in bladder cancer progression (Fujii 2013). This evidence, in addition to alterations in expression of homologues ALKBH3 and ALKBH8 resulting in increased cancer cell proliferation (Tasaki 2011), indicates a possible role for other ALKBH homologues and oxidative demethylation in cancer progression (Camps 2011).

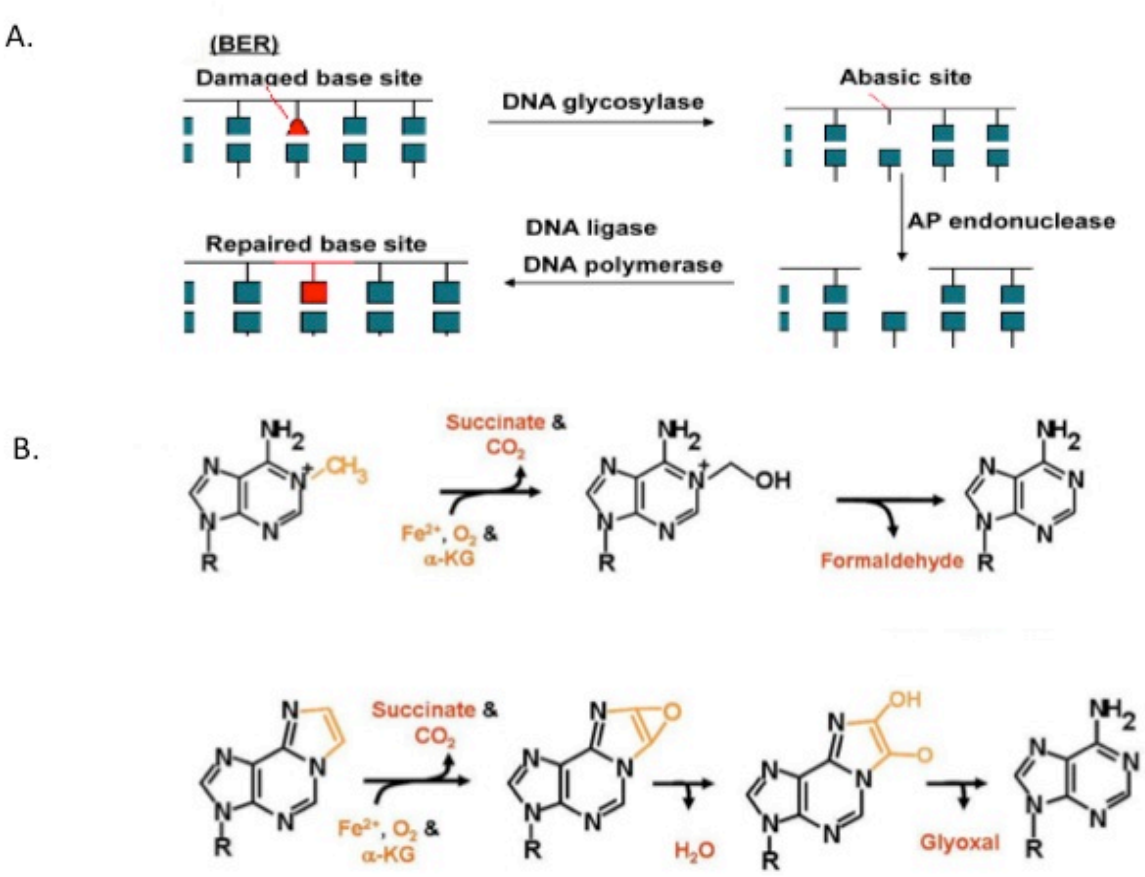

Fig 2. Mechanisms of DNA Repair. (A) The base excision repair (BER) mechanism is shown. BER involves the coordinated action of a DNA glycosylase, AP endonuclease, DNA ligase and DNA polymerase. (B) The direct repair mechanism carried out by oxidative demethylases, such as ALKBH2 is shown. Top panel shows the repair of 1-meA using Fe(II) and alpha-ketoglutarate to oxidize the damaged base, releasing succinate, CO_2, formaldehyde and the original base in tact. The bottom panel shows oxidative demethylation of an εA, released as glyoxyl (O'connor 2013).

Hypothesis and Specific Aims:

Chapter 2: Characterization of evolution of ALKBH2 in tumors

Hypothesis: ALKBH2 single point mutations will result in altered repair of ALKBH2 substrates, indicating potential sites for substrate discrimination by ALKBH2

Specific Aims:

1. Identify mutations in ALKBH2 that result in alterations in repair of either canonical or non-canonical substrates

2. *In vitro* confirmation that candidates seen *in vivo* have altered repair capabilities.

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Chapter 2:

Identification of determinants of ALKBH2 Evolvability

Abstract

The human DNA repair enzyme, ALKBH2, is an Fe(II), alpha-ketoglutarate dependent dioxygenase that repairs endogenous methylating (1mA and 3mC) and etheno (εA) lesions. Recently, ALKBH2 has been described as a tumor suppressor, and a number of somatic ALKBH2 mutations have been reported in tumors. To get insights into selective pressures driving the evolution of ALKBH2, we evolved an ALKBH2 random mutant library under MNNG selection. This drug generates cytotoxic methyl adducts in DNA that are not known ALKBH2 substrates including O6mG and 3mA. Active site mutants obtained from our selection show substantial overlap with tumor mutations. We used a panel of *E. coli* complementation systems to study how these active site mutations alter protection against both known methyl and etheno substrates, and against two additional cytotoxic lesions. We find that: 1) ALKBH2 activity against known substrates is typically decreased but not abolished by these mutations; 2) Activity against methyl adducts can be differentially modulated relative to etheno adducts. 3) Some of our MNNG-selected mutations (but not tumor somatic mutations) exhibit protection that extends to additional cytotoxic lesions. These findings open the door for genetic modulation of ALKBH2 substrate specificity, which has implications for alkylating agent therapy.

Introduction

Alkylating agents can react with DNA to form cytotoxic methyl lesions. These lesions, such as N^1-methyladenine (1-meA), N^3-methyladenine (3-meA) and N^3-methylcytosine (3-meC) protrude into the minor groove, blocking polymerases and result in primarily cytotoxic effects caused by replication arrest and transcription interruption (Sedgwick, 2007, Nieminuszczy 2007). Another methyl lesion, O^6-methylguanine (O^6-meG), is mutagenic due to its ability to mismatch with adenine, leading to G to T mutations. However, O^6-meG lesions can also cause significant cytotoxicity through the initiation of a futile mismatch repair cycles that eventually leads to cell cycle arrest, chromosomal aberrations and apoptosis (Kaina 1997, Karran 1992). Due to this cytotoxicity, methylating agents are often used as antitumor chemotherapeutic drugs (Segwick 2004).

The human homologue to the *E. coli* AlkB DNA repair enzyme, ALKBH2, is an Fe2+, α-ketoglutarate oxidative demethylase (Segwick 2007). The enzyme uses a hairpin motif to probe for unstable base pairs, and then uses an arginine finger to flip the damaged base into the active site. ALKBH2 then employs a one-step, direct repair mechanism where the damage is released leaving the original base intact (Yi 2013). ALKBH2 uses this mechanism to repair the methyl lesions 1-meA and 3-meC (Sedgwick 2004, Duncan 2002,), and the etheno adducts ethenoadenine and ethenocytosine (Ringvoll 2008, Fu 2012). Etheno adducts arise from both exogenous sources, such as cigarette smoke (Chen 2007) and a diet high in ω-6 fatty acids (Hagenlocher 2001), and endogenous sources such as lipid peroxidation (Berquist

2012, Dosanjh 1994, Nair 1999). These lesions are primarily mutagenic, and have also been found to be a potential drive of carcinogenesis (Bartsch 2005).

ALKBH2 has no repair capabilities for 3-meA or O6-meG. N^3-meA is repaired by the enzyme Tag (Bjelland 1993), while O^6-meG is repaired by the direct repair enzyme Methyl-guanine methyltransferase (MGMT), which is the sole O^6-meG repair enzyme in humans (Pegg 1990, Pegg 2000). The Camps' lab has used directed evolution to determine if ALKBH2 can evolve the ability to repair 3-meA, which is often induced by chemotherapeutic agents and is not a canonical ALKBH2 substrate. An ALKBH2 mutant library was generated using random mutagenesis with an error-prone PolI and selected on a gradient plate containing increasing amounts of the drug N--Methyl N'-nitro-N-nitrosoguanidine (MNNG), which induces lesions wild-type (WT) ALKBH2 cannot repair. Results showed that some mutants enhanced protection against MNNG compared to WT in oxidative demethylation deficient strains.

Alterations in ALKBH2 expression levels have been observed in human cancer cell lines. For example, ALKBH2 down-regulation has been observed in 68% of primary gastric cancers, and a study by Gao et. al. (2011) has shown that ALKBH2 overexpression significantly inhibits gastric cancer cell proliferation. Another study by Fujii et. al (2013) has shown that overexpression of ALKBH2 has pro-oncogenic effects in human bladder cancer. This evidence, in addition to alterations in expression of homologues ALKBH3 and ALKBH8 resulting in increased cancer cell proliferation (Tasaki 2011), indicates a possible role for ALKBH homologues and

oxidative demethylation in cancer progression (Camps 2011). In addition, there are also naturally occurring ALKBH2 mutations that have been found to arise in tumors. To our knowledge there are 33 single point mutations (at 26 unique positions) in ALKBH2 that have been found to occur naturally in tumors (Cerami 2012, Gao 2103). Of these 27 unique positions where mutations occur, 7 overlap with mutations that were discovered during our random mutagenesis selection, indicating that these mutations are possibly altering repair capabilities of ALKBH2, and to our knowledge none of the mutations discovered have been characterized (Figure 1).

In this chapter, I present the results of an *in vivo* screening of ALKBH2 mutants. In addition to the random mutagenesis library, we generated additional ALKBH2 mutants that naturally arise in tumors of various cancers, which were discovered in the cBioPortal Cancer Genomics database (Cerami 2012, Gao 2013). Mutants used in this study are listed in Table 2. We used an *E. coli* complementation system to test the ability of each mutant to protect against different alkylating agents to characterize the repair capabilities of each mutant. We use the GW7101 *E. coli* strain, which contains an *ada* deletion (see Chapter 1), and therefore is deficient in all direct repair capabilities. In additional, we use the PJ5 (MGMT deficient) and MV1932(ada/Tag) deficient, *E. coli* strains, which allowed us to look specifically at O^6-methylGuanine (O6-meG) and N^3-methyladenine (N^3-meA) repair, respectively. These strains are summarized in table 1.

Our results suggest that the positions P180, R193, G169, and E175 are important positions for repair of canonical methyl substrates, since all mutants lose

MMS protection compared to WT. In addition, our library produced a mutant, P180L, that appears to have O^6-meG repair capabilities, but not N^3-meA, although results need to be confirmed *in vitro*. In addition, P180L appears to show enhanced etheno adduct repair. The R193S and G169R mutants (from the random mutagenesis library) show protection against MNNG in both strains, which could suggest either repair of both N^3-meA and O^6-meG, but is more likely indicative of an alternative mechanism enhanced by these mutations. In naturally occurring tumor mutants, the P180, R193 and G169 positions are important for protection against canonical methyl substrates, which is similar to what was observed for our random mutation library screen. However, the H144 position appears to not be important in MMS protection, indicating that this position is not required for catalysis. No tumor mutants showed enhanced protection for non-canonical substrates.

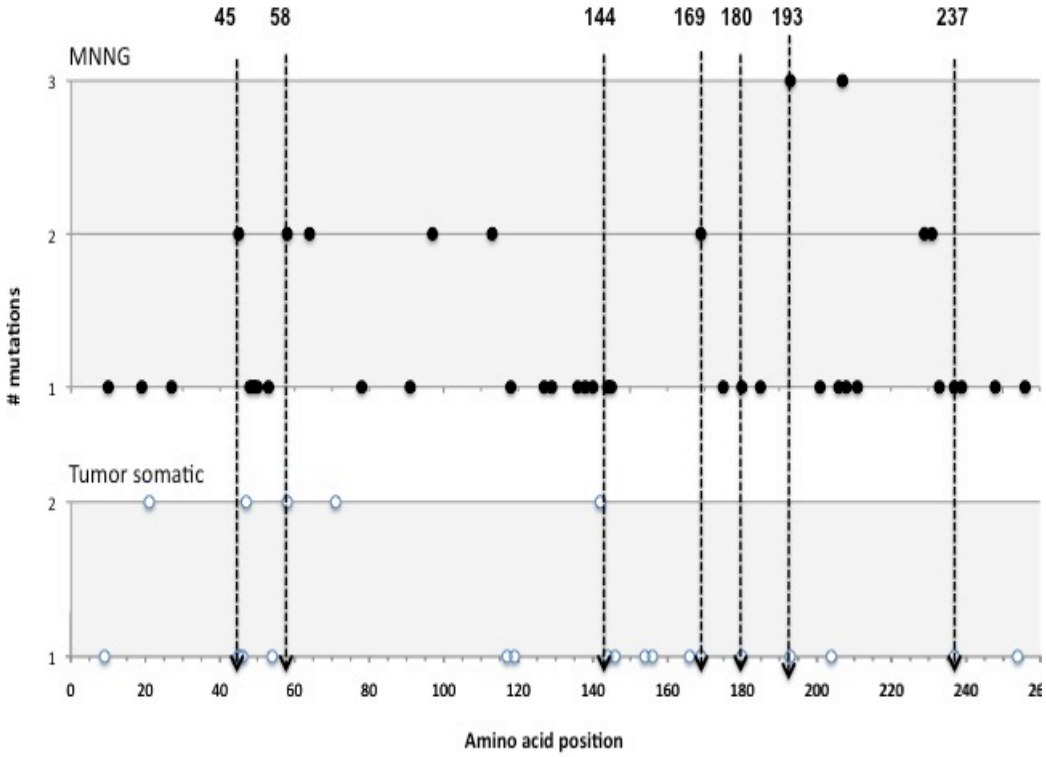

Figure 2.1. Overlap of mutations by MNNG selection and in tumors. This figure is a summary of ALKBH2 mutants that were discovered by our directed evolution of ALKBH2 under MNNG selection and as somatic mutations ALKBH2 tumors. White dots on the bottom panel represent mutations that were discovered as tumor mutants on cBioPortal. While most mutations were only isolated once, some were found to occur on two separate occasions, as indicated with either a 1 or 2 on the y-axis. The black dots in the top panel represent mutations isolated in our MNNG selection. Some mutants were isolated on either 2 or 3 isolations, although most were only isolated once. The arrows represent positions where the MNNG selection and tumor mutations overlap.

Materials & Methods

Bacterial Strains

This study uses three *E.coli* strains deficient in various repair capabilities. The GW7101 strains is deficient in *ada/AlkB*, which makes this strain deficient not only in oxidative demethylation, but the entire adaptive response as well. This strain is used to test the repair of canonical ALKBH2 substrates, 1-meA and 3-meC. The PJ5 strain is deficient in *ada*, and therefore deficient in alkyltransferase activity which would allow for testing of O^6-meG repair. The MV1932 strains is deficient in *ada/Tag*, which makes this strain incapable of repairing N^3-meA. Strains are summarized in table 1.

Generation of ALKBH2 Mutant Library

The ALKBH2 random mutant library was generated using error-prone Polymerase I *in vivo* mutagenesis (Camps 2003, Alexander 2014). ALKBH2 mutant libraries generated using this method were then transformed into *GW7101* cells (*E. coli* cell strain deficient in oxidative demethylation). Mutants were then selected for on LB agar containing a gradient of increasing 1-methyl-3-nitro-1nitrosoguanidine (MNNG) concentration, which is a drug that primarily induces O^6-meG and 3-meA lesions, with background levels of other methyl and etheno adducts. Wild-type ALKBH2 confers no protection against MNNG treatment in *GW7101,* although mutants were selected that exhibited high levels of protection, indicating the possibility that these mutants evolved the ability to repair new substrates.

Plasmid Constructs

ALKBH2 is in the pLitmus28i vector. Site directed mutagenesis and megapriming cloning techniques were then used to generate various ALKBH2 mutants (Ling 1997). Mutants discovered by random mutagenesis that were used in this study are G169W, E175K, P180L, and R193T based on overlap with somatic mutations in tumors. ALKBH2 mutants identified as somatic mutations in tumors used in this study are G119L, H144R, D166H, G169R, P180S, and R193S. A summary of mutants is shown in table 2.

MMS, CAA and MNNG Gradients

pLitmus 28i vectors containg ALKBH2 mutants were transformed into GW7101 (*ada/alkB* deficient), PJ5 (*O^6-meG repair deficient*) and MV1932 (*ada/Tag* deficient) cells and grown in 5mL cultures overnight, shaking at 215RPM at 37°C. In the morning, 40µL of the overnight culture was mixed with 2mL of top agar and stamped on a square agar plate containing a gradient of increasing concentration of either MMS, CAA or MNNG using a sterile glass microscope slide. The agar plates are poured by placing square petri dishes on a slope and pouring the bottom layer, 25mL of LB Agar with drug, and letting it set at a slope. Once dried, the plate is set flat, and a top layer of 25 mL of LB Agar (no drug) is poured onto the plate. The agar is allowed to harden and is then stamped with samples mixed with top agar. Once stamped, the gradients are grown 16-24 hours at 37°C. Gradient growth is measured

in centimeters and the highest point of growth is marked at the end of continuous culture growth and the beginning of growth is marked where the culture starts growing. Length of growth is proportional to protection against the drug tested. All experiments were done in triplicate, with each plate representing a separate clone. In addition to gradient plate stamping, each clone was plated (not under pressure by drug) at a 10^{-4} dilution as test for viability.

Strain name	Repair deficiency	Drug used	Lesion(s) tested for
AB1157	WT		
GW7101	*Ada/AlkB (Oxidative demethylation)*	MMS CAA	N^1-meA N^3-meC εA, εC
PJ5	*Ada (Alkyltransferase)*	MNNG	O^6-meG (leaky for N3mA)
MV1932	*Ada/Tag (N^3-meA repair deficient)*	MNNG	N^3-meA

Table 1. Summary of strains used in this study, drug tested on each strain, and what lesion would be tested for repair in each strain.

ALKBH2 Mutant	Location of mutation	Selection: (Random Mutagenesis or Tumor Mutation)	Tumor Sample ID	Additional location comments
H144R	α-3	Stomach Adenocarcinoma	TCGA-BR-8487-01	α-helix 3
G169R	Active site	Random mutagenesis	-	Active site loop
G169W	Active site	Lung adenocarcinoma	TCGA-05-4396-01	Active site loop
E175K	Active site	Random mutagenesis	-	Active site loop
P180S	Active site	Prostate adenocarcinoma	TCGA-KK-A6E8-01	Active site loop
P180L	Active site	Random mutagenesis	-	Active site loop
R193S	B-sheet 12	Random mutagenesis	-	Active site loop
R193T	B-sheet 12	Breast Invasive carcinoma	TCGA-C8-A26Y-01	Active site loop

Table 2. List of ALKBH2 mutants characterized in this study. Mutants were either evolved using random mutagenesis in the Camps' lab, as described above, or are found as somatic mutations in tumors. Tumor type, as well as sample ID, are listed for tumor mutations.

Results

Here we used a panel of *E. coli* complementation systems to determine the effect of ALKBH2 mutations on substrate specificity. Our panel includes strains deficient of oxidative demethylation (GW7101) to measure repair 1-meA and 3-meC (with MMS challenge) or ethenoA (with CAA challenge), alkyltrasnferase activity (PJ5) to monitor repair of O^6-meG (with MNNG challenge), or of N^3-meA repair (MV1932 with MNNG challenge). The complementation systems for measurement 1mA/3mC has been previously described (Dinglay 2000). Complementation systems for measurement of 3mA and O6mG have been extensively studied and characterized in our lab, showing that each strain is specific for the lesion of interest, and shows no or little decrease in protection against other lesions (characterized by Christopher Troll and Joshua Lilly in the Camps' Lab). I have developed the system for detection of ethenoA repair based on previous reports of CAA induction of etheno formation and of ALKBH2 repair of ethenoA (Ringvoll 2008, Fu 2012).

Characterization of mutants obtained by directed evolution

All characterized MNNG-selected mutants show a decreased ability to protect in the presence of MMS, and therefore are deficient in activity against 1mA/3mC.

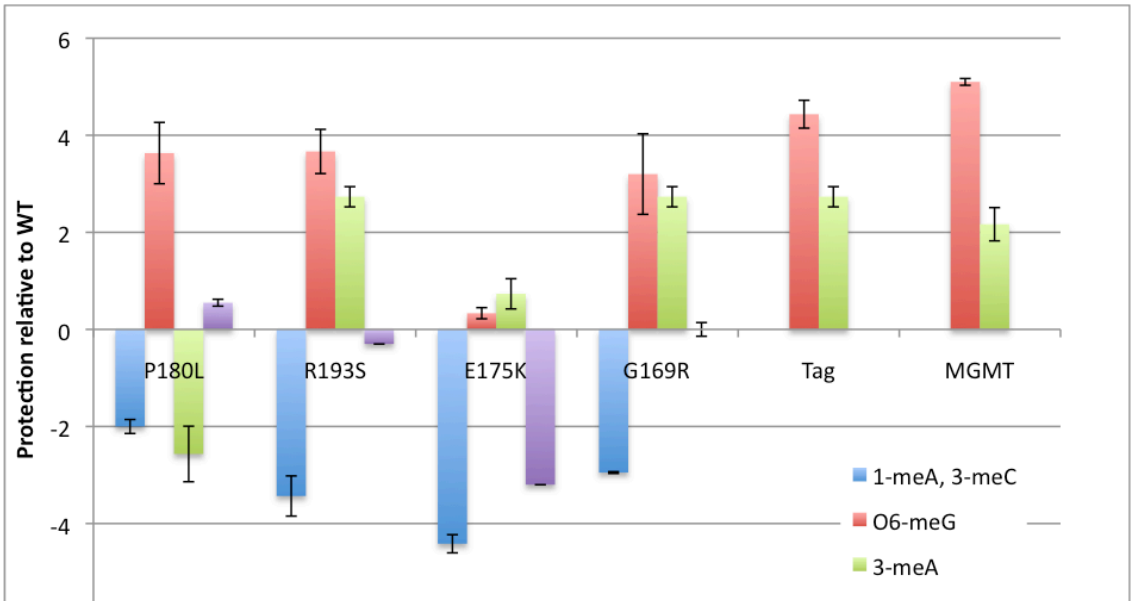

Fig. 2 MMS, MNNG and CAA Gradients for MNNG selected mutants. Each bar represents the protection by a mutant against a certain lesion relative to WT levels of protection. Blue column indicates mutants that were tested under MMS in oxidative demethylation deficient strains, therefore indicating repair canonical methyl substrates (1-meA, 3-meC). Red column indicates testing under MNNG in strains deficient in alkyltransferase activity, and therefore shows O^6-meG repair. Green indicates testing under MNNG in Tag deficient strains, indicating N^3-meA repair. Purple represents repair of etheno adducts in oxidative demethylation strain under CAA stress.

There are two sets of mutants with extended protection:

The P180L mutant showed increased protection relative to WT in the PJ5 strain, which points to O^6-meG repair. The P180L mutant is also the mutant that appears to exhibit enhanced protection to etheno adducts, as seen from the CAA

gradients in Figure 2. P180L shows decreased protection to MNNG in the MV1932 strain, indicating that this mutant is incapable of repairing N^3-meA. This result is strengthened by the conclusion that MNNG protection seen in the PJ5 complementation system is reflective of O6mG repair, since the PJ5 system is leaky for N3mA repair (see Tag control).

G169R and R193S mutants showed enhanced protection against MNNG in MV1932, suggesting protection against N3mA lesions. Viability of MV1932 cells is known to be affected by endogenous N3mA generation (Troll 2014). Consistent with our interpretation of increased N3mA tolerance, these two strains showed increased viability in the absence of any MNNG challenge, and similar to viability seen when Tag (a glycosylase that specifically repairs N3mA) is expressed (**Fig. 3**). These two mutants also showed the protection in PJ5 cells, but so did Tag, suggesting that in this case protection is likely due to N3mA repair, not O6mG repair (**Figure 2)**.

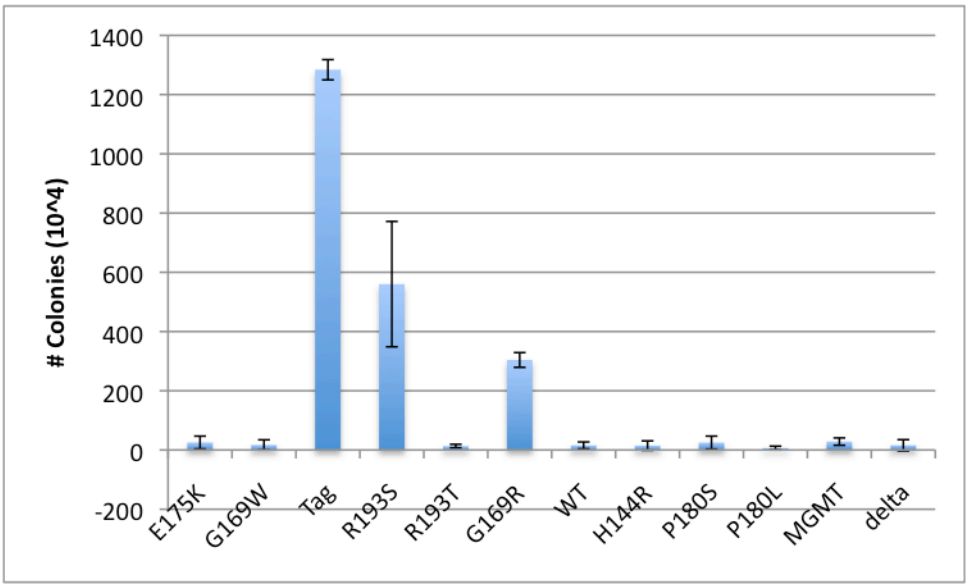

Figure 3. Viability from MV1932 gradients. In addition to the gradient plates, each mutant was plated (not under MNNG pressure) at a 10^4 dilution to check for mutant viability. Each mutant was plated in triplicate.

Characterization of naturally occurring ALKBH2 tumor mutants

We selected tumor mutants that overlapped with mutants isolated from our random mutagenesis located in positions at or close to active site (see **table 2** above). Of the mutants screened, only H144R had WT levels of protection against MMS (canonical methyl substrates), while in MNNG-selected mutants all characterized tumor mutations produced decreased protection against MMS (**Fig. 4)**. Unlike those, tumor mutants did not show any evidence of extended protection, neither against O6mG, nor against N3mA or ethenoA.

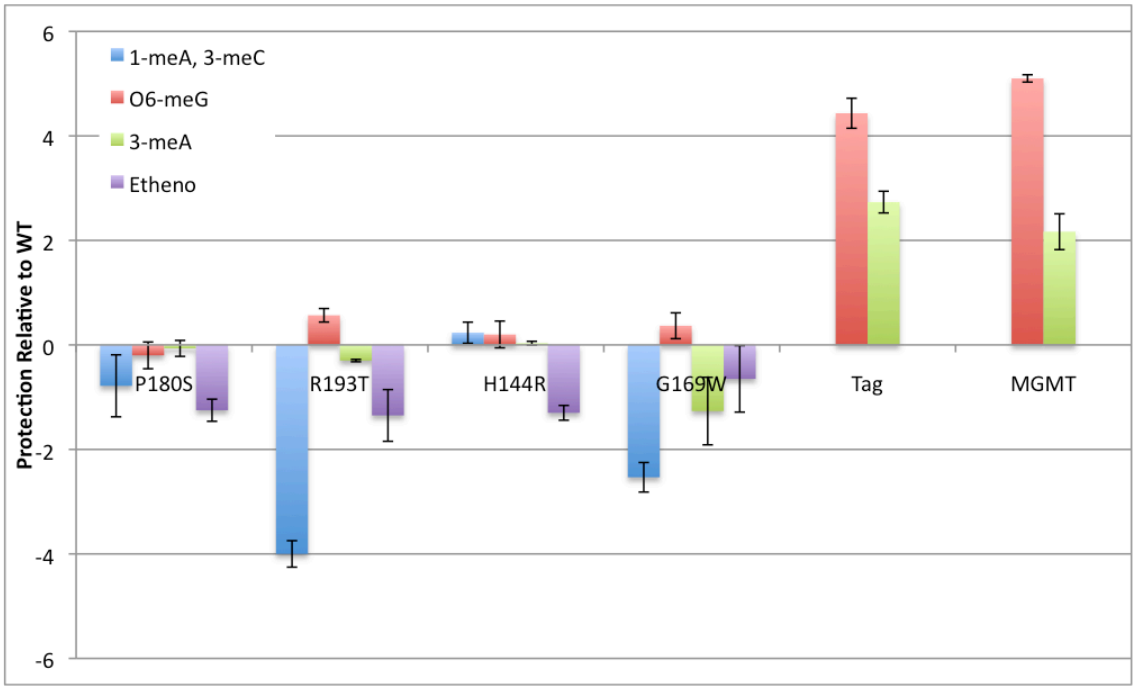

Figure 4. MMS, MNNG and CAA Gradients for Tumor Mutants. Each bar represents the protection by a mutant against a certain lesion relative to WT levels of protection. Blue column indicates mutants that were tested under MMS in oxidative demethylation deficient strains, therefore indicating repair canonical methyl substrates (1-meA, 3-meC). Red column indicates testing under MNNG in strains deficient in alkyltransferase activity, and therefore shows O^6-meG repair. Green indicates testing under MNNG in Tag deficient strains, indicating N^3-meA repair. Purple represents repair of etheno adducts in oxidative demethylation strain under CAA stress.

Discussion

Concordance between directed evolution and tumor somatic mutations

In this study, we tested ALKBH2 mutants evolved by random mutagenesis in our lab and mutants that have been isolated in various tumors for their ability to protect against agents inducing canonical and non-canonical ALKBH2 substrates. To our knowledge, this was the first characterization of ALKBH2 tumor mutants.

ALKBH2 is a 261 amino acid long protein, and there have been 33 ALKBH2 mutations isolated in tumors at 26 unique positions (Cerami 2012, Gao 2013). In our random mutagenesis isolation, we isolated 51 mutations at 39 unique positions. Of these two separate isolations, seven mutations overlap at position but not necessarily in amino acid substitution. Of these seven positions, four occur in the active site (**Fig. 1**). The four active site positions are G169, P180, R193 and S237. If the two sets were random, we would expect at most a single position overlap between the two isolations, so to have seven positions of overlap and the fact that four of them involved active site residues was a striking observation. Due to the similarity in lesions induced by MNNG with lesions induced during chemotherapy, as well as some induced endogenously (3-meA), it is possible that our selection may have mimicked the selective pressures ALKBH2 experiences in tumors.

Evolvability comes at a cost

Mutants isolated both by random mutagenesis and those found in tumors showed decreased protection to canonical methyl substrates 1-meA and 3-meC induced by MMS (**Fig 2**). This result indicates that P180, R193 and G169 are positions that play a critical role in oxidation of methyl substrates. The G169 residue is located in a β-sheet in the active site and near the flipped out damaged base (**Fig. 5**). Mutations at this residue could have altering effects on DNA interactions with ALKBH2, particularly considering the change in charge. The P180 residue is located in a loop that interacts with the flipped out, damaged base (**Fig. 5**). Mutations at this position, due to the flexible nature of loops, could cause conformation changes in the

active site that have the potential to explain an extended substrate (Dellus-gur 2013), which I will discuss further below. Lastly, the R193 position is located in a β-sheet near the active site. Interestingly, as seen from **Fig. 5b**, all of these mutations lie within the same looped structure of ALKBH2 (**Fig. 5b)**. We hypothesize that this loop is critical for maintaining the topology of the ALKBH2 active site, and that each mutation is causing a significant alteration of this loop that results in decreased repair of 1mA/3mC. The only mutant tested outside of this loop, H144R (Figure 3), did not result in the repair alterations (**Fig. 2**).

It is unclear whether the observed loss of function is selected for or simply tolerated for by tumors. ALKBH2 is important for protection against endogenous methylation and against the genotoxic effects of inflammation (Fu 2012, Falnes 2002, Trewik 2002, Duncan 2002). On the other hand ALKBH2 overexpression has been shown to decrease proliferation of some tumors (Gao 2011), pointing to an additional role of ALKBH2 as a checkpoint. Thus, activity many need to be down-regulated but not necessarily abolished. An alternative explanation is an evolutionary trade-off between repair of canonical substrates and protection against alternative substrates. Our identification of mutants protecting against N3mA and/or O6mG are consistent with this alternative hypothesis, although this does not appear to be the case in the selected tumor somatic mutations.

ALKBH2 mutants suggests etheno and methyl repair can be differentially modulated

Interestingly, the P180L mutant isolated from our random mutagenesis library appears to show enhanced protection against etheno adducts (**Fig. 2**). This mutant

exhibits decreased methyl protection (**Figure 2**, and confirmed *in vitro* by Christopher Troll in our laboratory), while showing enhanced etheno protection (**Fig. 2**). If confirmed *in vitro*, this mutant could show that canonical substrate preference can be modulated. While the main source of methyl lesions are chemotherapeutic drugs, etheno adducts are produced endogenously during periods of inflammation (Berquist 2012, Dosanjh 1994). In addition, etheno adducts are primarily mutagenic and the genetic instability caused by these mutagenic lesions could actually lead to the proliferation of cancer (Bartsch 2005). This P180L mutant could be a tool to study the differential effects allowing for inhibition of methyl repair versus etheno repair. However, this needs to be confirmed through *in vitro* analysis (see Chapter 3).

ALKBH2 Mutants Show Protection from S_N1 Toxicity

Three mutants that were isolated from our random mutagenesis selection appear to protect against alternative substrates. The P180L mutant appears to protect against O^6-meG, while R193S and G169R appear to protect against N^3-meA as seen from our gradient data in **Fig. 2**.

P180L selectively enhances repair in the PJ5 strain (orange bar, **Fig. 2**), which is deficient of alkyltransferase capabilities and therefore O^6-meG repair. This is in contrast to the MV1932 strain (green bar, **Fig. 2**), which is deficient of N^3-meA repair capabilities. The sole O^6-meG alkyltransferase in humans is MGMT, which was used as a control in our studies. Although P180L does not reach MGMT levels of protection, the fact that this mutant did not show protection in MV1932 (**Fig. 2**) and did not protect against endogenous N3mA lesions (**Fig. 3**), led us to conclude that the

protection observed is from O^6-meG protection, however O^6-meG repair will need to be confirmed *in vitro.*

The G169R and R193S mutants also show protection against N^3-meA, similar to Tag, which is an N^3-meA glycosylase used as a positive control in our study. This observation from the gradient plates is also confirmed by the viability measurements we did for these mutants. As seen in **Fig. 3**, G169R and R193 also enhance viability in comparison to other mutants. Since N^3-meA is often produced endogenously (Rydberg 1982), this further confirms that the gradient data represents N^3-meA protection by G169R and R193S. Due to this observation, we conclude that these two mutants appear to protect N^3-meA, which needs to be confirmed *in vitro*.

These lesions are not normally repaired by ALKBH2. This evolved capability could be due to the position of each mutant in the active site loop discussed above (**Fig. 5b**). Dellus-gur et. al (2013) have recently suggested that a proteins' ability to evolve is dependent on flexible, looped structures in the active site, built around a stable scaffold portion of the protein. Therefore, the positions of these mutations in an active site loop could be consistent with an evolved activity. Although our results suggest repair O^6-meG and N^3-meA *in vivo*, these results need to be confirmed biochemically to show ALKBH2 is repairing these lesions and not mediating protection by an alternative mechanism.

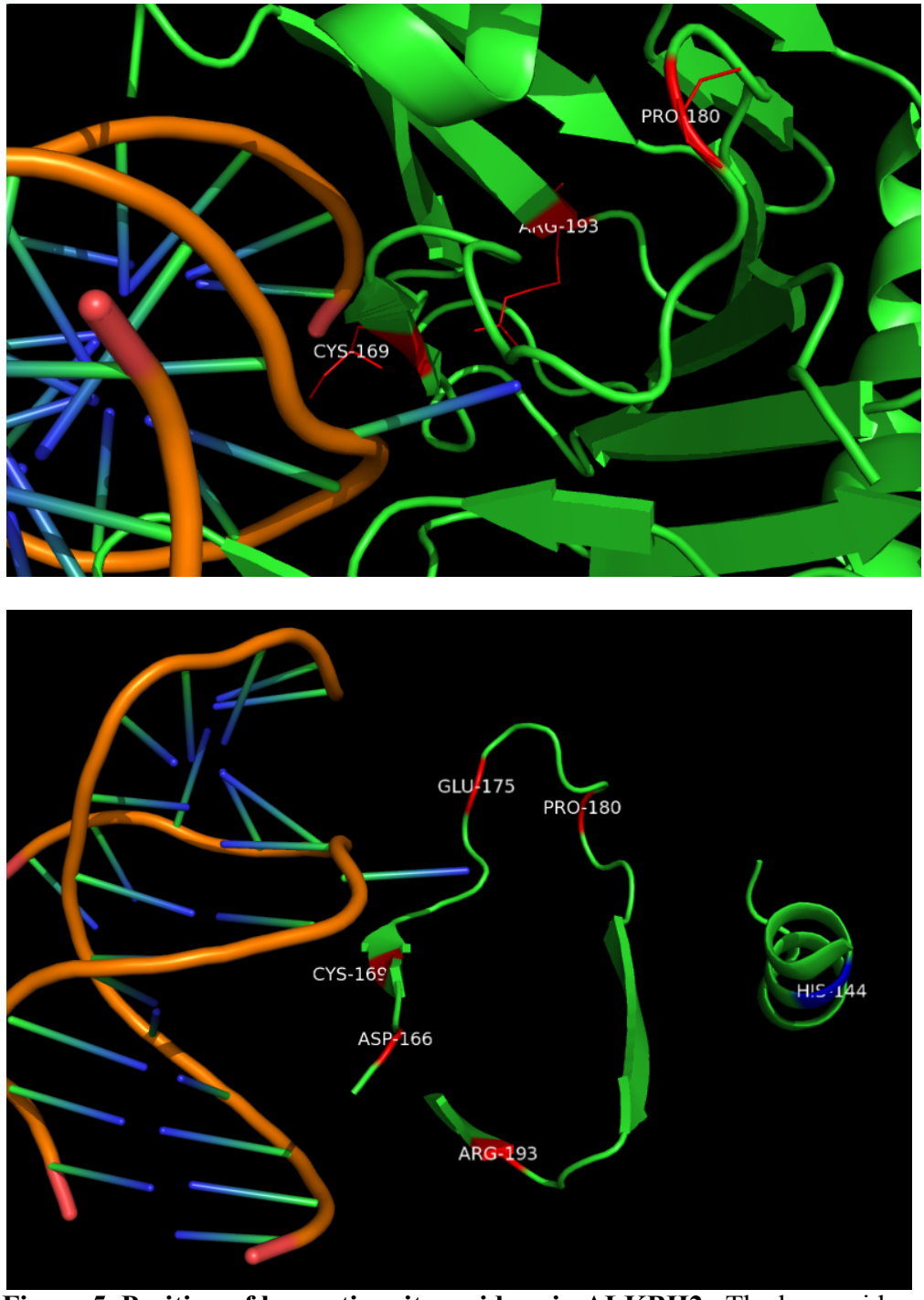

Figure 5. Position of key active site residues in ALKBH2. The key residues, P180, G169 and R193 have been highlighted in red. The G169 has been mutated to a cysteine for crystallization purposes. Each residue is shown to be positioned around a flipped out damaged base, in this case a 1-meA damaged base (Protein Databank, image 3BTY).

Conclusions

Alkylating agents can induce a variety of lesions with either cytotoxic or mutagenic effects and are used as antitumor agents, capitalizing on the high sensitivity of tumors to DNA damage (Sedgwick 2007, Nieminuszczy 2007, Segwick 2004). The human DNA repair protein ALKBH2 can be mutated in tumors. Here we show that a subset of these mutations (the ones that map to the active site and its vicinity) results in a loss of protection against canonical methyl substrates 1-meA, 3-meC. In addition, we have evolved a set of ALKBH2 mutants that have mutations at similar positions as tumor mutants, but different amino acid substitutions. We found a loss of protection against known ALKBH2 cytotoxic substrates in both sets of mutants. In addition, our selected mutants also show the potential of ALKBH2 to evolve protection against two additional cytotoxic substrates 3-meA and O^6-meG. These mutants (P180, G169, R193) are all in an active site loop. The critical role of loops for the evolution of extended substrate specificity has recently been reported as a general theme in protein evolution (Dellus-gur 2013). Finally, the increased protection by P180L against etheno adducts shows that activity against methyl adducts can be differentially modulated relative to etheno adducts.

ALKBH2 and other ALKBH homologues also play a role in cancer progression and suppression (Fujii 2013, Gao 2011, Tasaki 2011). Understanding the selections operating on ALBKH2, and the interplay between repair, pro- and anti-oncogenic effects of ALKBH2, should provide insights into the checks that keep cancer at bay.

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Chapter 3:
Future Directions and conclusions

The aim of this study was to characterize ALKBH2 mutants that were evolved in our random mutagenesis library and also that arise in tumors to gain insight into the selective pressures driving ALKBH2 evolution. To characterize the repair capabilities of each mutant, we used several *E.coli* complementation systems to test the ability of each mutant to protect against different alkylating agents. All mutants tested (P180L/S, R193T/S, G169W/R) were positioned in an active site loop that contacts the damaged DNA for repair, except for one (H144R) which is located in an alpha-helix away from the active site (Figure 5, Chapter 2). All these mutants, except H144R lost 1-meA and 3-meC protection, which are normal ALKBH2 substrates, to some degree. These results indicate that this active site loop is important for 1mA/3mC repair. In addition, mutants evolved through our MNNG selection (P180L, R193S, G169R) showed extended protection against other cytotoxic lesions. These include O^6-meG (P180L) and N^3-meA (R193S and G169R). These observations suggest the potential of ALKBH2 to evolve an extended spectrum of protection. In addition the P180L mutant also showed enhanced resistance to etheno adduct toxicity.

The main question remaining is the mechanisms involved in the observed extension of protection and an enhanced etheno adduct protection. Future research should focus on *in vitro* studies to characterize the nature of these effects.

First we need to establish whether protection is due to oxidative elimination of the adducts (methyl or etheno) or whether it is due to other mechanisms. This is especially true in the case of O^6-meG, since there is no known methylated oxygen

substrate for ALKBH2 and given that this lesion is normally repaired through the action of a different protein (MGMT, which is an alkyltransferase). Below I have proposed three sets of experiments to confirm the results observed in our *in vivo* studies (chapter 2) and discuss the implications of these findings in my concluding remarks.

Direct measurement of etheno adduct repair using the 2-hydrazinobenzothaizole-based etheno-adduct repair protocol (HERP)

This HERP method was developed by Shivange et. al (2015) as a way to measure direct repair of etheno adducts. When etheno adducts are repaired by the direct repair enzyme, ALKBH2, the original base is left in tact and the repaired etheno adduct is released as glyoxal (Yi et. al. 2013, Figure 1). The HERP assay is a colorimetric assay that uses 2-hydrazinobenzothiazole, which reacts with glyoxal to form a complex organic compound that has a yellow color and a peak absorption at 365nm. This reaction is specific for glyoxal, only, and therefore makes it a sensitive assay for etheno repair (Shivange et. al 2013).

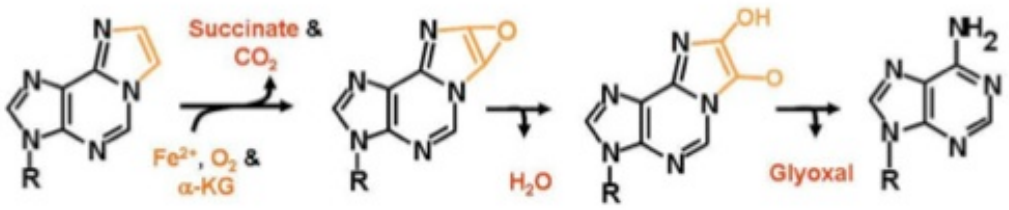

Figure 1. Direct repair by ALKBH2. ALKBH2 uses a direct repair mechanism to oxidize etheno adducts using Fe(II), alpha-ketoglutarate and oxygen and then releases the unstable intermediate as glyoxal.

We have already begun initial testing of this method by the generation of a standard curve for glyoxal detection, as seen in Figure 2. After showing successful glyoxal detection (Fig 2) we tested the ability to detect glyoxal released after etheno repair. This was carried out by treating dsDNA with varying concentrations of chloroacetaldehyde (CAA), which induces etheno adducts (Malaveille 1975). The CAA was removed by doing an ethanol precipitation, and the damaged DNA was then incubated with purified ALKBH2 and a reaction buffer containing Fe(II), alpha-ketoglutarate and ascorbate, which are substrates necessary for oxidative demethylation by ALKBH2 (Figure 1). The initial results show the ability to detect glyoxal after ALKBH2 repair (Figure 3). Future research should work to purify the mutants, specifically P180L, identified in Chapter 2, and characterize etheno adduct repair using the HERP method. This would allow us to confirm the slight increase in etheno protection by P180L observed in the CAA gradients shown in Figure 2, Chapter 2.

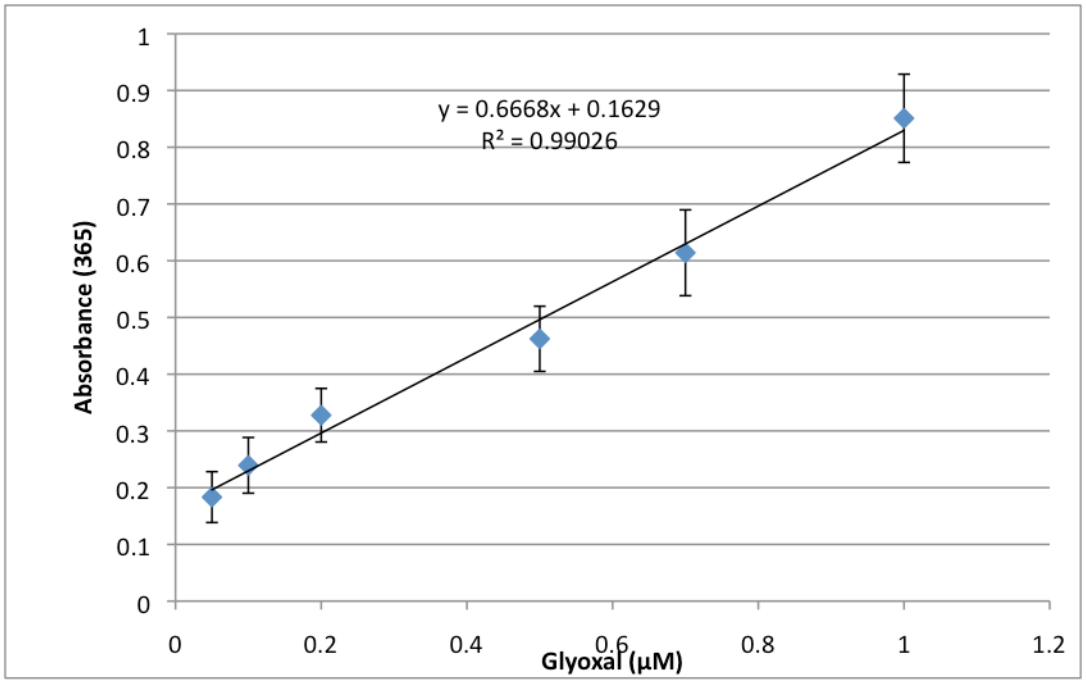

Figure 2. Glyoxal Standard Curve. This standard curve was generated using increasing concentrations of glyoxal at µMolar concentrations. Glyoxal has a peak absorbance at 365nm.

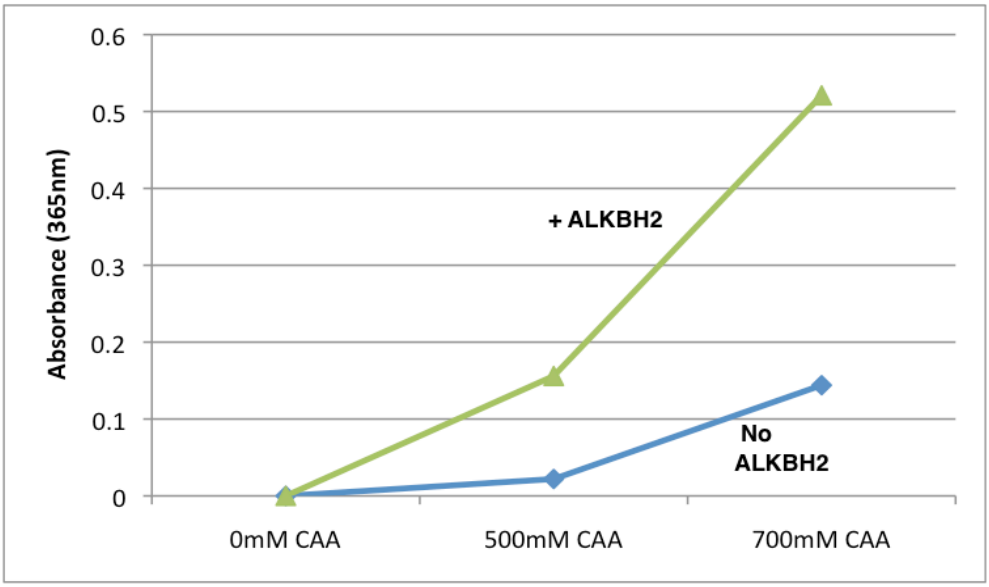

Figure 3. Direct repair of etheno adducts by ALKBH2. When ALKBH2 repairs etheno adducts, glyoxal is released. This assay is able to detect etheno adduct repair by measuring glyoxal release.

Measuring Repair of O^6-meG and N^3-meA

In order to investigate how the P180L mutant protects against O^6-meG and etheno adduct toxicity and how the G169R and R193S mutants protect against 3meA toxicity, we will inactivate catalysis by introducing the R110A mutation in each of these mutant backgrounds, which will knock out the base flipping mechanism that is essential for catalysis (Yi 2013). We will see whether the same panel of mutants with the R110A mutation can still protect, which would be indicative of an alternative mechanism (besides oxidative demethylation) of repair.

Abolishing protection through the R110A mutation would be consistent with catalytic removal of the lesions. If that is the case we have two collaborations in place that that would help confirm repair of O6meG or 3meA by oxidative demethylation.

Repair of O^6-meG

To measure O6mG repair *in vitro*, we have established a collaboration with Dr. Geoff Margison at the Unviersity of Manchester. He has an assay that measures O^6-meG alkyl transfer by MGMT in *E. coli* lysates. The assay developed by Dr. Margison's group involves incubating [^{3}H]-methylated DNA with limiting amounts of purified MGMT. After incubation, the radioactivity is determined by scintillation counting to determine the methyl-transfer to MGMT. This assay was used to validate the use of our PJ5 complementation system to characterize the impact of rare SNPs

on MGMT function (Figure 4). Therefore Dr. Margison is in a position to determine whether P180L can remove O6mG lesions and whether (unlike MGMT, which is a suicide enzyme) P180L ALKBH2 does so catalytically.

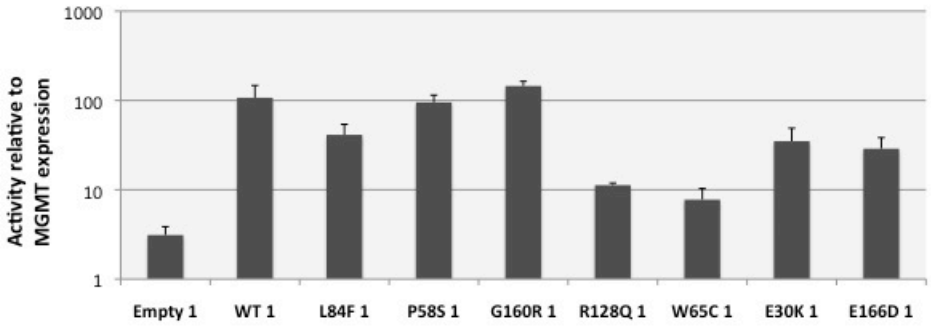

Figure 4. Biochemical Analysis of O^6-meG Repair by MGMT Polymorphisms. Dr. Geoff Margison has developed a biochemical assay to measure repair of O^6-meG by MGMT polymorphisms. The y-axis shows activity of each polymorphism relative to MGMT expression levels.

Although ALKBH2 primarily repairs damage using an oxidative demethylation reaction, we think there is the possibility that ALKBH2 could evolve methyltransferase ability. Another ALKBH homologue, ALKBH8, has tRNA methyltransferase activity (Kalhor 2003). This activity, however, requires a methyltransferase domain, which ALKBH8 has in addition to its oxidative demethylase domain (Songe-Moller 2010) and interaction with an accessory protein, TRM112 (Songe-Moller 2010).

Measuring N^3-meA Repair Using Nanopore Sequencing

In collaboration with Dr. Mark Akeson's group, we are developing a nanopore sequencing method for measuring etheno repair, which we believe can be adapted for measuring repair of N^3-meA as well. The method involves inducing etheno adducts by treating the DNA with CAA, and then incubating with the glycosylase AAG, which selectively releases etheno adducts, generating an abasic site in the process. This abasic site then has a strong signal that can be detected in the nanopore. We have already shown that this method is able to quantify etheno adduct formation in M13 DNA. We plan to use this method to measure ALKBH2 activity by pre-treating the CAA-treated DNA with ALKBH2 and measuring the associated decrease in abasic site generation.

This nanopore sequencing method should be adaptable for detection of N^3-meA repair as well. In this case M13 DNA will be sequentially treated with methyl iodide (a strong SN1 agent) and with Tag, an *E. coli* glycosylase that removes 3mA lesions, generating abasic sites at positions harboring N3mA lesions. This method could also be used to see if the G169R and R193S mutants remove the methyl lesions by oxidative demethylation, therefore reducing the number of abasic sites generated by sequential treatment with Tag glycosylase, as described above for etheno adduct repair. If it turns out that protection is not due to oxidative dememthylation of N3mA, nanopore sequencing can also be used to see whether G169R and R193S generate abasic sites by themselves, since N3mA is a labile lesion and spontaneous

depurination is the general mechanism by which N^3-meA is removed, facilitated by N3mA glycosylases.

Concluding remarks

To our knowledge this is the first analysis of the impact of ALKBH2 cancer somatic mutations on ALKBH2 repair. Our finding that individual mutants have differences in protection profile opens the door for epidemiological studies of cancer risk, progression, and drug resistance. A much larger somatic mutation database would be needed, though.

Our initial studies identified select ALKBH2 mutants that show enhanced repair of canonical substrates and/or an extended substrate profile. The P180L mutant isolated thorough our random mutagenesis library showed enhanced protection against etheno toxicity, as well as protection against O^6-meG, which is not a known ALKBH2 substrate. The R193S and G169R mutants, also evolved in our mutant library, showed enhanced protection against N^3-meA, another lesion not known to be an ALKBH2 substrate. We believe this extended ALKBH2 protection profile could be associated with shifts in the positioning of the active site loop (Chapter 2). This agrees with recent studies suggesting that loop structures concentrate the functional plasticity (and ability to evolve new activities) of proteins (Dellus-Gur 2013). This opens up possibilities for characterization of ALKBH2 orthologs for alternative substrate repair (other groups are already profiling ALKBH enzymes in prokaryotes and in Arabidopsis; Meza 2012), as well as rational

engineering of the R193-G169 loop for additional enhancements in repair (Figure 5B, Chapter 2). These studies may anticipate the evolution of ALKBH2 in tumors under selective pressure by chemotherapeutic alkylating agents.

The availability of ALKBH mutants with differences in O6mG vs N3mA repair can also be used as a tool to investigate the relative therapeutic contributions of N3mA vs. O6mG since both lesions are generated by chemotherapeutic alkylating agents and there is evidence that both mechanisms are important for tumor killing (Fu and Samson 2012)

The P180L mutant, if shown to have O^6-meG repair abilities, would have significant implications in cancer treatment. MGMT, the main methyltransferase that repairs O^6-meG, is used for selective enrichment of transduced stem cells (Davis et. al 2000, Bowman et. al 2003). Single point mutations in MGMT protect MGMT from inhibitors, but allow direct repair of O^6-meG to continue (Crone and Pegg 1993, Crone et. al 1994, Loktionova and Pegg 1996). Therefore, during treatment with chemotherapeutic agents (which induce O^6-meG and also inhibit MGMT repair), tumor cells are sensitive to alkylating agents while MGMT-mutant transduced stem cells are protected and allowed to repopulate hematopoietic compartments. If ALKBH2 P180L is shown to repair O^6-meG, this mutant also has the added capability of repairing endogenous, mutagenic etheno adducts, which are not only increased during periods of inflammation but are themselves believed to be carcinogenic. Therefore, ALKBH2 P180L could have the potential to enhance enrichment of transduced stem cells.

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