Genetic Analysis of Diverse Functions of DNA Mismatch Repair in Mammalian Cells

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CERTIFICATE OF APPROVAL

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6-TG-6-thioguanine

ATM-Ataxia telangiectasia mutated

ATP-adenosine triphosphate

ATR-Ataxia talengectasia and Rad3 related

CDA-cytidine deaminase

CHK-checkpoint kinase

CTP-cytosine triphosphate

dCMP-deoxycytidine monophosphate

DCTD-deoxycytidylate deaminase

DNA-deoxyribonucleic acid

dUMP-deoxyuridine monophosphate

EXO-exonuclease

GHL-gyrase b, heat shock protein 90, MutL

GTP-guanosine triphosphate

HNPCC-hereditary non-polyposis colon cancer

IDL-insertion/deletion loop

MLH-MutL homologue

MMR-mismatch repair

MNNG-N-methyl-N′-nitro-N-nitrosoguanidine

MSH-MutS homologue

MSI-microsatellite instability

MTS-Muir-Torre syndrome

Mut-mutator

PCNA-proliferating cell nuclear antigen

PCR-polymerase chain reaction

PMS-Post meiotic segregation

RFC-replication factor C

RNA-ribonucleic acid

RPA-replication protein A

RT-PCR-reverse transcriptase PCR

SAM-S-adenylmethionine

siRNA-small interfering RNA

TTP-thymidine triphosphate

WT-wild type

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Abstract

DNA mismatch repair (MMR) is a versatile cellular mechanism that corrects errors formed during replication, responds to certain types of DNA damage, and helps control recombination. When mismatches arise in DNA due to polymerase errors, MMR responds by assembling a multi-protein complex that detects and removes the mismatch. This complex is also necessary to signal apoptosis in response to certain types of DNA damage. Central to MMR's function in mutation avoidance is the heterodimer MutLα, composed of MLH1 and PMS2, which acts to couple mismatch recognition to downstream steps. Both MLH1 and PMS2 contain ATPase domains in their N-termini, and it had previously been shown in yeast and in human *in vitro* analyses that MutLα ATP binding and hydrolysis is necessary for repair. However, the relative contributions of each ATPase domain to repair had yet to be examined *in vivo* in mammalian cells. I analyzed the effect of mutations in the highly conserved ATPase domain of MutLα *in vivo* in mouse cell culture. I observed that mutations impacting ATP binding and hydrolysis in the MutLα protein MLH1 impact repair to a greater degree than the equivalent ATPase domain mutations in MLH1's binding partner PMS2, as measured by instability at microsatellite loci. I also examined the effect of the mutations on the cytotoxic response to the methylation mimetic 6-thioguanine (6-TG), which elicits a MMR-dependent apoptotic response. Consistent with the mutator results, mutations in the ATPase domain of MLH1 caused resistance to 6-TG, while mutations in the ATPase domain of PMS2 did not. These results indicate a functional asymmetry in the contributions of the ATPase domains of the MutLα partners to repair and that MLH1 and PMS2 may have distinct roles during repair.

In the second part of my research, I examined the role of the nucleotide pool regulator deoxycytidylate deaminase (DCTD) in the MMR-dependent response to 6-TG. Previous work with the yeast homologue *DCD1* had shown that *DCD1* plays a role in this response. DCTD catalyzes the conversion of dCMP to dUMP and thus helps maintain the dCTP:dTTP balance within the cell. I found that reduced expression of DCTD in human cells causes increased resistance to 6-TG, indicating DCTD is a necessary component in the MMR-dependent response to 6-TG in human cells. Surprisingly, cells derived from $Dctd^{ko/ko}$ mice do not display significant resistance to 6-TG. When nucleotide pool levels were measured, the cells derived from the $Dctd^{ko/ko}$ mice did not exhibit as significant dCTP:dTTP imbalance as previously reported in established rodent cell lines. In addition, the human cells displayed no significant imbalance. These results suggest an adaptive response to maintaining the dCTP:dTTP pool levels in the absence of *Dctd* in the mouse, and also suggest that resistance to 6-TG toxicity in human cells can occur without dCTP:dTTP pool imbalance.

CHAPTER ONE

Background and significance

Cellular replication fidelity

Every time a human cell divides, it must replicate 3×10^9 basepairs of DNA with high fidelity, yet only makes on average one mistake per 10^9 nucleotides (Drake, 1999). This accuracy is accomplished by a few mechanisms. First is the semi-conservative nature of DNA replication and Watson-Crick base pairing of nucleotides whereby the nucleotides in the parental strand provide a template for the new strand (Meselson and Stahl, 1958). If a nucleotide is placed incorrectly, two of the DNA polymerases responsible for chromosomal replication contain 3' to 5' proofreading exonuclease functions that can detect and repair an improperly placed base (Shcherbakova et al., 2003). Failing both of these, cells have a diverse repair mechanism called DNA mismatch repair that can correct errors missed by polymerase.

DNA mismatch repair

DNA mismatch repair (MMR) is a highly conserved process that maintains genome integrity by correcting errors during DNA replication that escape DNA polymerase proofreading exonuclease function, suppressing recombination between heterologous sequences, and signaling apoptosis in response to certain types of chemical damage (Kolodner and Marsischky, 1999; Li, 2008; Stojic et al., 2004a). In cells deficient for MMR, mutation rates can increase by as much as 1000-fold (Harfe and Jinks-Robertson, 2000), replication between repeated divergent sequences of DNA can increase and apoptosis in response to certain types of DNA damage can be compromised. The importance of MMR is highlighted by increased cancer incidence in humans and mice when MMR is not functional (Buermeyer et al., 1999a). In humans, inherited mutations in certain MMR genes cause hereditary non-polyposis colon cancer (HNPCC),

the most common form of inherited colon cancer (Lynch and de la Chapelle, 1999; Vasen, 2007). The relationship between MMR and cancer has helped to increase our understanding of the process of MMR.

MMR in *E. coli*

Due to a combination of biochemical and genetic approaches, the process of mismatch repair is best understood in the bacterium *Escherichia coli*. The defining of prokaryotic MMR started with the discovery of the MutH, MutL, and MutS mutator genes during screens for genes that when inactivated increase mutation rates in *E. coli* (Liberfarb and Bryson, 1970; Siegel and Bryson, 1967; Siegel and Kamel, 1974). Eventually, all elements of the repair process were described and a fully-reconstituted system was defined using purified proteins in an *in vitro* assay (Lahue et al., 1989) (Figure 1-1).

Initiation of repair by MutS

Mismatch repair initiation is facilitated by the homodimeric MutS protein, which in concert with the β-clamp of polymerase III, translocates along the DNA, likely in association with the replication apparatus (Lopez de Saro et al., 2006). MutS has as much as a 17-fold higher binding affinity for mismatched DNA than non-mismatched DNA (Jiricny et al., 1988), and initiates repair once it contacts base-base mismatches or small insertion/deletion loops (IDLs) (Parker and Marinus, 1992; Su and Modrich, 1986). MutS is a homodimer; however, asymmetry is seen in how the homodimer functions, as only one unit of MutS binds mismatched DNA (Sixma, 2001). Recognition of and binding to a mismatch causes an ATP-dependant conformational change in MutS that

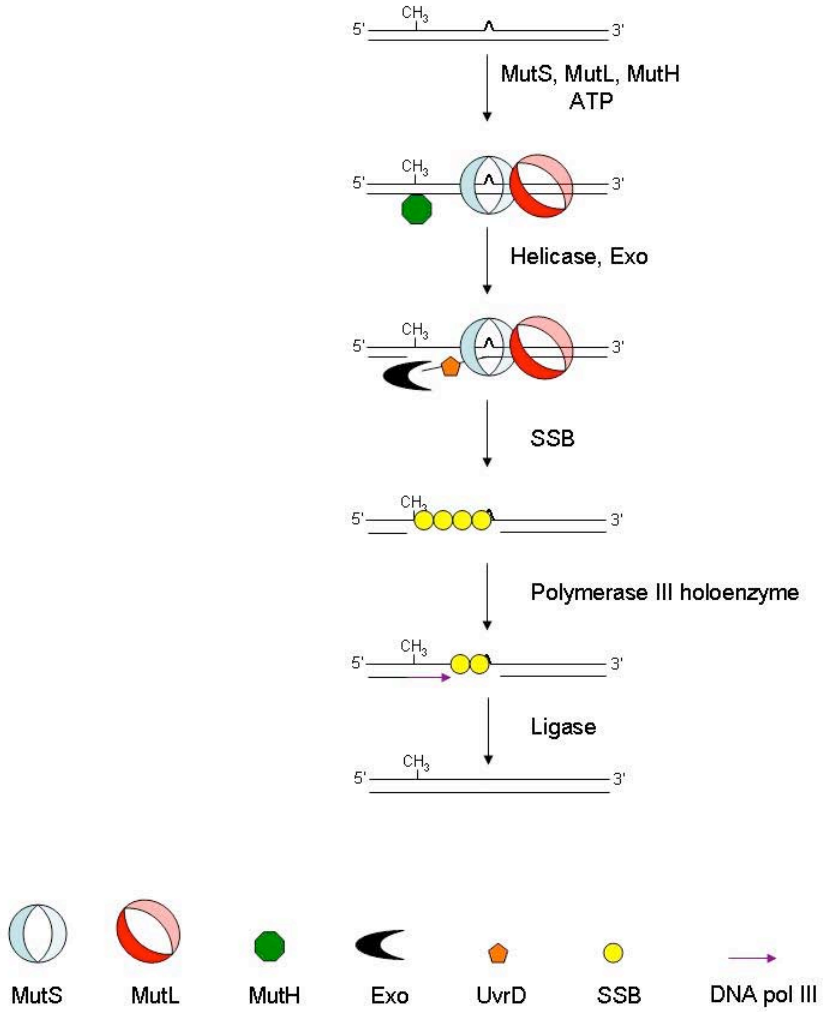


Figure 1-1. Reconstitution of MMR *in vitro* in *E. coli*. MMR is initiated by MutS binding to a mismatch or IDL. MutS undergoes an ATP-dependent conformational change to recruit MutL. MutL activates the endonuclease activity of MutH, which nicks the DNA at a hemi-methylated site, either 5′ or 3′ to the mismatch. Helicase (UvrD) unwinds the DNA and one of four exonucleases excises the DNA past the mismatch. SSB protects the single-stranded DNA. Resynthesis is carried out by DNA polymerase III.

exposes a domain for binding the MutL protein (Grilley et al., 1989; Mendillo et al., 2009).

MutL, the matchmaker

MutL is known as a molecular matchmaker and serves to couple mismatch recognition by MutS to downstream MMR events. Similar to MutS, MutL exists as a homodimer and possesses ATPase activity (Ban et al., 1999; Ban and Yang, 1998; Spampinato and Modrich, 2000), albeit much weaker than that of MutS. MutL also binds DNA, however in a mismatch independent manner (Junop et al., 2003). In addition to interacting with MutS, MutL has been shown to interact with downstream MMR factors, including the endonuclease MutH (Hall and Matson, 1999), the helicase UvrD (Hall et al., 1998), and the β clamp of DNA polymerase III (Lopez de Saro et al., 2006).

Strand discrimination

Together, MutS and MutL recruit MutH and activate its endonuclease activity (Grilley et al., 1990). Methyl-adenine directed nicking of the DNA by MutH occurs 5' to a GATC sequence either upstream or downstream of the mismatch. In bacteria, GATC sequences are symmetrically methylated at the N^6 position of the adenine, and mismatch repair takes advantage of this to discriminate between template and newly replicated DNA. MutH will not nick DNA at a fully methylated GATC, but will nick at a hemi-methylated sequence, on the daughter, or newly synthesized strand, which is transiently unmethylated during replication (Lu et al., 1983). This nicking allows MutS and MutL to load UvrD (Helicase II) onto the DNA to unwind it prior to excision (Dao and Modrich, 1998). MutL directly interacts with UvrD (Hall et al., 1998), and most likely directs it to

unwind in the direction of the mismatch. UvrD unwinds DNA 3′ to 5′ with respect to the strand it is bound to (Matson, 1986), so it must be given direction to bind to the correct strand to unwind towards the mismatch.

Excision and resynthesis

Once the DNA has been unwound, excision of the new strand is accomplished by one of four exonucleases, both 3′-5′ (RecJ, ExoVII) and 5′-3′ (ExoI, ExoX) (Burdett et al., 2001). The presence of any one of these exonucleases facilitates repair, as it requires mutation of all four of exonucleases to abolish repair (Burdett et al., 2001). After excision has occurred past the mismatch, single-stranded binding protein (SSB) coats and protects the template (parent) strand from excision, and DNA polymerase III holoenzyme carries out resynthesis of the DNA (Modrich and Lahue, 1996). Finally, DNA ligase seals the remaining nick to complete repair.

Contribution of MutL ATPase domain to MMR

As the component responsible for relaying mismatch recognition to the downstream steps of MMR, MutL plays a central role in MMR. A critical domain of MutL function is the ATPase domain, which has been shown to be necessary for most of the above steps requiring MutL. Once MutL has been recruited by MutS to the mismatch, the activation of MutH depends on ATP binding by MutL (Ban and Yang, 1998). In addition, loading of UvrD to the nicked DNA by MutL requires bound ATP (Matson and Robertson, 2006). Analysis with a strain of *E. coli* harboring an amino acid substitution in MutL at the residue responsible for ATP hydrolysis showed that MutL was competent for binding MutS and DNA, activating MutH, and loading UvrD even without the ability to hydrolyze ATP (Robertson et al., 2006). However, a strain mutant for

binding ATP was not able to interact with MutS, activate MutH, or load UvrD (Robertson et al., 2006). The role of ATP hydrolysis may lie in dissociation of the MutL/DNA complex, allowing the DNA polymerase III holoenzyme to load in order to start resynthesis. MutL has also been shown to interact with the β clamp and loader of the polymerase III holoenzyme, and this has been shown to depend on MutL ATP hydrolysis (Li et al., 2008).

Eukaryotic DNA mismatch repair

The mechanism of eukaryotic mismatch repair is similar to that of *E. coli,* albeit for strand discrimination, but has a higher level of complexity in terms of the proteins involved (Table 1-1). Both MutS and MutL have numerous homologues in eukaryotes, and these homologues function as heterodimers rather than the homodimers seen in *E. coli*.

MutS homologues

Mismatch recognition is facilitated by the MutS homologue (MSH) proteins, which form heterodimers of either MSH2/MSH6 (MutSα) or MSH2/MSH3 (MutSβ) (Acharya et al., 1996; Drummond et al., 1995). MutSα binds specifically to mismatched bases and 1-2 bp IDLs, while MutSβ binds to IDLs of ≥2 bp (Genschel et al., 1998). The MSH proteins, similar to MutS, possess ATPase activity (Alani et al., 1997; Iaccarino et al., 1998). While not necessary for mismatch binding, ATP binding and hydrolysis are thought to be needed for coordination of downstream events, including translocation along the DNA and protein/protein interaction with MutLα (Blackwell et al., 1998a; Blackwell et al., 1998b; Mendillo et al.).

Bacterial protein	Eukaryotic homologue	Function
MutS	MutSα (MSH2, MSH6) MutSβ (MSH2, MSH3)	Mismatch recognition
MutL	MutLα (MLH1, PMS2) MutLβ (MLH1, PMS1)	Endonuclease activity, Coordination of downstream steps
MutH	No homolog identified	Endonuclease
UvrD	No homolog identified	Helicase
ExoI, ExoVII, ExoX, RecJ	EXO1	Exonuclease
SSB	RPA	Single-stranded binding protein
β-clamp	PCNA	Replication clamp
	RFC	Clamp loader
DNA pol III	DNA pol δ	DNA Polymerase

Table 1-1. MMR proteins in *E. coli* and eukaryotic homologues.

MutL homologues

MutL function in eukaryotes also is accomplished by multiple homologues that act as heterodimers (Li and Modrich, 1995; Prolla et al., 1994a). The main MutL activity in eukaryotes is carried out by MutLα, which is comprised of MLH1 and PMS2 (Pms1 in yeast) (Prolla et al., 1994b). Two other MutL complexes exist, MutLβ (MLH1 and PMS1) (Pang et al., 1997; Raschle et al., 1999) and MutLγ (MLH1 and MLH3) (Lipkin et al., 2000) but their contribution to mutation avoidance is minimal and less well defined than MutLα. Both components of MutLα contain ATPase domains that are critical for proper MMR function, although the details of how the ATPase domains of MLH1 and PMS2 contribute to repair have not been fully resolved. The ATPase activity of MutLα will be discussed in full in Chapter 3.

Strand discrimination

A prominent difference between *E. coli* and eukaryotic mismatch repair is the mechanism of faithfully choosing the newly replicated strand for repair, referred to as strand discrimination. Unlike *E. coli* DNA, yeast DNA is not methylated, so methyl-directed strand discrimination cannot be the mechanism for strand discrimination. Mammalian DNA is methylated, primarily at CpG dinucleotides. However, no evidence exists to support methyl-directed strand discrimination in mammalian cells (Drummond and Bellacosa, 2001). In fact, cells lacking the major maintenance DNA methylase reduce tumor incidence in a mismatch repair deficient background (Trinh et al., 2002). How eukaryotic MMR discriminates between parental and daughter strands is not fully understood. *In vitro*, single-strand nicks direct repair to that strand, and it is thought that nicks from Okazaki fragments during lagging strand replication may create a signal for

MMR to direct repair to the nascent strand (Erdeniz et al., 2005). However, this explanation is not an easy fit for strand discrimination for the largely nick free leading strand synthesis. Here, the eukaryotic β clamp, PCNA (proliferating cell nuclear antigen) may serve as a possible interface between the replication machinery and MMR to help distinguish the new from the old strand (Umar et al., 1996).

Excision and resynthesis

Only one exonuclease, EXO1, has been identified to be clearly involved in eukaryotic MMR (Tishkoff et al., 1998; Tishkoff et al., 1997). Interestingly, EXOI shows a strict 5'-3' polarity *in vitro* (Szankasi and Smith, 1992). However, eukaryotic MMR, like bacterial MMR, is capable of bidirectional mismatch repair. In addition, neither yeast *ExoI* mutants nor mouse *ExoI* knockouts show a mutator phenotype characteristic of MMR deficiency, strongly suggesting that a yet-undiscovered redundant exonuclease may be active in MMR (Amin et al., 2001; Tishkoff et al., 1997; Wei et al., 2003).

No specific DNA helicase has been implicated in eukaryotic MMR. The helicase SGS1 has been shown to interact with MLH1 (Pedrazzi et al., 2001), but yeast cells deficient in SGS1 do not display the major characteristics of MMR deficiency (Myung et al., 2001), suggesting it is either involved in other aspects of DNA metabolism or is functionally redundant with another DNA helicase. No MMR-specific ligase has been identified in eukaryotic cells.

***In vitro* repair**

Reconstitution of repair *in vitro* has been described for human MMR, for both 5' and 3' nick-directed repair (Figure 1-2) (Constantin et al., 2005; Dzantiev et al., 2004). These

studies show that only four protein complexes, MutSα, MutLα, RPA (a single-stranded DNA binding protein) and EXO1, are required for excision on a mismatched DNA substrate when the single-stranded nick is located 5′ to the mismatch (Dzantiev et al., 2004). MutSα is necessary for mismatch recognition and ATP-dependent activation of EXOI. RPA enhances excision and creates smaller excision tracts. Although not required to initiate excision, MutLα suppresses excision on homoduplex DNA substrates, ensuring that the reaction is dependent on a mismatch. When the substrate contains a single-strand nick 3′ to the mismatch, PCNA and RFC (replication factor C) are also required (Dzantiev et al., 2004). PCNA has been implicated in the both the early and later steps of MMR (Johnson et al., 1996; Umar et al., 1996). Its interaction with MutS homologues suggests a role for PCNA in localizing MutS complexes to the mismatch (Kleczkowska et al., 2001). In addition, PCNA was shown *in vitro* to be necessary to suppress 5′ to 3′ excision activity that would lead excision away from the mismatch, when repair is being directed from a 3′ nick (Dzantiev et al., 2004). RFC, the clamp loader, is necessary for the PCNA-mediated suppression of 5′to 3′ excision. In both 5′ and 3′ nick-directed MMR, polymerase δ, along with PCNA, RPA, and RFC, are necessary for the resynthesis step following the excision reaction (Constantin et al., 2005).

MutLα endonuclease activity

Interestingly, MutLα is required for the initiation of excision when the ss nick resides 3′ to the mismatch, while it is not required for initiation of excision when the nick resides 5′ to the mismatch. Additionally, EXOI is also required for 3′ nick-directed repair, even though it is only capable of excision with 5′ to 3′ polarity. These

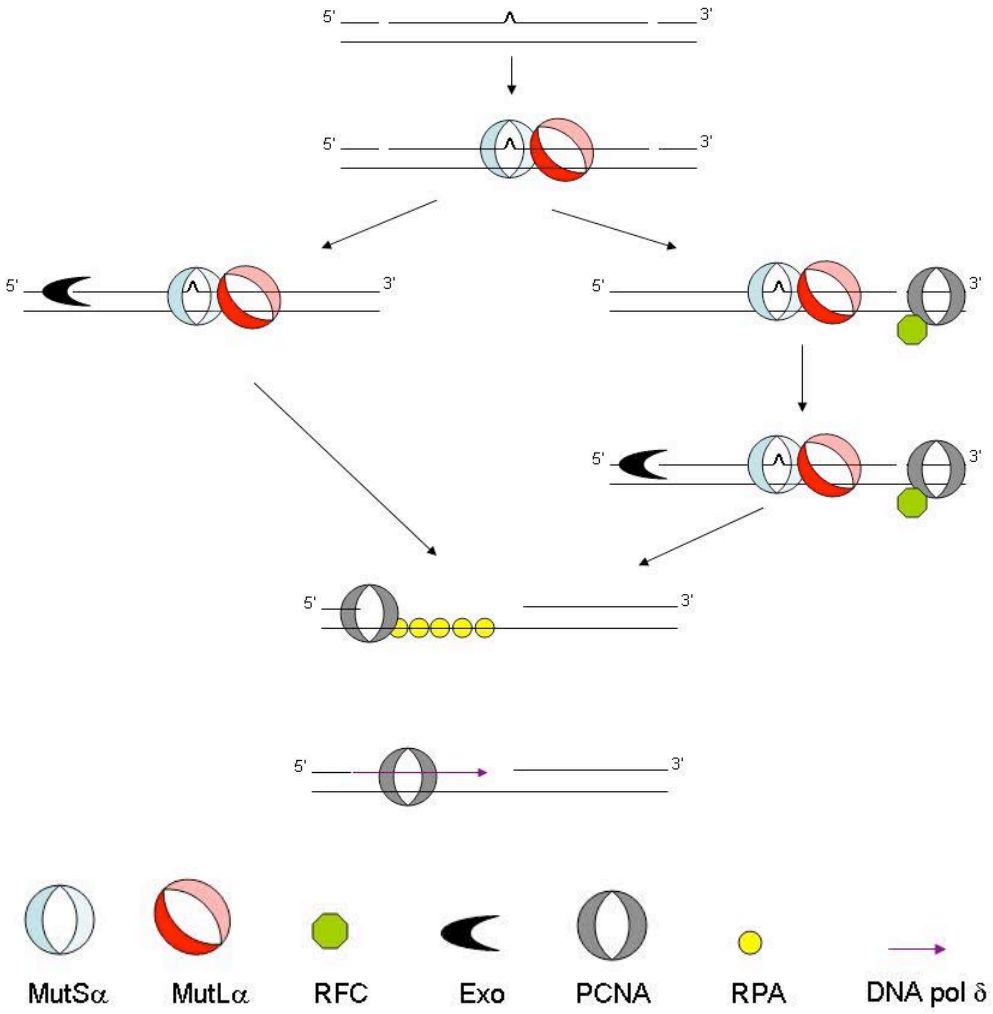


Figure 1-2. Reconstitution of repair *in vitro* in eukaryotic cells. MMR is carried out on a synthetic DNA substrate designed to contain a nick either 5′ or 3′ of the mismatch. MutSα binds the mismatch and recruits MutLα. If the nick is located 5′ to the mismatch, EXO1 is loaded at the nick and excises past the mismatch. If the nick is located 3′ to the mismatch, the endonuclease function of MutLα is activated, nicking the DNA 5' of the mismatch. PCNA and RFC ensure excision won't lead away from the mismatch. EXO1 can then load at the 5′ nick and carry out excision past the mismatch. RPA coats the ssDNA to protect it, and PCNA and polδ complete repair synthesis.

discrepancies can be explained by a recently described latent endonuclease function of MutLα (Erdeniz et al., 2007; Kadyrov et al., 2006). As described above, MutLα is needed for initiation of repair only when the nick resides 3′ to the mismatch. It was later shown that latent MutLα endonuclease function is activated when the nick is located 3′ to the mismatch. This endonuclease activity creates nicks on the DNA, eventually creating ss nicks 5′ to the mismatch. Presumably, this allows the excision complex, including EXOI, to load at a nick 5′ to the mismatch. Therefore, EXO1 can excise with 5′ to 3′ polarity even when the initial nick is on the 3′ side of the mismatch. The endonuclease domain resides in *PMS2*, and is dependent on ATP hydrolysis of MutLα, as double mutations in the *MLH1* and *PMS2* ATP hydrolysis residues together abolishes endonuclease activity, as measured by an *in vitro* endonuclease assay (Kadyrov et al., 2006).

MMR and recombination

MMR is involved in several aspects of both mitotic and meiotic recombination. MMR suppresses recombination between divergent, homeologous sequences to maintain genome integrity (Surtees et al., 2004). Homeologous sequences are regions of DNA that contain a high level of sequence similarity, e.g. a gene from two closely related species. Additionally, the genomes of most organisms contain many regions of similar but not exact repeated DNA, and recombination between these sequences could result in genomic instability. The resultant mismatches that occur between divergent DNA in the early stages of homeologous recombination are substrates for MMR, which acts to suppress the potential recombination. In bacteria, sequences that diverge by as little as 3% are prevented from recombining by MMR (Worth et al., 1994). In yeast deficient for MMR,

recombination between two sequences with 91% similarity went up 100-fold compared to MMR-proficient cells (Datta et al., 1996).

MMR is also involved in regulating gene conversion, which is the unidirectional transfer of genetic information from one allele to another. Gene conversion can occur during double strand break (DSB) repair by homologous recombination in both mitotic and meiotic cells. DSBs can be repaired by invasion of the 3' ends of the broken DNA into donor sequences of homologous chromosomes (Meselson and Radding, 1975). Synthesis is primed from the ends of the invading sequences using the donor as template. Heteroduplex DNA is formed where the invading sequence anneals to the donor sequence. If these sequences are not an exact match, mismatches arise that are substrates for MMR. MMR can either abort the strand exchange process and allow the break to be repaired by non-homologous end joining, or it can process the mismatch. Repair of these mismatches by MMR can result in gene conversion (Surtees et al., 2004).

In contrast to mitotic recombination, different MMR complexes are needed for meiotic recombination. MutSα and MutSβ are most likely not needed for meiotic recombination, as mouse knockouts of *Msh2, Msh6,* and *Msh3* display no defects in meiosis (Wei et al., 2002). Instead, the MutS homologues MSH4 and MSH5 are needed for meiotic recombination. Mouse knockouts of *Msh4* are sterile, and chromosomal analysis indicates reduced chromosome pairing and alignment in MSH4-deficient sperm (Kneitz et al., 2000). A similar phenotype was observed in *Msh5* mouse knockouts (de Vries et al., 1999; Edelmann et al., 1999). MutL homologues *Mlh1* and *Pms2* are also important for meiosis. Sperm deficient in MLH1 show normal chromosome pairing, but premature chromosome separation and a ~10-fold reduction in chiasmata, suggesting a

role in crossing-over (Baker et al., 1996). MLH1 has been localized to discrete foci on meiotic chromosomes, again consistent with a role in cross-over (Baker et al., 1995). Sperm from *Pms2*$^{-/-}$ mice exhibit abnormal chromosomal pairing, which may account for infertility observed in *Pms2*-deficient males (Baker et al., 1995).

MMR and DNA damage response

In addition to increasing replication fidelity by correcting errors during replication, MMR also is necessary for signaling apoptosis in response to certain DNA damaging agents. MMR has been shown to play a role in the response to S_N1-type methylating agents, cisplatin, and gamma irradiation (Stojic et al., 2004a). MMR is necessary to signal apoptosis in response to these agents, as cells deficient for MMR are more resistant to the cytotoxic effects of the damage than cells with intact MMR (Anthoney et al., 1996; Buermeyer et al., 1999b; Kat et al., 1993). The most well understood type of MMR-dependent damage response is to S_N1-type methylating agents.

S_N1-type methylators include N-methyl N-nitrosourea (MNU), N-methyl N'-nitro N-nitrosoguanidine (MNNG), and temozolomide. S_N1-type methylators create O^6-methylguanine lesions in DNA (Karran and Bignami, 1992). ^{Me}G in the template strand can base pair with either C or T (Patel et al., 1986a; Patel et al., 1986b), and the resultant mismatch is recognized by the MMR apparatus (Duckett et al., 1996). Treatment with these methylating agents induces rapid recruitment of MMR proteins to the chromatin (Schroering and Williams, 2008), eventually leading to G_2/M arrest and apoptosis. Two models have been proposed to explain how mismatch repair responds to the lesions (Figure 1-3) (Karran, 2001). In the first model, termed the direct signal model, MMR recognizes the $^{O6\text{-}Me}G$ lesion and directly signals to downstream effectors to initiate

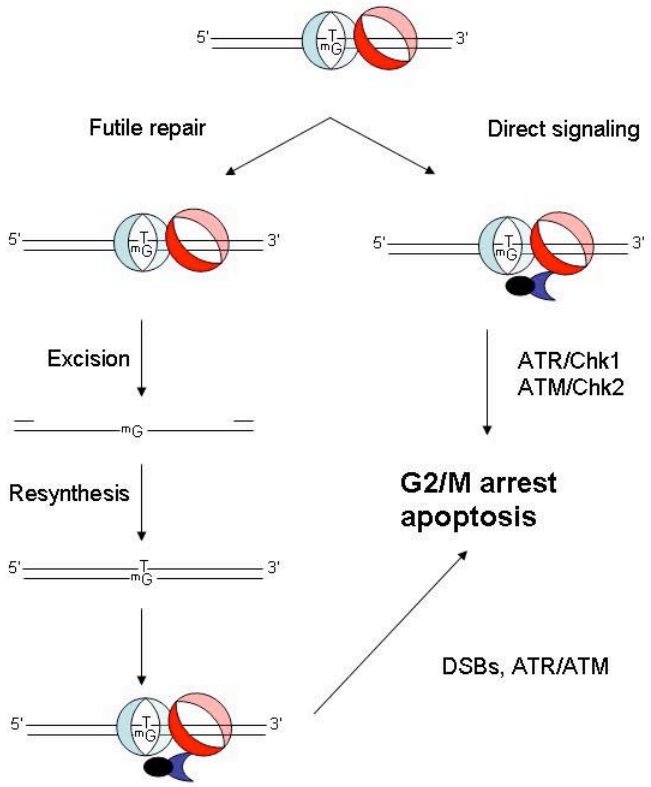


Figure 1-3. MMR-dependent response to methylation damage. Treatment with methylating agents such as MNNG creates $^{O6\text{-}Me}$G lesions in the DNA. MMR recognizes these lesions and responds, eventually signaling cell cycle arrest and apoptosis. Two models are shown for how MMR responds to the damage. The models are not mutually exclusive, and evidence exists to support both models. In the direct signaling model, MMR senses the damage and directly signals to ATM/ATR to induce G2/M arrest and apoptosis (ref). In the second model, futile repair, MMR attempts to correct the lesion but because it is in the template strand, repair is futile and eventually causes double-strand breaks, signaling G2/M arrest and apoptosis through ATM/ATR (ref).

apoptosis (Yoshioka et al., 2006). The second model, termed futile repair, involves repeated attempts by MMR to correct the lesion in the DNA. However, because the $^{O6\text{-}Me}$G is in the template strand, the lesion cannot be corrected by MMR. Repeated futile attempts to repair the lesion results in replication fork collapse, double strand breaks, cell cycle arrest, and apoptosis (Stojic et al., 2004b; Yamane et al., 2004). The two models are not mutually exclusive, and evidence exists to support both models. Separation of function mutants exist for some MMR genes in which their capacity to function in mutation avoidance is abolished, but yet they are still capable of responding to DNA damaging agents (Lin et al., 2004; Yang et al., 2004). This suggests that excision repair is not necessary to signal apoptosis in response to damaging agents, supporting the direct signaling model. In support of the futile repair model is the observation that *ExoI* deficiency causes partial resistance to methylating agents in mouse cells, indicating excision is involved in the MMR-dependent response (Schaetzlein et al., 2007). In addition, the endonuclease activity of MutLα is also necessary for the MMR-dependent DNA damage response, further substantiating the role of futile repair in mediating the damage response (Erdeniz et al., 2007).

Human cancer associated with MMR gene mutations

Inherited mutations in several MMR genes cause hereditary nonpolyposis colon cancer, a cancer syndrome defined by intestinal, uterine, endometrium, and biliary and pancreatic cancers (Lynch and de la Chapelle, 1999; Vasen, 2007). HNPCC, also known as Lynch syndrome, is a dominant disease with high penetrance; it is thought to account for approximately 2-3% of all colon cancers (Cunningham et al., 2001; Rustgi, 2007). Mutations in *MSH2* and *MLH1* account for greater than 90% of HNPCC cases, although

a few mutations in *PMS2*, *MSH6*, *PMS1*, and *MLH3* have been reported (Lynch and de la Chapelle, 2003). Afflicted individuals inherit one non-functional copy of the gene. Over time, function of the remaining gene can become lost due to a somatic event in a single cell. Because MMR functions in mutation avoidance, cells with deficient MMR have an increased mutation rate. Mutation can then occur in tumor suppressor and oncogenes, some of which contain sequences in their open reading frames prone to mutation in a MMR-deficient background (Duval and Hamelin, 2002).

The correlation between MMR genes and HNPCC was first observed through microsatellite analysis. Microsatellites are short segments of repeated DNA that are located throughout the genome of many organisms. They are used as molecular markers in genetic and population studies, as the repeat length number can vary from person to person. When they were utilized in HNPCC kindreds, it was observed that the microsatellites were changing length in the individuals afflicted with HNPCC, and this was attributed to repair defects arising from deficiency in MMR (Aaltonen et al., 1993; Thibodeau et al., 1993; Umar et al., 1994). Microsatellite instability (MSI) is now a hallmark of deficiency in MMR proteins.

Two other cancer syndromes are associated with deficiency in MMR genes. Muir-Torre syndrome (MTS) is defined by the presence of both sebaceous skin tumors and internal malignancies (Ponti et al., 2005). Muir-Torre can be caused by mutations in *MSH2* or *MLH1*. About half of patients with MTS develop intestinal tumors. Turcot syndrome is characterized by colorectal cancers and tumors of the central nervous system (Hamilton et al., 1995). Mutations, some reported to be bialleleic, have been detected in *MSH2*, *MLH1*, *MSH6*, and *PMS2* (Hamilton et al., 1995; Ostergaard et al., 2005).

In addition to causing hereditary cancer, *MLH1* and *MSH2* have been implicated in sporadic colon cancers. Approximately 15% of sporadic colorectal cancers display MSI, with the majority attributed to epigenetic silencing of the *MLH1* promoter (Herman et al., 1998; Kane et al., 1997).

Mouse knockouts of MMR genes

Mouse knockouts have been created for *Msh2*, *Msh6*, *Msh3*, *Mlh1*, *Pms1*, *Pms2*, and *Exo1*. These knockouts have proven to be good models for human cancer. The most severe "cancer" phenotypes are observed in knockouts of *Msh2* (de Wind et al., 1995; Reitmair et al., 1995) and *Mlh1* (Prolla et al., 1998; Yao et al., 1999). This is not surprising, given that both MSH2 and MLH1 are common to each of their respective heterodimers and therefore have no proteins that can compensate in their absence. Both knockouts display increased mutation rate, increased tolerance to certain DNA damaging agents, increased instability at microsatellite loci, and enhanced tumorigenesis. Both *Msh2* and *Mlh1* mouse knockouts develop adenomas and adenocarcinomas of the small and large intestine. *Pms2* knockout mice develop sarcomas and lymphomas at an accelerated rate and show features of hypermutability, but do not develop intestinal tumors (Prolla et al., 1998; Yao et al., 1999). Mice deficient for *Msh6* develop a range of tumors, including B- and T-cell lymphomas and epithelial tumors (Edelmann et al., 1997). Cells from *Msh6*$^{-/-}$ mice do not display microsatellite instability but do show defects in the *in vitro* MMR assay, indicating MSH6 is needed to correct base-base mismatches but not IDLs (Edelmann et al., 1997). *ExoI* mice have an increased susceptibility to lymphomas and slightly elevated mutation rate, but only at mononucleotide and not dinucleotide runs (Wei et al., 2003). The low mutation rate

observed in the *ExoI* mice indicates the possibility for a yet undiscovered redundant exonuclease. *Msh3* (de Wind et al., 1999), *Pms1* (Prolla et al., 1998), and *Mlh3* (Lipkin et al., 2002) deficient mice do not display significant mutator phenotypes.

Thesis prospectus

In my thesis research, I investigated two diverse functions of MMR: the role of the MutLα ATPase domains in MMR-mediated mutation avoidance *in vivo*, and the MMR-dependent DNA damage response. To better understand the role of the highly conserved MutLα ATPase domains, I examined the effect of mutations impacting ATP binding and hydrolysis of *MLH1* and *PMS2*. MutLα is responsible for coordinating events downstream of mismatch recognition, and previous work had implicated its ATPase activity as a necessary component of this coordination. While studies had been carried out in yeast cells and mammalian cell-free extracts, the effect of mutations in the ATP hydrolysis and Mg^{2+} binding residues *in vivo* in mammalian cells had not been examined. I observed that mutations abolishing ATP binding and hydrolysis in *MLH1* had a greater effect on repair of microsatellite sequences and response to DNA damage than the equivalent mutations in *PMS2*, supporting a functional asymmetry between the N-terminal domains of MLH1 and PMS2 comprising the ATPase activity of MutLα.

I also examined the role of deoxycytidylate deaminase (DCTD) in the MMR-dependent response to DNA damage caused by the S_N1-type methylation mimetic compound 6-thioguanine. To determine the effect of DCTD deficiency, I used siRNA knockdown in human cells and also studied a *Dctd* knockout mouse and cells derived therefrom. While DCTD knockdown in human cells resulted as expected in resistance to

6-TG, the mouse knockout cells surprisingly did not display significant resistance, indicating a potential adaptive response in the whole mouse.

CHAPTER 2

Materials and Methods

ATPase mutant analysis

Cell lines and media

MC2 *Mlh1*$^{-/-}$ and WT complemented cell lines were created as described (Buermeyer et al., 1999b). *MLH1-E34A*, and *MLBH1-N38A* cells were created by transfecting plasmids expressing *MLH1-E34A* and *MLH1-N38A* human *MLH1* cDNA under the CMV promoter into *Mlh1*$^{ko/ko}$ mouse embryonic fibroblasts (MEFs). 20 μg linearized DNA was electroporated into 1x10^{7} cells at 0.32 kV and 500 μF. Stably expressing colonies were selected using neomycin.

C18 *Pms2*$^{-/-}$ and WT complemented cells were created as described (Deschenes et al., 2007). *PMS2-E41A*, and *PMS2-N45A* cells were created as above, only plasmids were of *PMS2-E41A* and *PMS2-N45A* human *PMS2* cDNA transfected into *Pms*$^{ko/ko}$ MEFs. Colonies were selected using puromycin.

All cell lines were maintained in DMEM supplemented with 10% BCS, 1xNEAA, and 50 μg/mL gentamycin sulfate and grown in a humidified incubator with 5% CO_2.

For the microsatellite analysis, all cell lines were subcloned, passaged at 1:10 for two weeks, and then 300 cells were plated on 150 mm plates for colony formation. For each cell line, at least ten clones stably expressing *MLH1* and *PMS2* were expanded and harvested for genomic DNA.

Plasmid construction

MLH1-E34A, and *PMS2-E41A* were constructed as described (Tomer et al., 2002), only the cDNA containing the mutation was cloned into the expression vectors pCMV-neo (for *MLH1*) and pCMV-puro (for *PMS2*). *MLH1-N38A* and *PMS2-N45A* were created by site-directed mutagenesis (Stratagene) of pBluescript plasmids containing the *MLH1* or

PMS2 cDNA. Primers containing the desired mutations were used for PCR with the plasmids as template: 5'CAAAGAGATGATTGAGGCCTGTTTAGATGCAAAATCC3' and 5'GGATTTTGCATCTAAACAGGCCTCAATCATCTCTTTG3'
for *MLH1-N38A* and
5'GGTAAAGGAGTTAGTAGAAGCCAGTCTGGATGCATGCTGG3' and
5'CCAGCATGCATCCAGACTGGCTTCTACTAACTCCTTTACC3' for *PMS2-N45A*. PCR was carried out at 16 cycles of 95º 30 sec, 55º 1 min, 68º 12 min. Once mutagenized, the DNA was digested with Dpn1 to remove methylated, unmutated template DNA. The remaining mutated DNA was transformed into bacteria. Colonies were picked, screened by restriction digest, and the mutation confirmed by sequencing. The cDNA was then cloned into either pCMV-neo or pCMV-puro.

Western blots

Cells were lysed in 1x SDS sample buffer, heated at 95º for 5 min, and run on an 8% SDS-PAGE gel. The protein was then transferred to a PDVF membrane and probed with mouse anti-human MLH1, PMS2, and MSH6 antibodies (BD Biosciences) at 1:1000, 1:500, and 1:2500. Membranes were then probed with goat anti-mouse HRP secondary antibody at 1:1000. Bands were visualized using enhanced chemilumenescense from Perkin Elmer.

DNA preparation

Genomic DNA was prepared by trypsinizing cells, washing in PBS, and resuspending in DNA lysis buffer containing 10mM Tris pH 7.5, 10mM EDTA pH 8, 10mM NaCl, and 0.5% sarkosyl, plus 1 mg/mL proteinase K. Lysate was incubated overnight at 55º and the proteinase K was heat inactivated at 95º for 5 min.

Dinucleotide analysis

Six loci containing dinucleotide (CA) repeats were utilized (see Table 2-1). DNA was diluted 1:10 and subject to PCR using a Robocycler under the following conditions: 95°C 5 min, 64° 1 min, 72° 1 min, 95° 1 min, 62°/60°/58° 1 min, 72° 1 min, 95° 1 min, 56°/52° 1 min, 30 cycles of 95° 1 min, 52° 1 min, 72° 1 min, with a final extension of 72° 3 min. PCR products were loaded onto a 7% denaturing acrylamide gel. The DNA was then transferred to a nitrocellulose membrane and hybridized using a biotin-labeled $(CA)_{10}$ oligodeoxynucleotide. P-values were calculated using fisher's exact t-test on Prism software.

Mononucleotide analysis

Six loci containing poly-A runs were analyzed. (see Table 2-1). A universal system of fluorescently tagged M13 primers was used to label PCR products for analysis. All forward primers contained a 5' M13 tail of the following sequence: 5'CACGACGTTGTAAAACGAC3'. Fluorescently labeled M13 forward primer was included to produce a 3 primer reaction resulting in amplification of the mononucleotide loci with a fluorescent labeled M13 end. PCR conditions are as follows: 95° 5 min, 20 cycles of 94º 15 sec, 65º 20 sec (-1º per cycle), 72º 20 sec, 35 cycles of 92º 15 sec, 50º 15 sec, 72º 15 sec, and a final extension of 72º for 10 min. PCR products were subject to fragment size analysis by the OHSU MMI Sequencing Core using an ABI Prism Sequencer and analyzed using Peak Scanner software.

A run primers	Sequence
uPAR	F:5′CACGACGTTGTAAAACGACGCTCATCTTCGTTCCCTGTC3′ R:5′GTTTCTTCATTCGGTGGAAAGCTCTGA3′
JH116	F:5′CACGACGTTGTAAAACGACCTCTGGGCATACCAGCTACTG3′ R:5′CTCCATCCTGTGAGGTTAAACACAT3′
27A	F:5′CACGACGTTGTAAAACGACCTATTGGATAAGTATGAGGTACTG3′ R:5′GTTTCTTCACCATAGTGCTAGCAATCAAGTGG3′
23A	F:5′CACGACGTTGTAAAACGACTTGCTGAATTGGTGAGCTTC3′ R:5′GTTTCTACGTCAAAAATCAATGTTAGG3′
M-BAT 26	F5′CACGACGTTGTAAAACGACTCACCATCCATTGCACAGTT3′ R:5′GTTTCTCTGCGAGAAGGTACTCACCC3′
M-BAT 37	F:5′CACGACGTTGTAAAACGACTCTGCCCAAACGTGCTTAAT3′ R:5′GTTTCTCCTGCCTGGGCTAAAATAGA3′
CA run primers	Sequence
D4MIT27	F:5′ GCACGGTAGTTTTTCCAGGA3′ R:5′TGGTGGGCAGGCAATAGT3′
D19MIT41	F:5′AGCCCTCCACCCAGTTTC3′ R:5′TCTGGGGAAAAAGGATGAGA 3′
D13MIT67	F:5′TTTCATGGAGTCGAGTATTTTGG 3′ R:5′ATCTTGCATAGAACCTTTGGATG3′
D16MIT4	F:5′AGTTCCAGGCTACTTGGGGT 3′ R:5′GAGCCCTCATTGCAAATCAT 3′
D17MIT123	F5′CACAAGGAGGGAGCCTGTAG 3′ R:5′CACCGTAAGAGTCTAATAATAAGGGG 3′
D13MIT139	F:5′AGAATAAGTCAAGGCTATGATGTGG3′ R:5′TTGTTTGTTTGTTTGAAGTAGAACG 3′

Table 2-1. Primers used for microsatellite analysis.

6-TG colony formation assay

Cells were plated out onto 100 mm plates at a density of 300 or 500 cells per plate. After 24 hours, 6-thioguanine (Sigma) was added and left on for 24 hours. The cells were then

washed and fresh media was added. 7-10 days after addition of 6-TG the media was removed and the plates were fixed in 25% ethanol, 50% methanol, 0.25% methylene blue to stain the colonies. For each dose of 6-TG, three plates were stained, and the experiment was repeated three times.

Human DCTD analysis

Cell culture

HeLa cells were grown in a 5% CO2 humidified incubator and maintained in Dulbecco's Modified Eagle Media (DMEM) with 10% Bovine Calf Serum (BCS), 1x non-essential amino acids, and gentamycin sulfate. SUPB-15 and K562 cells were maintained in RPMI 1640 with 10% FBS, 1x NEAA, and 1x pen/strep. HEK293 cells were maintained in DMEM with 10% FBS.

siRNA treatment

Validated siRNA oligonucleotides against *DCTD* as well as a scrambled control were obtained from Ambion with the following sequence for DCTD: sense 5'-CCAAAUACCCGUACGUGUGtt-3' and antisense 5'-CACACGUACGGGUAUUUGGtg-3'. For the HeLa and HEK293 cells, oligonucleotides were transfected using Lipofectamine according to manufacturer's protocol. For transfection into a 24-well plate, 30-50 nM RNA was complexed with 1 µL lipofectamine per well. Complexes were left on cells for 24 hours, at which point the cells were washed and fresh media was added. For the SUP-B15 and K562 cells, 5 µg RNA was electroporated into $2.5x10^6$ cells suspended in 500 µL Opti-Mem media at 250 kV and 1050 µF.

Western blots

HeLa, 293, K562 and SUPB-15 cells were lysed in 1x SDS sample buffer, heated at 95º for 5 min, and run on a 12% SDS-PAGE gel. The protein was then transferred to a PDVF membrane and probed with chicken anti-human DCTD antibody (Ambion) at 1:250 dilution. Mouse anti-chicken HRP secondary antibody from Ambion was used at 1:1000. Bands were visualized using enhanced chemilumenescense from Perkin Elmer.

6-TG response curves

For the HeLa cells, 10,000 cells were plated per well in 24 well plates. Twenty-four hours later, siRNA transfections were performed as described. 48 hours after transfection, 6-TG was added and left on for 24 hours. For each time point, two wells per dose 6-TG were trypsinized, spun down, resuspended in 100 µL media plus trypan blue, and counted using a hemocytometer. Each experiment was repeated three times.

For the SUP-B15 and K562 cells, cells were electroporated as described. 48 hours later, 100,000 cells were aliquoted per well of a 12 well plate and 6-TG was added. 24 hours later, the cells were washed and resuspended in fresh media. Cells were stained with trypan blue and counted using the hemocytometer; cells were counted every 24 hours. For each dose of 6-TG, two wells were counted, and the experiments were repeated at least two times.

Colony formation assay

HeLa and HEK293 cells were transfected with siRNA and the next day cells were plated out onto 100 mm plates at a density of 300 or 600 cells per plate. The subsequent steps were carried out as described for the 6-TG colony formation assay for the ATPase mutant analysis above.

dNTP pool measurement

dNTPs pools were measured on log-phase cells. The cell lines were sent to the laboratory of Dr. Chris Mathews at Oregon State University, where three extractions of each cell line were taken according to the protocol described in (Song et al., 2005), and each of these was measured in duplicate as described (Sherman and Fyfe, 1989). Results are given as the average of all trials +/- standard deviation.

Dctd mouse analysis

Mice

Dctd knockout mice were obtained from Texas Genomic Institute (Houston, TX). Mice were genotyped by PCR according to manufacturer's protocol using the following primers: TG0017-5' 5'TCTCGAATGAAAGACACGCACA3' and LTR-rev 5'ATAAACCCTCTTGCAGTTGCATC3' for the mutant band, and TG0017-5' and TG0017-3' 5'GTGAAGAAGCAGAGCGAGTGAGG3' for the wild type band. PCR conditions were as follows: 94º 4 min, 10 cycles of 94º 15 sec, 65º 30 sec (decrease by 1º per cycle), 72º 40 sec, 30 cycles of 94º 15 sec, 55º 30 sec, and 72º 40 sec.

RT-PCR

Lack of *Dctd* mRNA was confirmed by RT-PCR. Mouse embryonic fibroblasts (MEFs) from $Dctd^{+/+}$ and $Dctd^{ko/ko}$ mice were treated with Trizol (Invitrogen) according manufacturer's protocol to isolate total mRNA. RT was carried out using oligo dT primers (Promega) and reverse transcriptase (Roche). PCR was then performed using the following primers: Dctd F 5'GTGACGTATGACTTCTACACCC3' and Dctd R 5'GATGATGAGTTTAGCACATTCGTTA3' for *Dctd* expression, and c-myc F

5'TGTACCTCGTCCGATTCCACG3' and c-myc R

5'GATGGAGATGAGCCCGACTCCG3' for control.

Cell culture

Primary MEFs from *Dctd*$^{+/+}$ and *Dctd*$^{ko/ko}$ mice were harvested from day E13.5 embryos and maintained in DMEM (Invitrogen), 10% fetal bovine serum (Hyclone), 1x non-essential amino acids (Invitrogen), and 50 μg/ mL gentamycin sulfate (Sigma). To spontaneously immortalize the cells, cells were split 1:3 every 2-3 days until they reached crisis. Colonies that continued to grow were picked and expanded. Immortalized cells were maintained in DMEM 10% bovine calf serum, 1x NEAA, and gentamycin sulfate.

Lymphocytes were harvested from the spleens of 2- to 3-month old *Dctd*$^{ko/ko}$ and *Dctd*$^{+/+}$ mice. Spleens were homogenized using frosted coverslips. Red blood cells were lysed with ACK buffer (Lonza) and cells were passed over a 70 micron mesh to promote a single-cell suspension. To isolate lymphocytes, the cell suspension was passed over the Lympholyte-M column (Cederlane Laboratories). Lymphocytes were maintained in RPMI 1640 medium (Invitrogen) containing 20% HL-1 (Lonza), 10% dialyzed fetal bovine serum (Hyclone), 2mM glutamine, 1x NEAA, 20 mM Hepes, 1 mM pyruvate, 1x pen/strep, and 50 μM 2-mercaptoethamine. Cells were primed for 24 hours in 5μg/mL concanavalin A (Sigma) and 10 units/mL IL-2 (BD Biosciences). After 24 hours, cells were washed and maintained in the above medium without conconavalin A.

6-TG resistance assays

For the MEFs, the experiment was conducted as described for the HeLa cells above.

For the lymphocytes, 100,000 cells were plated per well in a 12-well dish. 24 hours after stimulation with conconavalin A and IL-2, 6-TG was added and left on for

one population doubling. Cells were then washed and plated in fresh medium. Cells were stained with trypan blue and counted every 24 hours using a hemacytometer. For each dose of 6-TG, two wells were counted, and the experiments were repeated three times.

CHAPTER 3

Analysis of ATPase mutants of MutLα *in vivo*

Abstract

The DNA MMR protein dimer MutLα is comprised of the MutL homologues MLH1 and PMS2, which each belong to the family of GHL ATPases. These ATPases undergo important comformational changes, including dimerization of their NH_2-termini associated with ATP binding and hydrolysis. Previous studies in yeast and biochemical studies with the mammalian proteins established the importance of this domain of MutLα for overall MMR function. I investigated the effect of mutation in the highly conserved ATP hydrolysis and Mg^{2+} binding residues of MLH1 and PMS2 in mammalian cell lines. Similar to what was observed in yeast, amino acid substitutions in MLH1 intended to impact either binding or hydrolysis of ATP disabled MMR as measured by instability at microsatellite sequences, to an extent similar to the *MLH1* null. In addition, cells expressing these *MLH1* mutations were resistant to the MMR-dependent cytotoxic effect of 6-thioguanine. In contrast, hydrolysis and binding mutants of PMS2 displayed no measureable increase in microsatellite instability, nor resistance to 6-TG. My findings suggest the MLH1 ATPase domain is more critical for normal MMR functions than the PMS2 ATPase domain.

INTRODUCTION

MutL ATPase domain

While described as the "molecular matchmaker" necessary for coupling MutS to downstream events such as strand discrimination and excision, the exact biochemical function of the MutL homologues had remained elusive until the determination of the crystal structure of the first 400 amino acids of its amino terminus (Ban and Yang, 1998; Dutta and Inouye, 2000). This crystal structure placed MutL into the GHL superfamily of proteins, all of which contain a similar ATPase domain comprised of four motifs in their NH_2-termini (Dutta and Inouye, 2000) (Figure 3-1). Other members of the GHL family include gyrase B and Hsp90. Gyrase B utilizes ATP to promote the homodimerization of its subunits that are needed to properly supercoil DNA (Sugino and Cozzarelli, 1980). In addition, Hsp90 ATPase activity is needed for dimerization of its subunits to achieve substrate binding and protein/protein interaction (Obermann et al., 1998; Panaretou et al., 1998). MutL was shown to have a similar ATPase cycle whereby binding of ATP induces homodimerization at its NH_2-termini (Ban and Yang, 1998). The binding and hydrolysis of ATP was not needed to form the MutS/MutL/DNA ternary complex, but was needed to activate MutH (Spampinato and Modrich, 2000). As discussed in Chapter 1, MutL ATP binding is necessary for interaction with MutH and UvrD, and it is thought ATP hydrolysis is necessary for complex dissociation (Polosina and Cupples).

A

```
Gyrase b 38  MVFEVVDNAIDEA----VSVQDDGRGL----VSGGLHGVGVGV--GTMV
Hsp90    25  FLRELISNSSDAL----LTIVDTGIGN----SMIGQFGVGFYG--GTKV
MutL     43  VVKELVENSLDAG----IRIRDNGCGI----ISLGFRGEALAS--GTTL
MLH1     31  AIKEMIENCLDAK----IQIQDNGTGI----STYGFGGEALAS--GTQI
PMS2     37  AVKELVENSLDAG----AVKSDNGCGV----ETFGFRGEALSS--GTTV
```

Motif I Motif II Motif III Motif IV

B

Figure 3-1. Sequence alignment and ATP binding pocket of GHL family members. A) High sequence similarity is seen among the GHL family members in the four motifs comprising the ATPase domain. Conserved residues are highlighted in red. The hydrolysis and Mg2+ binding residues mutated in this study are in bold. Adapted from (Tran and Liskay, 2000). B) ATP bound in the ATP binding pocket of MutL. Reprinted with permission from Raschle M, et al. JBC (2002) 277: 21810-21820.

Yeast MutLα ATPase domain

The MutL homologues *MLH1* and *PMS2* also contain this ATPase domain (Figure 3-1). To elucidate if a similar ATPase cycle functions in MutLα, studies were undertaken in the budding yeast *Sacchoramyces cerevisae*. Mutations were created in Mlh1 and Pms1 in the residues predicted to abolish ATP binding and/or hydrolysis (Tran and Liskay, 2000). The ATP binding mutations, *mlh1-G98A* and *pms1-G128A*, when present together in the same cell, abolished repair to the same extent as an *mlh1Δ* or *pms1Δ* strain. The same was observed for the ATP hydrolysis mutants, *mlh1-E31A* and *pms1-E61A*. However, when the mutants were examined singly, it was shown that a functional asymmetry existed. Mutation in the Mlh1p ATP binding or hydrolysis residues impacted repair to a greater extent than the equivalent mutations in Pms1p, as reported at the *hom3-10* locus, which reports frameshift mutations, and in forward mutations at the *CAN1* locus. The mutation rate for the Mlh1p ATP binding mutant was greater than the Pms1p mutant by 9-fold at *hom3-10* and 5.5-fold at the *CAN1* locus; the ATP hydrolysis mutant was greater by 16-fold and 7-fold, respectively. Importantly, the mutation rate of the Pms1p mutants did not increase in an *Mlh3Δ* background, indicating the redundant function of Mlh3p does not account for the difference seen between mutations in Mlh1p and Pms1p. Instead, a functional asymmetry exists in the ATPase domains of Mlh1p and Pms1p. This could represent differing roles for Mlh1p and Pms1p during repair.

Limited proteolysis and yeast-two hybrid experiments with Mlh1p and Pms1p demonstrated an ATPase cycle for MutLα similar to other GHL family members (Tran and Liskay, 2000). ATP binding causes a conformational change in the NH_2-terminal of

Mlh1p, and this promotes dimerization of the amino termini of Mlh1p and Pms1p. Analysis with the double-hydrolysis mutants showed that while this interaction is usually transient, the inability to hydrolyze ATP keeps Mlh1p and Pms1p in a prolonged state of dimerization, suggesting that hydrolysis of ATP causes dissociation of the NH_2-termini. Additional studies showed that Mlh1 binds ATP with much higher affinity than Pms1, and that it is likely that Mlh1 ATP binding is necessary for Pms1 ATP binding and subsequent steps in the MMR pathway (Hall et al., 2002).

Human MutLα ATPase domain

Studies with human MutLα further substantiated the ATPase cycle's importance to MMR. Limited proteolysis studies with purified human MutLα showed that MLH1 binds ATP and undergoes a conformational change as seen in yeast (Raschle et al., 2002; Tomer et al., 2002). The impact of hMutLα ATP hydrolysis and binding mutants on repair was also examined. Purified MutLα containing mutations in the ATP hydrolysis residues (*MLH1-E34A* and *PMS2-E41A*) was used to supplement a MutLα-deficient cell extract in an *in vitro* repair assay. When both MLH1 and PMS2 hydrolysis mutations were present, repair was abolished. However, when examined singly, the individual mutations affected repair to the same extent, about 50% of wild type MutLα (Raschle et al., 2002; Tomer et al., 2002). The effect of ATP binding was examined in a similar manner. MutLα requires Mg^{2+} as a cofactor to coordinate ATP binding, and a conserved arginine residue in the binding pocket is necessary for this coordination. Mutation of this arginine residue prevents binding of Mg^{2+} and therefore ATP (Raschle et al., 2002). The mutants *MLH1-N38A* and *PMS2-N45A*, deficient in ATP binding, totally abolished repair whether alone or in combination.

These *in vitro* results were at odds with the *in vivo* studies with budding yeast. This could represent a functional difference in MutLα's contribution to MMR between yeast and human cells. Another possibility is that the yeast studies were carried out *in vivo* while the human studies were conducted *in vitro*. MMR most likely operates closely with the replication apparatus, and the *in vitro* studies were carried out in a minimal system operating in the absence of replication. Whether the disparity seen between the yeast and human cells is due to actual differences between the organisms, or whether it is due to the studies being conducted *in vivo* versus *in vitro* remained to be answered.

Experimental Rationale

To understand better the relative importance of the MLH1 and PMS2 ATPase activities *in vivo,* I determined the effect of synonymous ATP binding and hydrolysis mutations of human MLH1 and PMS2 in mammalian cells. To achieve this, I obtained mouse cell lines knocked out for endogenous MLH1 or PMS2 and then expressed *MLH1-E34A, MLH1-N38A, PMS2-E41A* or *PMS2-N45A* human cDNA. After establishing that protein expression of the mutant lines corresponded to a level of exogenously expressed WT protein that fully complemented knockout cells, I performed microsatellite instability assays on the cell lines at both dinucleotide and mononucleotide repeat loci. Microsatellite instability (MSI) assays are an established method for testing the status of mismatch repair in cells, with increased MSI being reflective of defective MMR. DNA is harvested from cells and PCR is performed at sites of either mononucleotide (A)n or dinucleotide (CA)n repeats, where n is typically in the range of 15-25 repeats. These repeats are prone to slippage by polymerase (Figure 3-2), but the resultant "loop" mismatches are normally efficiently corrected by MMR. However, without proper MMR

function, these mismatches will not be repaired and lead to additions or deletions of repeat units that can be detected by PCR.

Another method for determining the status of mismatch repair in cells is by resistance to the drug 6-thioguanine (6-TG). As described in Chapter 1, cellular response to certain DNA damaging agents is dependent on MMR. 6-TG is a methylation mimetic that elicits the same MMR-dependent cytotoxic response as S_N1-methylating agents (Swann et al., 1996). Resistance to the cytotoxic effects of 6-TG can be used as a measure of faulty MMR function. To determine how equivalent ATPase mutants of MLH1 and PMS2 impacted 6-TG resistance, I measured the cytotoxic response to 6-TG using colony formation assay.

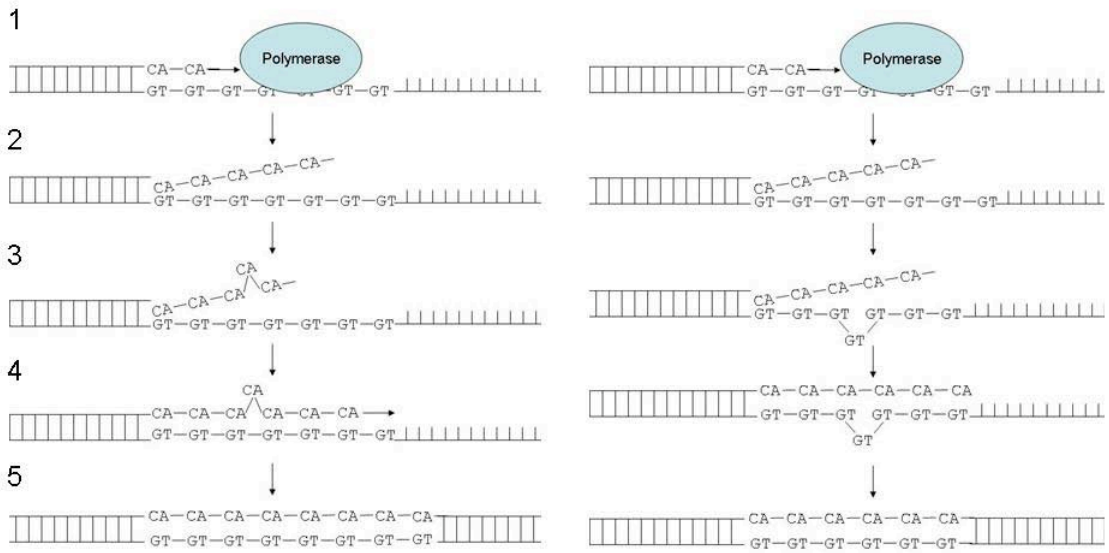


Figure 3-2. Slipped strand mispairing during replication. When polymerase replicates across a series of repeats, the strands can transiently breathe and "slip" out of register (steps 2 and 3). The left panel shows the top strand slipping, which results in an extra CA repeat in a loop (4). This will be corrected by MMR, or upon the next round of replication, it will be replicated and an extra CA repeat will be present in the DNA (5). The right panel shows the bottom strand slipping, resulting in a deletion of a CA repeat.

RESULTS

Generation of Cell Lines and Protein Expression

Stable cell lines were generated by transfecting *MLH1* or *PMS2* cDNA into spontaneously established *Mlh1*$^{-/-}$ or *Pms2*$^{-/-}$ MEFs and selecting for stably expressing colonies. To ensure similar levels of protein expression among the cell lines, Western blots were performed. The level of protein expression of the ATPase mutants corresponded to a similar level of exogenous WT protein expression that fully complemented knockout cell lines. As seen in Figure 3-3, the MLH1 and PMS2 ATP binding and hydrolysis mutant proteins are expressed at approximately the same level as their corresponding exogenously expressed WT proteins.

Microsatellite Instability Assays

Once proper expression level was determined, microsatellite instability assays were carried out. Cells were subcloned, passaged for two weeks, and then plated out to establish individual clones. At least 10 clones per cell line were expanded, checked for stable protein expression, and DNA prepared for PCR analysis. PCR was conducted using primers specific for di- or mononucleotide runs that had previously been established to show high instability in MMR-deficient cells (Buermeyer et al., 1999b; Yao et al., 1999). For the dinucleotide runs, the PCR products were run out on denaturing acrylamide gels and probed with a CA-biotin oligonucleotide. DNA polymerase stutters when replicating across these repeats, and creates a pattern of PCR products differing in size by two nucleotides. If a mutation has occurred in the cell clones compared to the parental cell line, this pattern shifts (Figure 3-4). A shifted pattern is counted as a slip. The total number of slips at each loci is combined for each

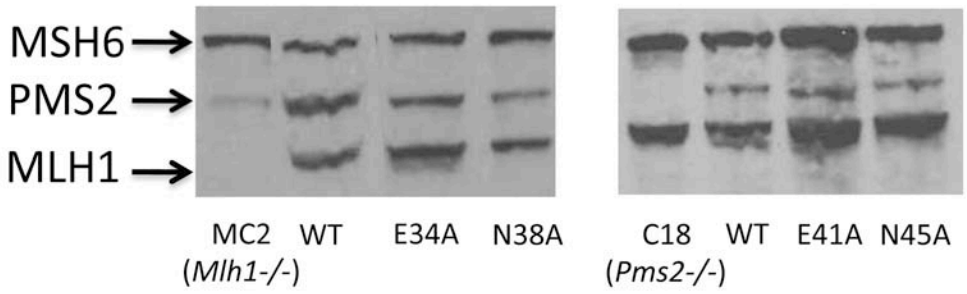


Figure 3-3. Protein expression in MC2 and C18 complemented cells. Western blots of MC2 (*Mlh1-/-*) cells expressing WT MLH1, *MLH1-E34A*, and *MLH1-N38A* in the left panel and C18 (*Pms2-/-*) cells expressing WT PMS2, *PMS2-E41A*, and *PMS2-N45A* in the right panel. MSH6 is used as a loading control. MC2 cells display reduced PMS2 expression, as PMS2 depends on MLH1 for protein stability.

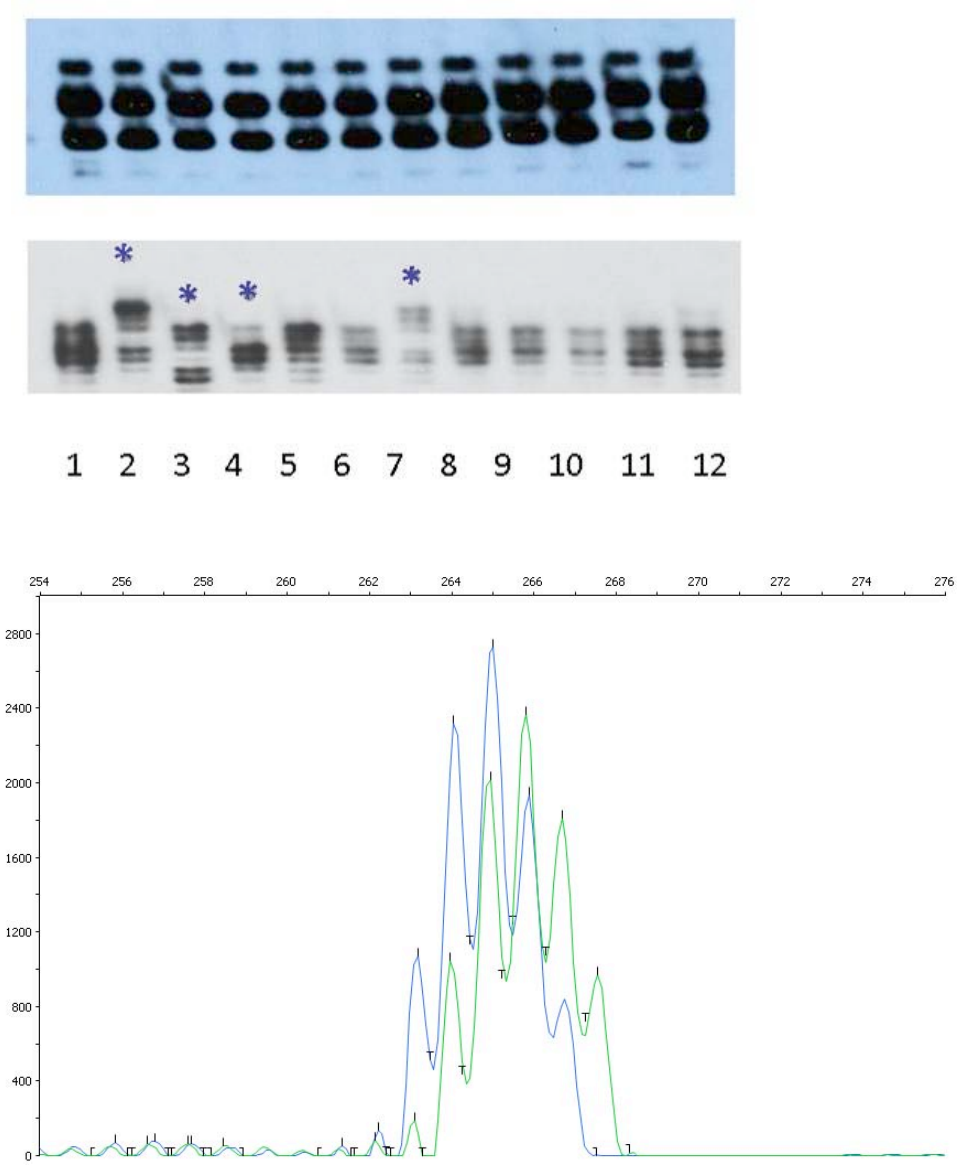
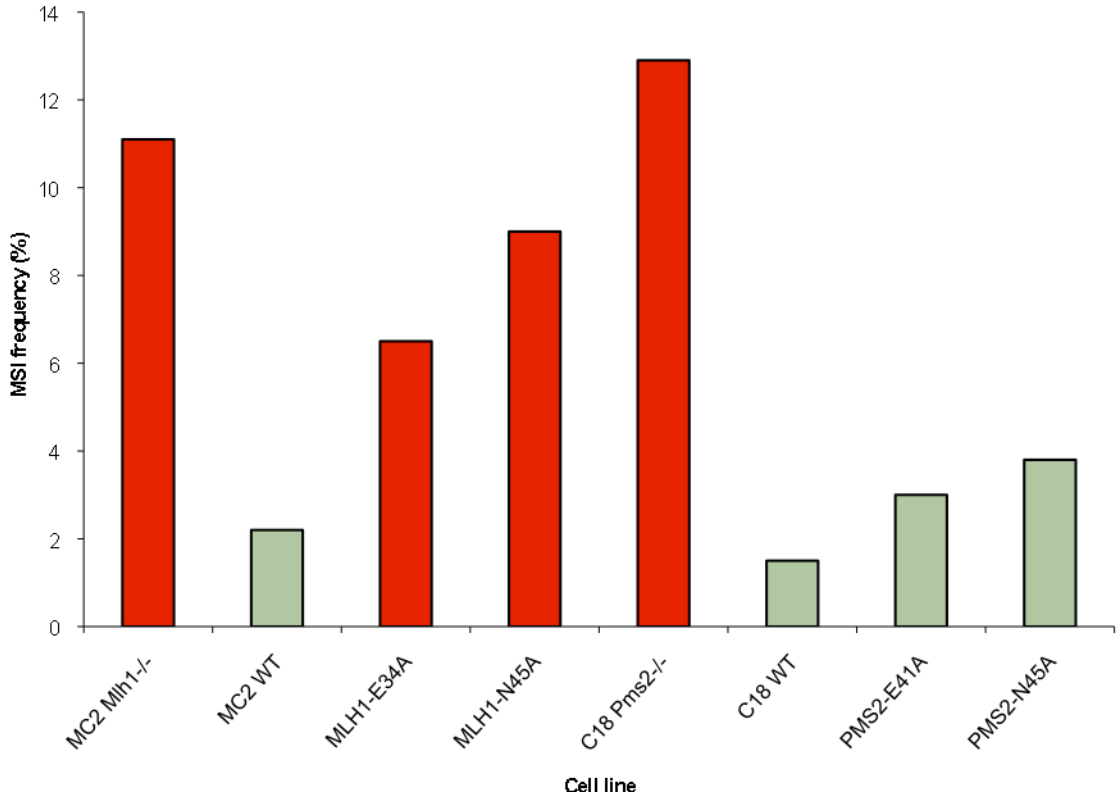


Figure (3-4). MSI at a di- and mononucleotide repeats. MSI was assessed at a CA repeat in WT (top) or *Mlh1*$^{-/-}$ (bottom) cells by PCR followed by separation on a 7.5% acrylamide gel (top). The pattern created by polymerase stutter across the repeat in the parental cell line is seen in lane 12; the subclones are in lanes 1-11. Shifts in the pattern indicating expansion or contraction of the repeat are denoted by asterisks.
Bottom, expansion of an A run. The chromatogram from a fluorescently-labeled PCR product of a 27-A run produces a specific pattern of peaks. The parental cell line (*Mlh1*$^{-/-}$) is in blue. A subclone of the parent is in green. The pattern of the clone has shifted one base pair to the right, indicating an expansion of the A run.

Cell line	#slips/total	Frequency (%)	p-value vs. WT	p-value vs. null
MC2 *Mlh1*$^{-/-}$	23/224	11.1	0.001	
MC2 WT	8/360	2.2		0.001
MLH1-E34A	15/230	6.5	0.015	0.176
MLH1-N45A	12/132	9	0.003	0.58
C18 *Pms2*$^{-/-}$	48/372	12.9	0.001	
C18 WT	2/126	1.5		0.001
PMS2-E41A	4/132	3	0.68	0.007
PMS2-N45A	6/156	3.8	0.43	0.001

Table 3-1. MSI at dinucleotide runs in *MLH1* or *PMS2*-expressing cells.



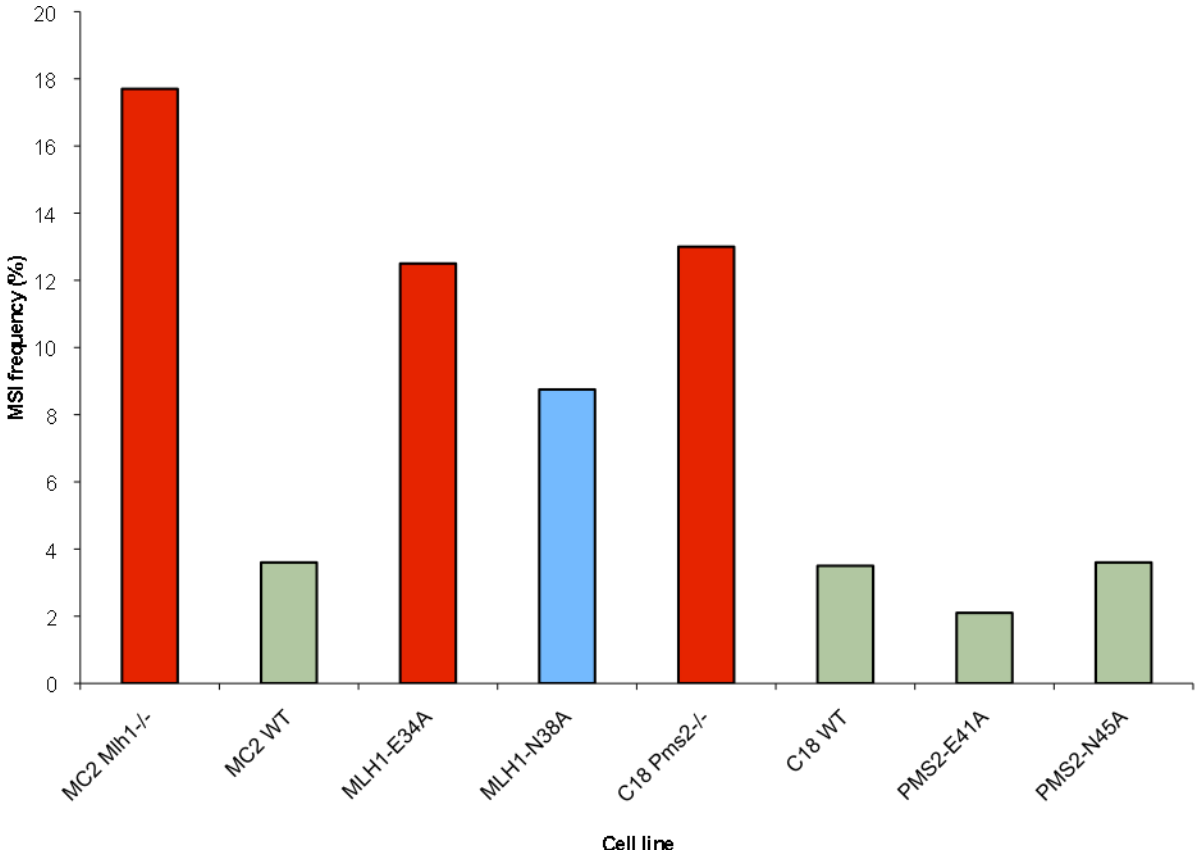
cell line and compared to the knockout and WT complemented cell lines. As seen in Table 3-1 the knockout cell lines for both *Mlh1* (MC2) and *Pms2* (C18) had a significantly higher frequency of slippage than the WT complemented lines, with 11.1% for MC2 cells and 2.2% for the *MLH1* WT cells (p=0.001, fisher's t-test) and 12.9% and 1.5% for the C18 and *PMS2* WT cells, respectively (p=0.001). These results are consistent with previous reports showing an increase in microsatellite instability when MMR is compromised (Baker et al., 1996). In addition, both *MLH-E34A* and *MLH1-N38A* showed a frequency of slippage that was equivalent to the MC2, *Mlh1* $^{-/-}$ cells. *MLH1-E34A* had a frequency of 6.5% (p=0.015 vs WT) and *MLH1-N38A* had a frequency of 9% (p=0.003 vs WT). On the other hand, *PMS2-E41A* and *PMS2-N45A* had a frequency of slippage that was equal to the C18 + WT *PMS2* complemented cell line (3% and 3.8%, p=0.68 and p=0.43 vs WT). These results suggest that at dinucleotide runs, the *MLH1* ATPase mutants impact repair to a greater degree than the *PMS2* mutants.

I next examined mononucleotide MSI at A runs. Since the pattern of slippage products produced by mononucleotide runs only differs by one nucleotide, the products are not easily analyzed on an acrylamide gel. Instead, the PCR products were fluorescently labeled and separated by capillary electrophoresis and analyzed by an ABI Sequencer by the OHSU MMI Core Facility. The readout is a series of peaks, the pattern of which will shift if there is a slip in the nucleotide run (Figure 3-4).

Again, the *Mlh1*- and *Pms2*-deficient cells had a frequency of slippage significantly different than the WT cells (19.7% and 13% vs 1.4% and 3.5%, p=0.002 and 0.01). As seen in Table 3-2, MC2 *MLH1-E34A* had a frequency of slippage that was

Cell line	Slips	Frequency (%)	p-value vs. WT	p-value vs. null
MC2 *Mlh1*$^{-/-}$	17/86	19.7	0.0002	
MC2 WT	1/74	1.4		0.0002
MLH1-E34A	16/128	12.5	0.007	0.2
MLH1-N38A	10/120	8.3	0.05	0.02
C18 *Pms2*$^{-/-}$	16/124	13	0.01	
C18 WT	4/114	3.5		0.01
PMS2-E41A	2/96	2.1	0.69	0.005
PMS2-N45A	4/110	3.6	1	0.017

Table 3-2 MSI at A runs in *MLH1* and *PMS2*-expressing cell lines.



equivalent to the *Mlh1* $^{-/-}$ cells (12.5% p=0.007 vs WT), while the *PMS2-E41A* and *PMS2-N45A* cells had a frequency that was similar to the *PMS2* WT complemented line (2.1% p=0.69 and 3.6% p=1). The *MLH1-N38A* cells, however, appeared to display an "intermediate" phenotype, with 8.3% slippage. The frequency of slippage of the *MLH1-N38A* cells is statistically different from both the complemented (p=0.05) and null cell lines (p=0.02). Overall, the *MLH1* mutants displayed a greater degree of instability at mononucleotide runs than the *PMS2* mutants.

Taken together, the mono- and dinucleotide MSI data suggest that mutations disabling MLH1 ATPase function impact repair *in vivo* to a greater extent than the equivalent mutations of PMS2.

Cytotoxic response to 6-thioguanine in ATPase mutant cells

MMR has been shown to be necessary to signal apoptosis in response to the DNA methylation mimetic 6-TG. To assess whether the ATPase mutations of *MLH1* and *PMS2* disrupt MMR's response to 6-TG, I performed 6-TG response assays with MC2, C18, WT complemented, ATP hydrolysis, and Mg^{2+} binding mutant cell lines of *MLH1* and *PMS2*. As seen in Figure 3-5, *Mlh1*$^{-/-}$ cells are more resistant than the WT complemented cells to 6-TG at every drug dose examined except the highest dose. Both the hydrolysis and Mg^{2+} binding mutants of *MLH1* displayed a resistance to 6-TG similar to that of *Mlh1*$^{-/-}$ cells. At only the highest dose of 6-TG did the cells form colonies at a frequency equal to WT. It was previously shown that high doses of methylating agents trigger a cellular response independent of MMR (Stojic et al., 2005).

Similar to *Mlh1*$^{-/-}$, *Pms2*$^{-/-}$ cells were more resistant to 6-TG than *PMS2* WT complemented cells, as seen in Figure 3-6. In contrast to the *MLH1* ATPase mutants,

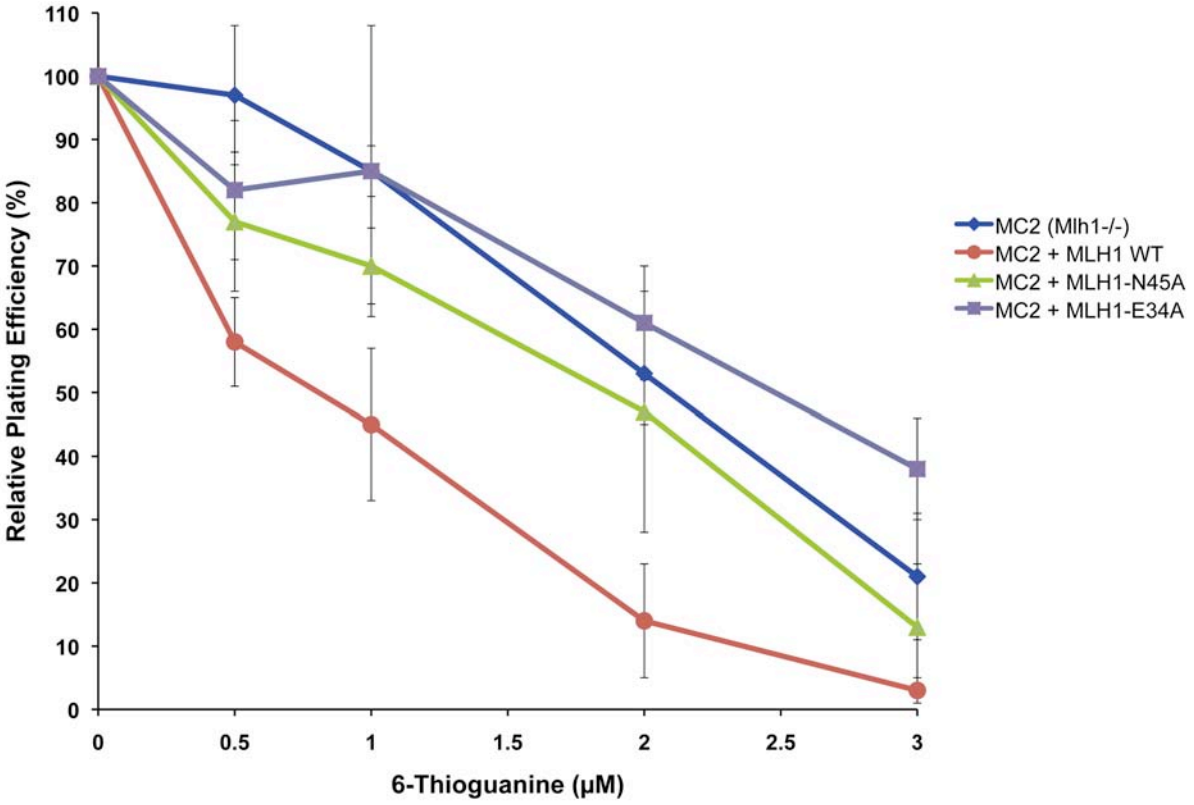


Figure 3-5. 6-Thioguanine response in MLH1-expressing cells. MC2, *MLH1*-WT, *MLH1-E34A*, or *MLH1-N38A* cells were plated out and exposed to varying doses of 6-thioguanine for 24 hours. Colonies were stained with methylene blue and counted 8-10 days after plating.

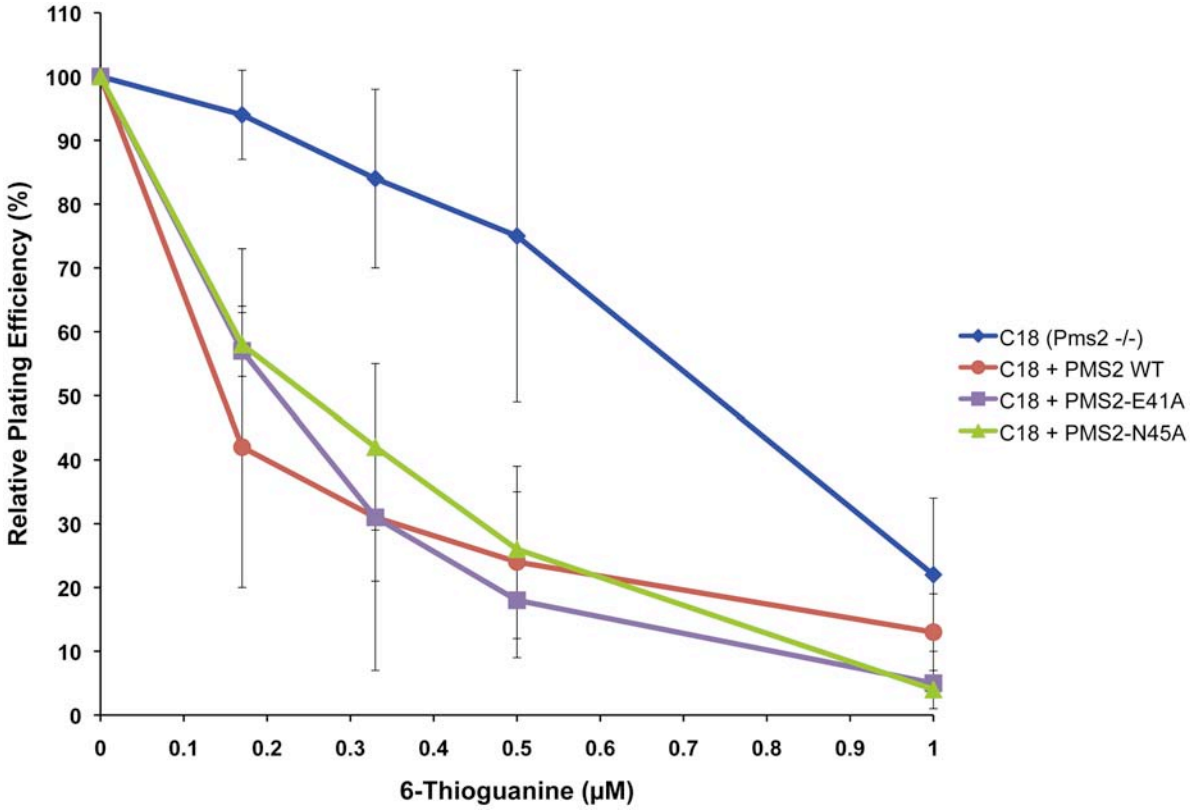


Figure 3-6. 6-Thioguanine response in PMS2-expressing cells. C18, *PMS2-WT*, *PMS2-E41A*, or *PMS2-N45A* cells were plated out and exposed to varying doses of 6-thioguanine for 24 hours. Colonies were stained with methylene blue and counted 8-10 days after plating.

however, the *PMS2* ATP hydrolysis and Mg^{2+} binding mutants were not more resistant to 6-TG than the WT complemented cells. At every 6-TG dose examined, the *PMS2* ATPase mutant cell lines had equivalent rates of colony formation as the *PMS2* WT cells.

These results are consistent with the MSI results. Both the *MLH1* ATP hydrolysis and Mg^{2+} mutants disabled repair *in vivo* as measured by increased microsatellite instability. In addition, the mutant cell lines are resistant to the cytotoxic response to 6-TG, providing further evidence that mutations disabling the MLH1 ATPase domain disrupt overall MMR function. The *PMS2* ATP hydrolysis and Mg^{2+} binding mutant cell lines do not display instability at microsatellite loci, and they are sensitive to the cytotoxic effect of 6-TG, suggesting that normal PMS2 ATPase domain function is less critical for MMR function.

DISCUSSION

The MutL homologues MLH1 and PMS2 belong to the family of GHL proteins, which contain an ATPase domain in their N-termini. The ATPase function is critical for biochemical activity of each family member (Dutta and Inouye, 2000). The importance of the ATPase domain for MutLα function has been demonstrated *in vivo* in yeast (Hall et al., 2002; Tran and Liskay, 2000) and *in vitro* with cell extracts and human purified protein (Raschle et al., 2002; Tomer et al., 2002). These studies indicated that ATP binding induces a conformational change in MLH1 that is necessary for MLH1 and PMS2 dimerization at their N-termini. In addition, double mutation of the residues critical for ATP binding or hydrolysis in MLH1 and PMS2 abolished repair. However, in yeast it was observed that mutations that abolished Mlh1 ATP binding and/or hydrolysis affected repair to a greater extent than the equivalent mutations in Pms1 (Tran and Liskay, 2000). This result was not manifested in the human *in vitro* analyses using purified proteins. Rather, mutations in the ATP binding or hydrolysis residues of *MLH1* impacted repair to the same extent as those in *PMS2,* indicating that *in vitro* in the human proteins, the MLH1 and PMS2 ATPase domains are equally critical for repair.

The effect of mutation in the ATP and hydrolysis and Mg^{2+} residues had yet to be examined *in vivo* in mammalian cells. In order to elucidate the contribution of the ATPase domains to repair in mammalian cells, I analyzed mutations impacting ATP binding and hydrolysis of MLH1 and PMS2 by MSI analyses and response to 6-TG.

The *MLH1* ATP hydrolysis mutant, *MLH1-E34A*, was disabled for mutation avoidance as indicated by increased microsatellite instability both mono- and dinucleotide repeat loci. In addition, it was resistant to the cytotoxic effect of 6-TG. The

effect of disabled ATP binding in MLH1 was also examined with the mutant *MLH1-N38A*. Similar to the hydrolysis mutant, *MLH1-N38A* expressing cells displayed increased MSI at mono and dinucleotide repeats and increased resistance to the cytotoxic effects of 6-TG. These results indicate that without the ability to bind or hydrolyze ATP, MLH1 was not able to function in mismatch repair. Since previous work showed that ATP hydrolysis of MutL was not necessary for association with MutS at a mismatch (Robertson et al., 2006), the ATPase function of the N-terminus of MLH1 is most likely contributing to downstream steps.

In contrast to the ATP binding and hydrolysis mutants of MLH1, the equivalent mutations in PMS2 had little if any impact on either mutation avoidance or the damage response. The ATP hydrolysis mutant, *PMS2-E41A*, showed wild-type levels of mono and dinucleotide repeat mutation and a normal response to the cytotoxic effects of 6-TG. The ATP binding mutant, *PMS2-N45A*, was similarly stable at MSI loci and sensitive to 6-TG. By *in vitro* analysis, *PMS2-N45A* had abolished repair function (Raschle et al., 2002). However, unlike the equivalent *MLH1* mutant, *PMS2-N45A* was still able to bind ATP. The authors of the study theorized that PMS2 only bound ATP transiently while MLH1 bound it consistently, highlighting the asymmetry present in the MutLα ATPase domain at the biochemical level.

Additional physical analyses conducted *in vitro* demonstrated the asymmetrical nature of ATP binding of both human and yeast MutLα (Sacho et al., 2008). In the absence of ATP, MutLα exists in an extended state, where MLH1 and PMS2 are dimerized at their C-termini, yet their N-termini are not dimerized and are thus open and extended. Low concentration of ATP induces a semi-extended one-arm state where one

unit of the dimer is bound by ATP and condenses to interact with the C-termini. Higher concentrations of ATP bring about a fully condensed state where both units of the MutLα dimer are bound by ATP and condensed down near the C-termini. As MLH1 has been shown to have >10-fold higher affinity for ATP than PMS2 (Hall et al., 2002), it is likely that MLH1 is the component of MutLα that initially binds ATP. This asymmetry in the order in which ATP binds could explain the unequal contributions of the MLH1 and PMS2 ATPase domains to repair (Figure 3-7).

Because the MutL ATPase domain is needed to activate downstream steps including activation of MutH (Spampinato and Modrich, 2000), the ATPase domains of human MutLα are likely to be necessary for the recruitment/activation of downstream players. My data suggest that the MLH1 and PMS2 ATPase domains contribute differentially to these functions (Figure 3-7). One possibility is the activation of the latent endonuclease function of MutLα, necessary for 3'-nick directed repair. This activity was shown to be dependent on the ATPase function of MutLα (Kadyrov et al., 2006). However, only the double hydrolysis mutant, *MLH1-E34A*/*PMS2-E41A*, was studied in the analysis. It is possible that if the mutations were analyzed independently of each other, the MLH1 ATP hydrolysis mutant may impact the endonuclease function more than the PMS2 ATP hydrolysis mutant, giving rise to the asymmetry observed. Additionally, the MLH1 ATPase domain could be critical for recruitment of other downstream factors, such as PCNA, EXO1 and RFC. In yeast, it was shown that the ATP-dependent association of the N-termini of MutLα is transient and depends on hydrolysis of ATP for dissociation (Tran and Liskay, 2000). Therefore, the complex is "locked in" when the ATP hydrolysis mutations are present in the cell, and other

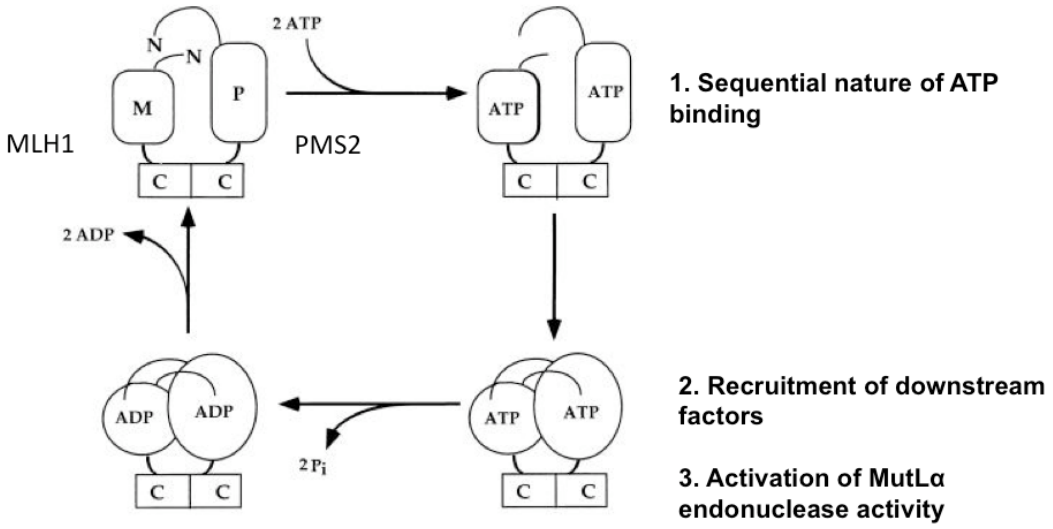


Figure 3-7. Model of asymmetry in MutLα ATPase domain. The observed asymmetry in the MutLα ATPase domain may be due to 1. The sequential binding of ATP, whereby MLH1 binds ATP first. 2. The MLH1 ATPase domain may be responsible for recruitment of downstream factors such as PCNA or EXO1. 3. The MLH1 ATPase domain may be responsible for activating the endonuclease activity of MutLα. Figure reprinted with permission from Tran and Liskay (2000) MCB 20:6390-6398.

components of MMR may be part of this complex. Immunoprecipitations with the MutLα ATP hydrolysis mutants could help identify previously undescribed components of MMR, such as a redundant exonuclease. Furthermore, comparisons of immunoprecipitations with the ATPase mutants of MLH1 versus PMS2 may help shed light on which downstream MMR proteins are dependent on the ATPase domains of MutLα for their assembly.

While the asymmetry observed between the affect of mutations in the ATPase domains of human MLH1 versus PMS2 is similar to what was observed in yeast (Tran and Liskay, 2000), it is in contrast to results obtained in biochemical studies with human cell extracts (Raschle et al., 2002; Tomer et al., 2002). The studies utilizing human MutLα were conducted in a cell-free system in the absence of replication and may not accurately reflect the complexities of *in vivo* MMR. However, because the ATP binding and hydrolysis residues of PMS2 were critical *in vitro* but not *in vivo*, this indicates something can compensate for the lack of PMS2 ATPase activity *in vivo* that is not found in the *in vitro* system. One explanation for this is the tethering of replication to MMR. *In vivo*, the MMR apparatus likely is in close association with the replication apparatus, through interaction with PCNA. *In vitro*, the DNA substrate does not undergo replication and the MMR complex must assemble at the mismatch without replication. It is possible that *in vivo*, PCNA is initially located to the site of the mismatch due to its involvement with replication, but *in vitro* it must be recruited to the mismatch by components of MMR. Potentially, the ATPase domain of PMS2 could be necessary for this recruitment *in vitro*, but *in vivo*, since PCNA is already at the site, the PMS2 ATPase domain is less critical. To test this, a chromatin immunoprecipitation could be performed. The

mismatched DNA substrate could be incubated with cell extract supplemented with either WT or ATP hydrolysis or binding mutant PMS2. After cross-linking, an IP against PCNA would be carried out, and then PCR could be performed to determine if PCNA is located at the mismatch. Its presence in the experiment with WT PMS2 but not mutant PMS2 would indicate the ATPase domain of PMS2 is recruiting PCNA to the mismatch.

There are a number of possibilities to explain the observed asymmetry in the ATPase domains of MutLα *in vivo*, and investigations to resolve the basis of the asymmetry will further our knowledge of the role of MutLα during repair. Additionally, understanding why asymmetry was observed *in vivo* but not *in vitro* is critical to assigning a role for the ATPase domain of PMS2.

CHAPTER 4

The role of DCTD in the MMR-dependent DNA damage response to 6-thioguanine

Abstract

Cellular response to S_N1-type methylating agents depends on the DNA mismatch repair system to signal apoptosis. It was recently shown in yeast that the enzyme deoxycytidylate deaminase (*DCD1*) also plays a role in this DNA mismatch repair dependent response to methylation damage. Based on previous studies in yeast and cultured mammalian cell lines, DCD1 and its mammalian homologue DCTD, are the enzymes primarily responsible for controlling the levels of dCTP and dTTP in cells. I analyzed the effect of deficiency of mammalian DCTD in human cells and cells derived from *Dctd*$^{ko/ko}$ mice. When DCTD was diminished by siRNA in HeLa and HEK293, cells displayed resistance to the methylation mimetic 6-thioguanine. In contrast, cells derived from *Dctd*-deficient mice remained sensitive to 6-TG. Analysis of the nucleotide pool levels showed that *Dctd*$^{ko/ko}$ mice displayed only slightly increased dCTP:dTTP pool ratio. However, the HeLa and HEK293 cells showed no significant change in the dCTP:dTTP levels upon reduction of DCTD expression. These results suggest that mammalian cells can compensate for the absence of deoxycytidylate deaminase, thereby maintaining a balance of the dCTP and dTTP pools. In addition, reduction of DCTD expression in human cells causes resistance to 6-TG without measurable changes in total pool levels.

INTRODUCTION

As discussed in Chapter 1, MMR function is necessary to signal apoptosis in response to S_N1-type methylating agents, and the methylation-mimetic drug 6-thioguanine (Buermeyer et al., 1999b; Karran and Marinus, 1982; Swann et al., 1996). Understanding how cells respond to this type of DNA damage is important, as 6-TG is used clinically to treat a number of types of cancer, including leukemia (Gee et al., 1969). These cancers can lose their responsiveness to 6-TG, and MMR status has been hypothesized to play a role in this resistance (Fink et al., 1998).

Methylation damage and MMR

Treatment with S_N1-type methylating agents leads to the formation of O^6-methyl guanine adducts in the DNA (Karran and Bignami, 1992). These lesions alone are not good substrates for MMR. However, upon the next round of replication, DNA polymerase will incorporate in approximately equal frequency either a C or a T across from O^6-methyl guanine in the template (Patel et al., 1986b). The $^{O6\text{-}Me}$G/T mispairs are good substrates for MMR, and their formation triggers the MMR-dependent damage response via direct signaling and/or futile repair (see Chapter 1 for more detail). In addition, the methylation mimetic 6-TG induces a MMR-dependent damage response (Swann et al., 1996). 6-TG must first get converted to 6-thioGTP and then incorporated in the DNA. Subsequently, its sulfur group is methylated by S-adenosylmethionine transferase (Swann et al., 1996), creating methylated DNA analogous to S_N1-type methylators. During subsequent replication, the $^{6\text{-}Me}$TG in the template strand can pair with either C or T creating $^{6\text{-}Me}$G:T mispairs that provoke a MMR-dependent damage response. 6-TG is frequently used in cell culture studies rather than S_N1-type methylators

for the following reasons. First, in contrast to MNNG, 6-TG is non-volatile thus facilitating experiments requiring prolonged time periods. Second, O^6-MeG lesions, but not $^{6\text{-Me}}$TG lesions, can be removed by cellular methyl guanine methyl transferase (MGMT) (Karran and Bignami, 1992), the levels of which can vary greatly among cell lines, thus potentially complicating interpretation of results.

Cell cycle arrest and apoptosis

Two major classes of cell cycle checkpoints have been implicated in mediating the G_2 arrest following treatment with S_N1-type methylators or 6-TG. The kinase ataxia telangiectasia and Rad3 related (ATR) and its effector checkpoint kinase 1 (CHK1) are known to be necessary to signal G2 arrest following many types of damage, including UV irradiation, alkylation damage, and abortive DNA replication resulting from treatment with aphidicolin (Zhao and Piwnica-Worms, 2001). Upon treatment with 6-TG in cells with an intact MMR system, CHK1 becomes phophorylated at S317 (Yamane et al., 2004). Phosphorylated CHK1 in turn phosphorylates CDC25C phosphatase, inactivating it (Adamson et al., 2005). Without activated CDC25C phophatase, CDC2 is kept in its phosphorylated, inactive state. Inactive CDC2 eventually leads to G2/M arrest. Another kinase important for the MMR-dependent response to methylation damage is ataxia telangiectasia mutated (ATM) and its effector checkpoint kinase 2 (CHK2). ATM/CHK2 is known to be necessary for arrest following strand breaks induced by ionizing radiation (Bakkenist and Kastan, 2003). While the induction of arrest in response to S_N1-type methylating agents does not require ATM/CHK2, cells that escape G2 arrest induced by ATR/CHK1 will be arrested by ATM/CHK2 (Yan et al., 2004). Hence, activation of CHK1 is seen within 24 hours of treatment with methylating agents,

while activation of CHK2 is more gradual, becoming apparent at days 2-6 following treatment (Stojic et al., 2004b; Yan et al., 2004). In addition to inactivating CDC2, both ATM and ATR phosphorylate p53 and p73 following damage by DNA methylating agents (Stojic et al., 2004b).

Deoxycytidylate deaminase and methylation tolerance

To gain a better understanding of the MMR-dependent response to methylating agents, our lab conducted a screen in budding yeast to uncover additional genes that play a role in the response. This yeast screen identified the deoxycytidylate deaminase gene, *DCD1*, as a necessary component in the DNA mismatch repair dependent response to methylating agents (Liskay et al., 2007).

The deoxycytidylate deaminase protein catalyzes the conversion of dCMP to dUMP, which is the main precursor to dTTP (McIntosh and Haynes, 1984). Previous work with yeast showed that when *DCD1* function is compromised, dCTP levels rise and dTTP levels fall (McIntosh and Haynes, 1984). The resulting dCTP/dTTP pool imbalance, an approximately 127-fold increase over WT, leads to an increased mutation rate (Kohalmi et al., 1991). In addition, our lab showed that this pool imbalance causes cytotoxic resistance to the methylating agent nitrosoguanidine (Liskay et al., 2007). Epistasis analysis assigned *DCD1* to the MMR-dependent S_N1-methylation damage response pathway. As explained above, treatment with methylators or methylation mimetics creates ^{Me}G:T mispairs in the DNA. In the futile repair model, the MMR machinery recognizes and attempts to repair the $^{O6\text{-}Me}$G:T mismatch (Massey et al., 2002). However, because the $^{O6\text{-}Me}$G is in the template strand, repair is futile, resulting in iterative rounds of attempted repair and eventually a double-strand break and apoptosis

(Stojic et al., 2004b; Yamane et al., 2004). Because the formation of $^{O6\text{-}Me}$G:T mispairs ultimately triggers apoptosis, *DCD1*-deficient cells that have low levels of dTTP and high levels of dCTP are predicted to place a C across from the $^{O6\text{-}Me}$G at a greater rate than normal cells and be resistant to methylation-induced cytotoxicity (see Figure 4-1) (Liskay et al., 2007).

Deficiency in the rodent homologue of *DCD1*, *Dctd*, was reported to result in an imbalance in the dTTP:dCTP pool ratio, associated with an increase in the spontaneous mutation rate in mouse lymphoma and Chinese hamster cell lines (Bianchi et al., 1987; Meuth, 1981; Weinberg et al., 1981). Chinese hamster ovary cells deficient for *Dctd* appeared to display an increased resistance to the methylating agent MNNG (Meuth, 1981). However, in neither study were isogenic wild-type parental cells available for direct comparisons to the *Dctd* mutant cells. Further complicating interpretations, the mouse lymphoma line contained an additional mutation in *purine nucleoside phosphorylase* (*PNP*) (Weinberg et al., 1981), which might influence deoxynucleotide pools (Snyder et al., 1997).

Experimental rationale

To better address the consequences of *Dctd* deficiency in mammalian cells in an isogenic system, I utilized both siRNA in human cells and cells from a mouse knockout of *Dctd* to determine the effect of *Dctd* deficiency on the dCTP/dTTP pools and in the cytotoxic response to 6-thioguanine. Initially, I utilized siRNA-mediated knockdown of DCTD activity in HeLa cells, two human leukemia cell lines, and HEK293 cells and measured the cytotoxic response to 6-TG using cell viability and colony formation assays. Next, I compared the 6-TG cytotoxic responses in primary MEFs from *Dctd*$^{+/+}$

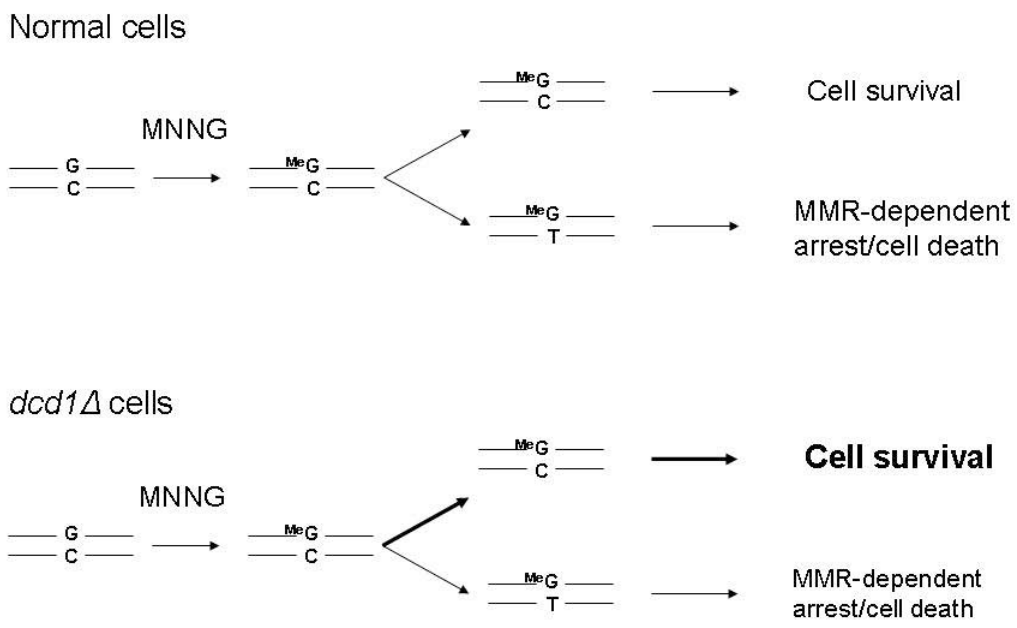


Figure 4-1. Response to S_N1-type methylating agents in wild type and *dcd1Δ* yeast cells. Treatment with the S_N1-type methylator MNNG creates O^6-MeG lesions in DNA. Upon the next round of replication, polymerase places either a C or T across from the lesion. In wild type cells, the C and T are incorporated with approximately equal frequency, leading to MMR-dependent cell death. In *dcd1Δ* cells, the dCTP:dTTP imbalance causes polymerase to place a C across from the lesion at an elevated rate, resulting in fewer $^{O6\text{-}Me}$G:T mismatches and increased cell survival over wild type cells. Adapted from (Liskay et al., 2007).

and *Dctd*$^{ko/ko}$ mice as well as established MEFs and lymphocytes from the *Dctd*$^{+/+}$ and *Dctd*$^{ko/ko}$ mice. Finally, I measured the nucleotide pool levels in primary cells derived from *Dctd*$^{+/+}$ and *Dctd*$^{ko/ko}$ mice and the HeLa and HEK293 cells.

RESULTS

DCTD siRNA

To assess the effect of diminished *DCTD* expression in human cells, I utilized siRNA to reduce expression of DCTD. siRNA stands for small interfering RNA and is a widely-used technique to temporarily reduce expression of, or "silence", target genes (Fire et al., 1998). Short RNA oligonucleotides complimentary to the mRNA sequence of the target gene are transfected into cells. Silencing of gene expression occurs when the siRNA binds to endogenous mRNA. This complex is then degraded by the protein dicer, creating more siRNAs that go on to silence the gene for approximately 72-120 hours (Bernstein et al., 2001). Validated siRNAs against a range of target genes are commercially available, and I performed siRNA against *DCTD* in the human cancer cell line HeLa using a validated oligonucleotide from Ambion. Maximum knockdown of ~90% was achieved 48-96 hours after transfection. As a control for experimental manipulations, a scrambled oligonucleotide was also transfected. A Western blot of cells transfected with Scrambled or DCTD siRNA using anti-human DCTD antibody can be seen in Figure 4-2. Knockdown was also performed on HEK293 epithelial cells and K562 and SUP-B15 leukemia cells, with maximum knockdown following the same timeline. siRNA-mediated knockdown of DCTD was achieved in all cell lines examined.

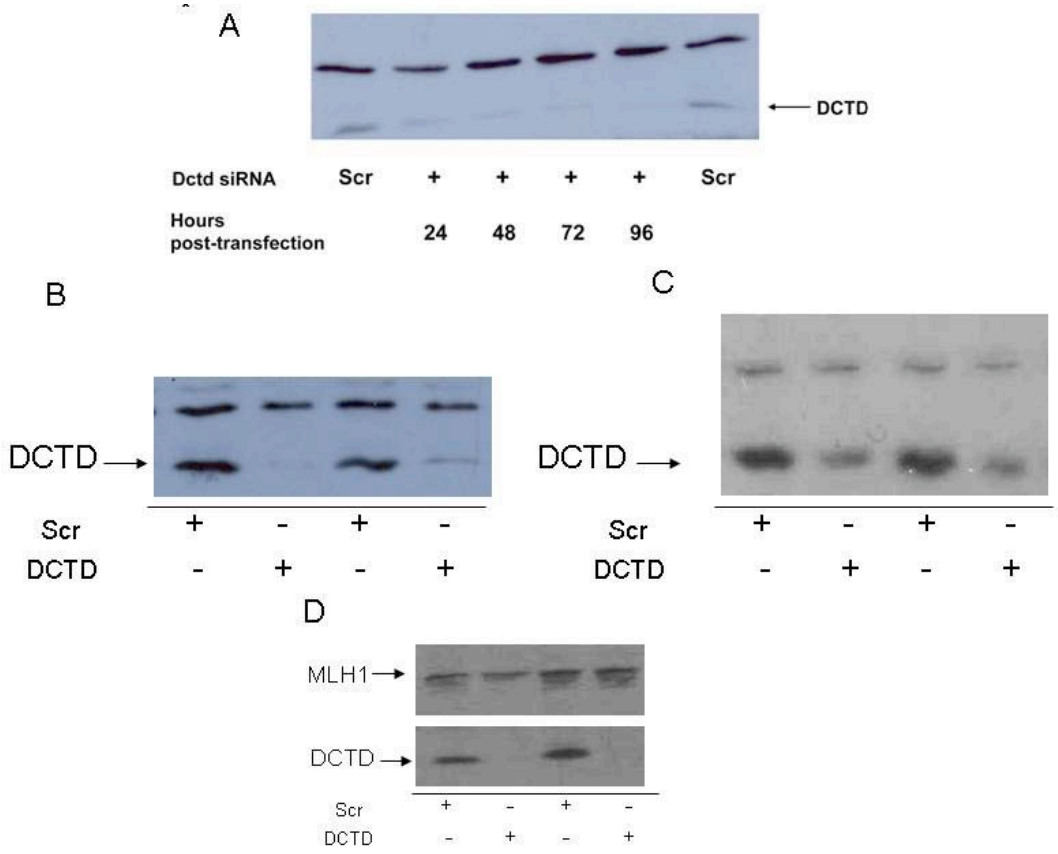


Figure 4-2. DCTD expression in cells transfected with siRNA. A, HeLa cells. B, K562 cells. C, SUP-B15 cells. D, 293 cells. All cells are at 48 and 72 hours following siRNA transfection, except HeLa cells, which are at 24, 48, 72, and 96 hours post-transfection. In panels A, B, and C, the background band served as a loading control; in panel D, MLH1 is used a loading control.

Cytotoxicity assays in HeLa cells with normal and reduced *DCTD* expression

To determine whether silencing *DCTD* expression affects the cytotoxic response to 6-TG, response curves were performed on transfected cells as described in Materials and Methods. 6-TG was added to cells at 48 hours post-siRNA transfection, when maximal mRNA repression is starting to occur. Toxicity from 6-TG requires two rounds of DNA replication, one to incorporate the 6-TG into the DNA and have SAM methylate the thio group, and one for the polymerase to place either the correct or incorrect nucleotide across from the lesion. At this point, DNA mismatch repair recognizes the lesion and signals to downstream factors to initiate apoptosis. In order to assess whether *DCTD* expression affects resistance to 6-TG, *DCTD* repression must persist long enough for multiple rounds of DNA replication. HeLa cells double once every 20-24 hours, and DCTD repression is seen even 120 hours post-siRNA transfection. Therefore, the duration of reduced *DCTD* expression should have been sufficient to detect 6-TG resistance. Figures 4-3 and 4-4 show that when *DCTD* expression was silenced by siRNA, cells displayed resistance to the cytotoxic effect of 6-TG compared to cells transfected with the scrambled control. The resistance to 6-TG was apparent at both 72 and 96 hours post-6-TG addition. The maximal level of resistance of DCTD-knockdown cells over control cells was observed at 96 hours post-drug addition, in 0.3 μM 6-TG. The cells with diminished DCTD expression displayed an approximately 8-fold resistance to 6-TG at this dosage compared to cells with normal DCTD expression. This suggests that when HeLa cells lack DCTD, their ability to signal arrest and apoptosis in response to 6-TG is compromised.

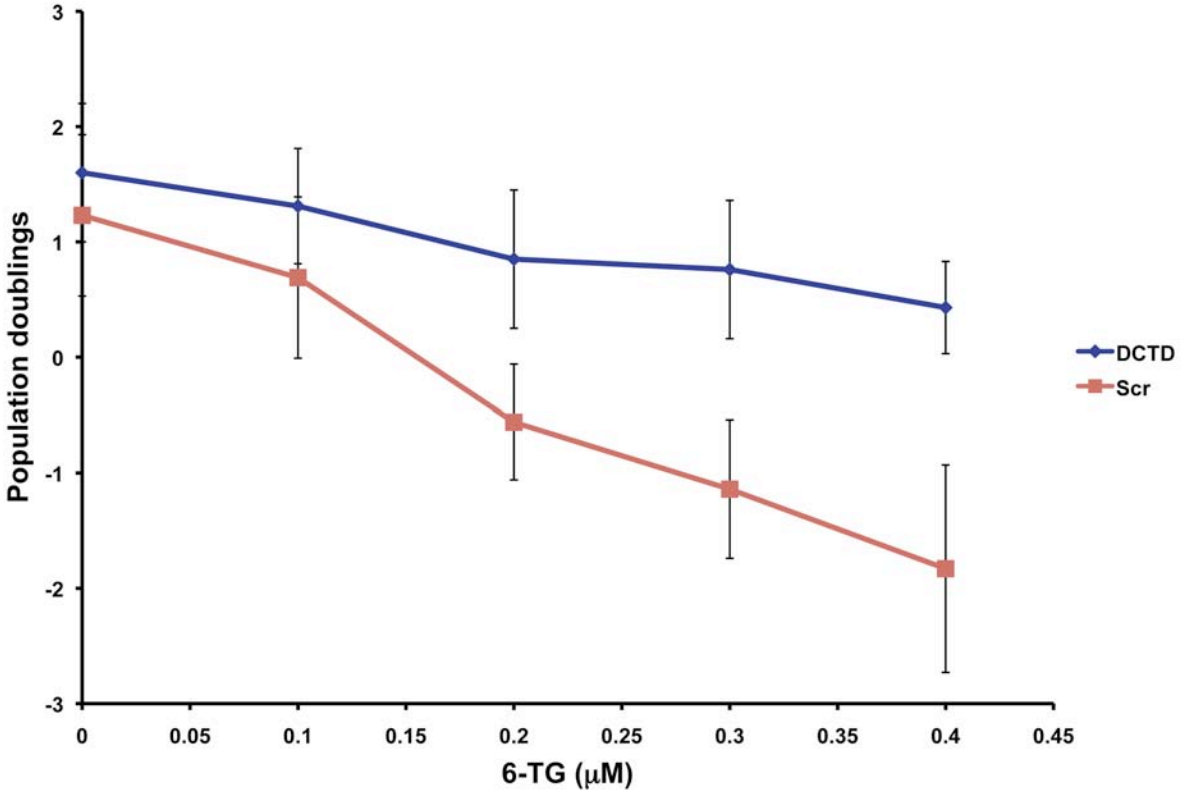


Figure 4-3. 6-TG cytotoxicity in HeLa cells. HeLa cells were transfected with siRNA against Dctd or Scr control. Cells were treated with 6-TG and counted 72 hours after drug addition.

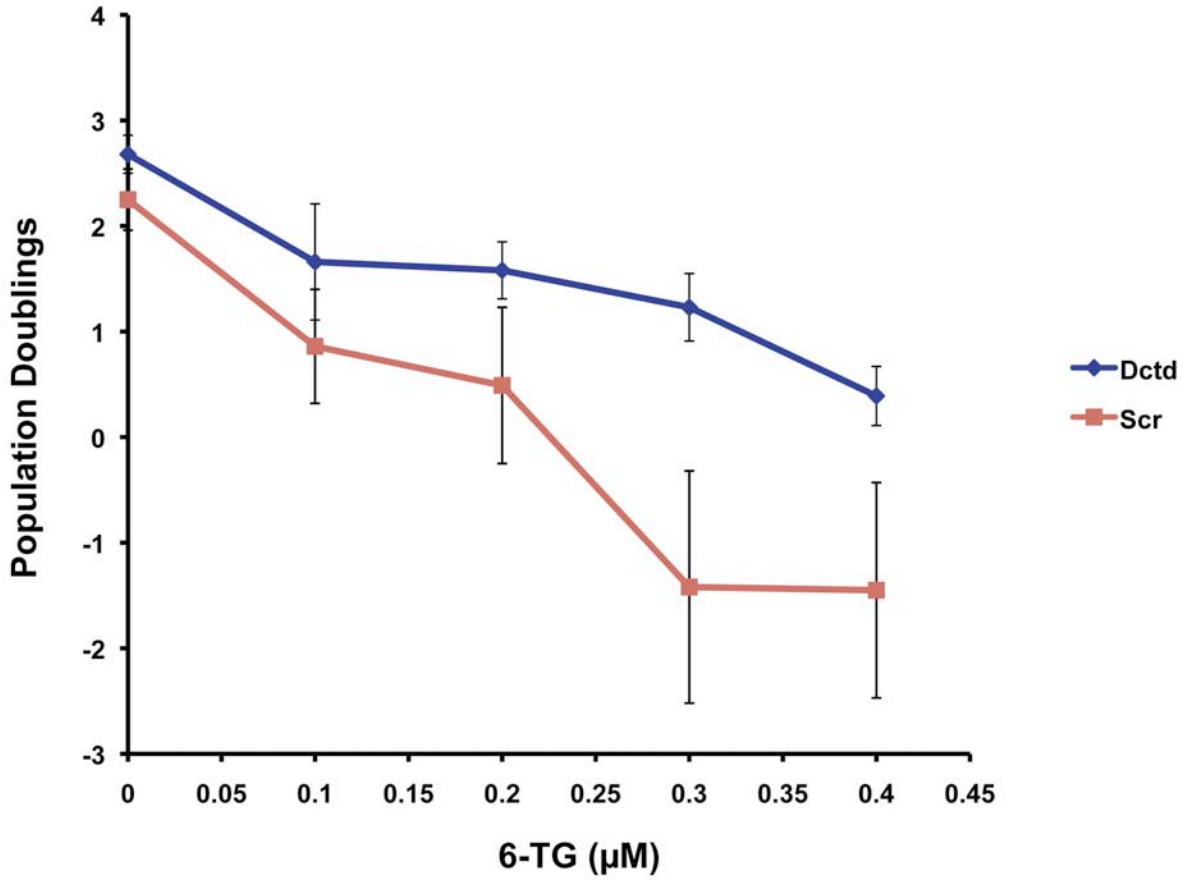


Figure 4-4. 6-TG cytotoxicity in HeLa cells. Cells were transfected with siRNA against either Dctd or Scr control. Cells were treated with 6-TG and counted 96 hours after drug addition.

6-TG clonogenic assay in HeLa cells

Next, I examined the effect of *DCTD* knockout on the ability of cells to form colonies in the presence of 6-TG. HeLa cells were transfected with either the *DCTD*-specific or scrambled oligonucleotide and assayed for colony formation as described in Materials and Methods. As seen in Figure 4-5, cells with diminished *DCTD* expression were able to form more colonies in the presence of 6-TG than cells with normal *DCTD* expression. At the highest dose of 6-TG (0.5 μM), the level of resistance of the cells knocked down for DCTD was approximately 5-fold higher than the cells transfected with the scrambled oligonucleotide. The colony formation assay, together with the cell viability curves, suggested that silencing *DCTD* expression in HeLa cells enhanced resistance to 6-TG treatment.

6-TG cytotoxicity assays in leukemia cells

Since methylators and methylation mimetics are frequently used to treat leukemias, I examined the effect on knocking down *DCTD* in human leukemia cell lines. Two cell lines were tested, K562 and SUPB-15. K562 is a human erythroleukemia cell line derived from a patient with chronic myelogenous leukemia. SUP-B15 is lymphoblast line derived from a patient with acute lymphoblastic leukemia. As with the HeLa cells, knockdown of DCTD was maintained long enough to perform cytoxicity assays as described. As seen in Figure 4-6, K562 cells do not appear to become resistant to 6-TG upon knockdown of DCTD; at every 6-TG dose examined, cells grew at the same rate as the scrambled oligonucleotide. However, SUP-B15 cells did show resistance compared to scrambled control. At 1 μM 6-TG, the DCTD-transfected cells

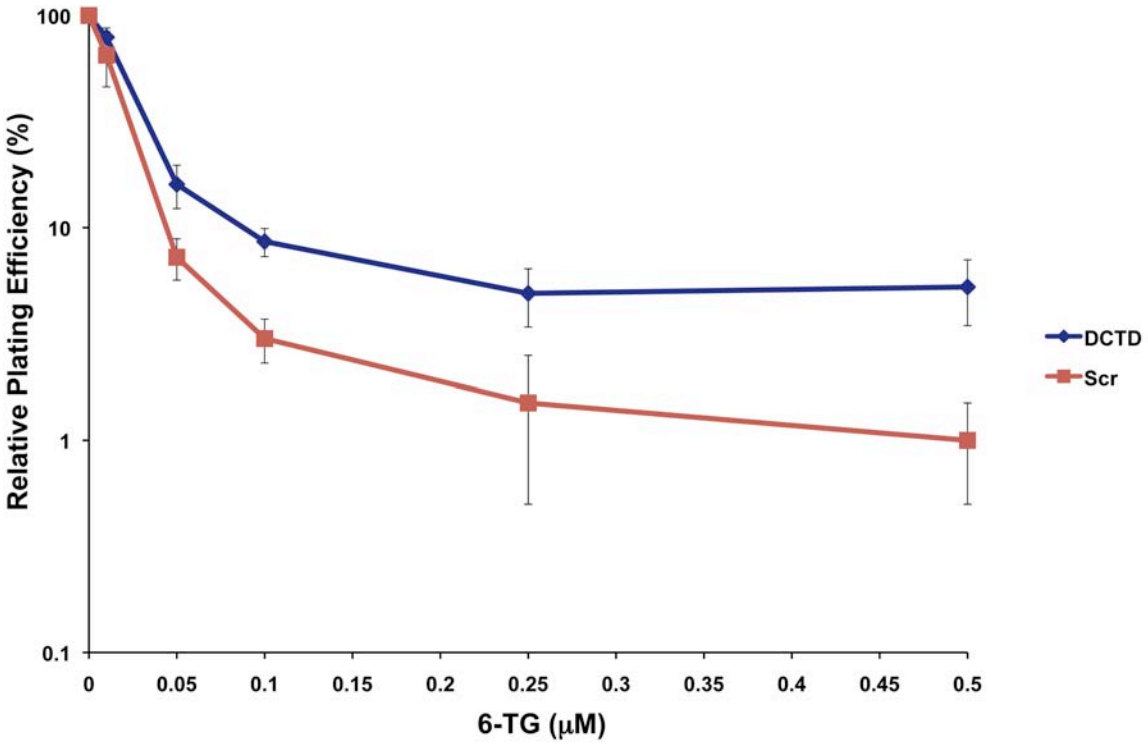


Figure 4-5. 6-TG Colony formation assay. HeLa cells were transfected with DCTD or Scrambled oligonucleotide and plated out for colonies in various concentrations of 6-TG.

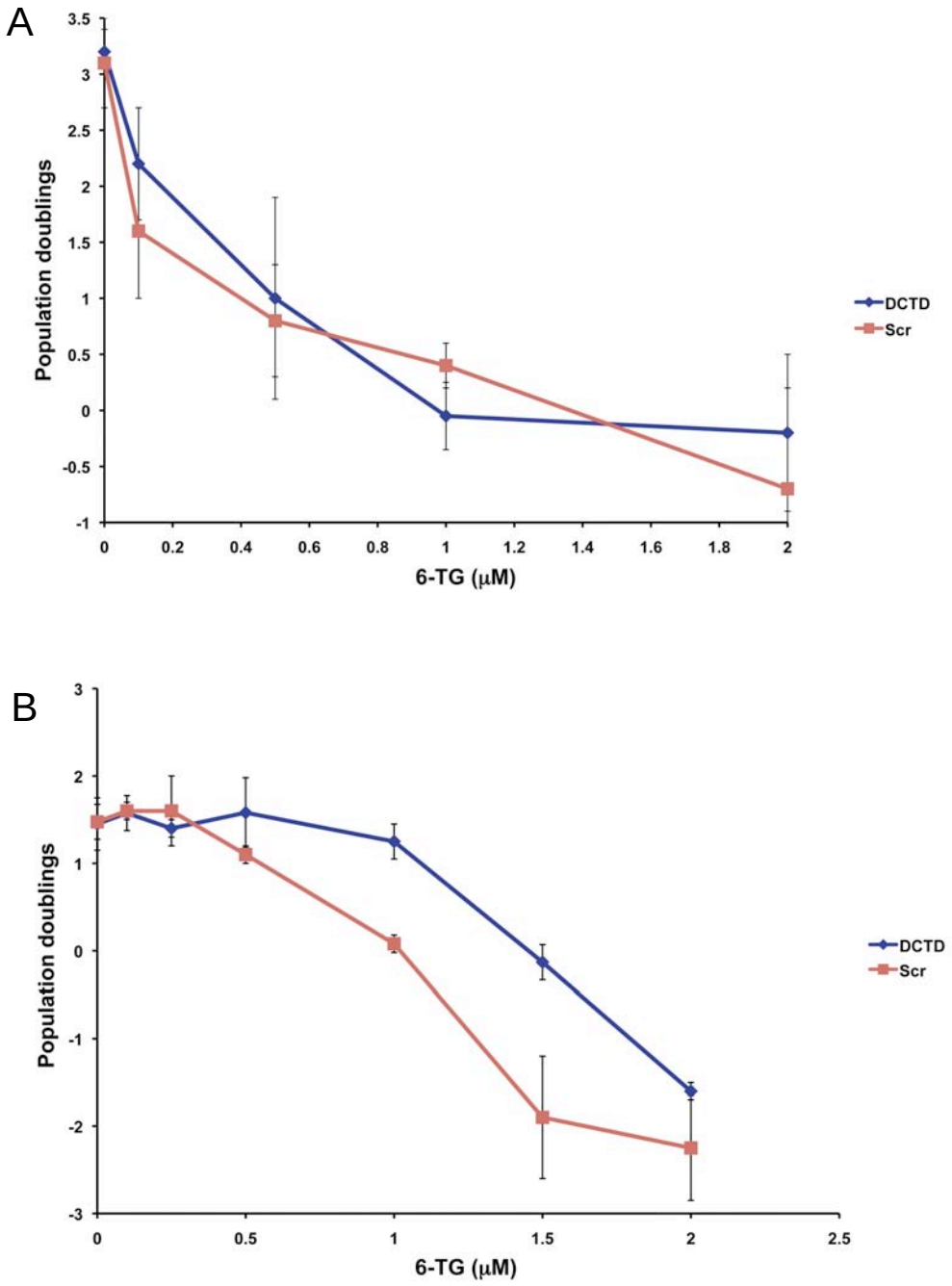


Figure 4-6. 6-TG cytotoxicity response in K562 and SUP-B15 cells. Cells were transfected with siRNA against either Dctd or a Scr control. Cells were treated with 6-TG and counted 72 hours after drug addition. A, K562 cells. B, SUP-B15 cells.

display a 2-fold resistance over the control cells. Why resistance was observed in SUP-B15 cells but not the K562 cells is unknown and will require further investigation.

6-TG cytotoxicity assays with HEK293 cells

All cytotoxicity assays described above to address the effect of *DCTD* knockdown on tolerance to 6-TG were conducted with tumor cell lines. In order to test the effect of DCTD-knockdown in a "normal" cell line, I repeated the assays using HEK293 cells, which are a non-tumor human embryonic kidney cell line. I transfected the cells with

either *DCTD* or scrambled siRNA and assayed for 6-TG tolerance by colony formation. Figure 4-7 shows that at the highest dose administered, 2.5 μM, the 293 cells with reduced *DCTD* expression are more resistant to 6-TG than those treated with the scrambled oligonucleotide, about a 6-fold increase in resistance. Therefore, in two tumor and one non-tumor human cell lines, *DCTD* expression levels appeared to be necessary for the cytotoxic response to 6-TG.

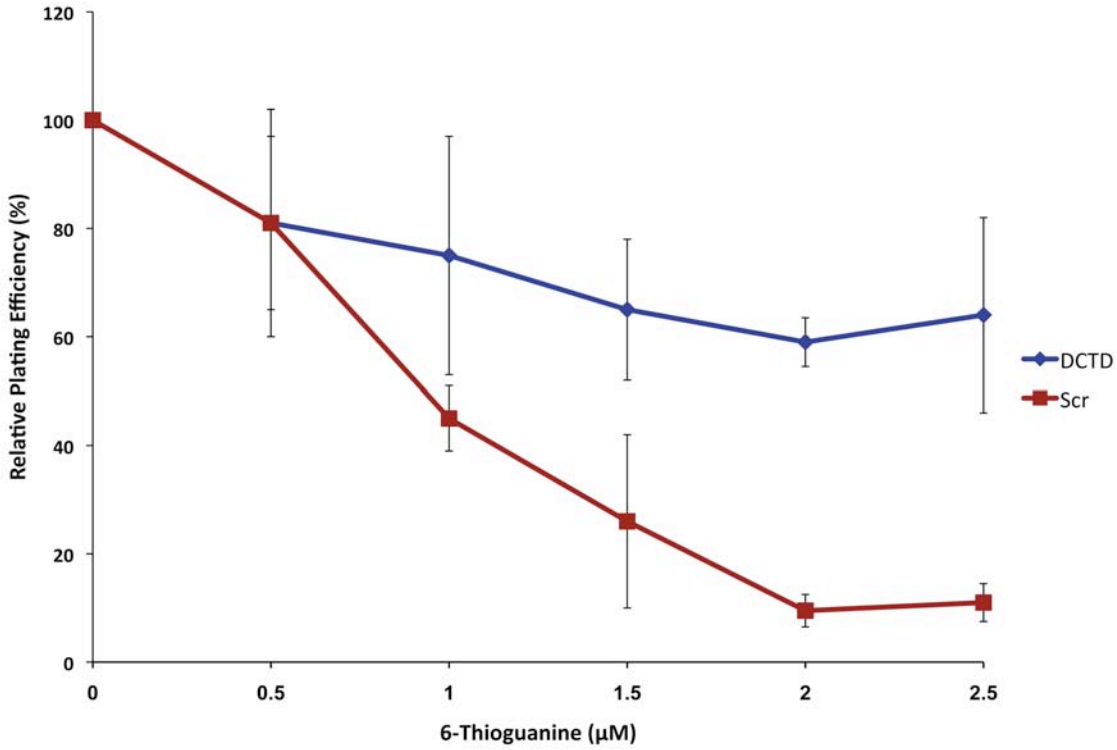


Figure 4-7. 6-TG Colony formation assay. HEK293 cells were transfected with DCTD or Scrambled oligonucleotide and plated out for colonies in various concentrations of 6-TG.

Dctd mouse knockout

While the siRNA-mediated knockdown provided a good method for isogenic comparison of the effects of deficiency of DCTD, it did not cause a complete absence of *DCTD* expression. A mouse knockout of *Dctd* is available commercially, and because the $Dctd^{ko/ko}$ mouse is a true null, it represents the cleanest system for evaluating the effects of *Dctd* deficiency on the cytotoxic response to 6-TG. In addition, the mouse knockout would allow me to observe phenotypes not possible using cell culture assays alone, such as increased tumorigenesis. Therefore, I obtained *Dctd* knockout mice from Texas A&M Institute for Genomic Medicine to further my studies. Because no antibody that recognizes mouse DCTD was commercially available, I performed RT-PCR on mouse embryonic fibroblasts (MEFs) from $Dctd^{+/+}$ and $Dctd^{ko/ko}$ mice. Results in Figure 4-8 show no detectable *Dctd* mRNA expression in cells from $Dctd^{ko/ko}$ mice when compared with cells from *wild-type* littermates.

Comparisons of the 6-TG response in $Dctd^{+/+}$ and $Dctd^{ko/ko}$ MEFs

Next, I compared the cytotoxic response to 6-TG in primary MEFs in $Dctd^{+/+}$ and $Dctd^{ko/ko}$ cells cultured from day E13.5 embryos. Figure 4-9 shows that there was no significant difference in the response to 6-TG between the $Dctd^{+/+}$ and $Dctd^{ko/ko}$ cells. This result is in contrast to the resistance seen in other studies with *Dctd*-deficient mammalian cell lines. Since the previous work showing more striking dNTP pool imbalances associated with *Dctd* deficiency was conducted using established rodent and mouse lymphoma cell lines, I next tested the 6-TG response in spontaneously established

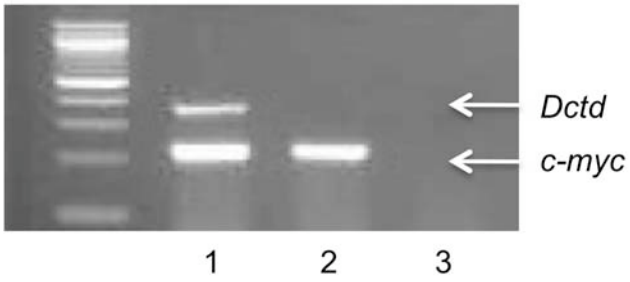


Figure 4-8. RT-PCR of *Dctd*$^{+/+}$ and *Dctd*$^{ko/ko}$ cells. Total RNA was isolated from *Dctd*$^{+/+}$ and *Dctd*$^{ko/ko}$ cells and cDNA was made using reverse transcriptase. PCR was performed using primers specific to *Dctd* cDNA and c-myc cDNA as a control. Lane 1, *Dctd* $^{+/+}$; Lane 2, *Dctd*$^{ko/ko}$; Lane 3, No transcriptase.

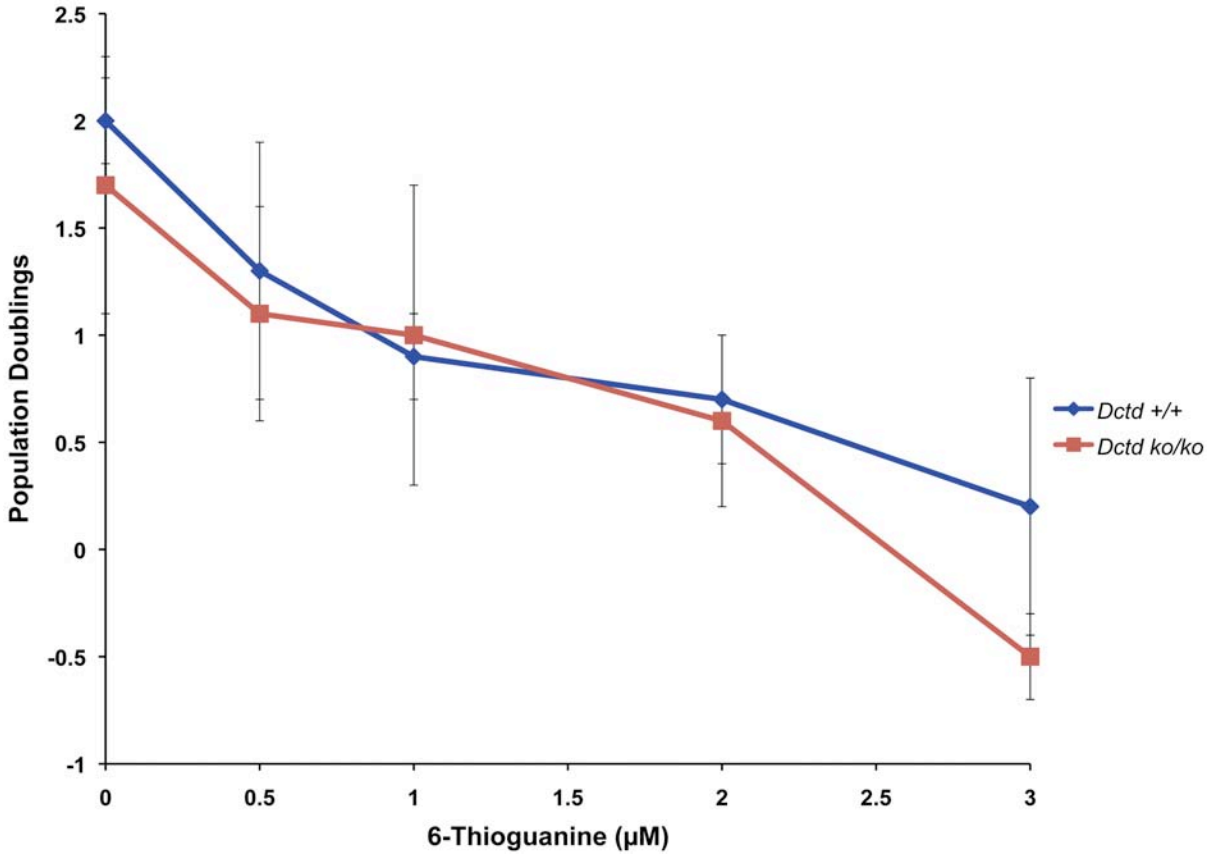


Figure 4-9. 6-TG resistance in *Dctd*$^{+/+}$ and *Dctd*$^{ko/ko}$ primary MEFs. Cells harvested from day E13.5 mice were plated and exposed to 6-TG for one population doubling. Cells were counted at 72 hours after addition of 6-TG.

Dctd$^{+/+}$ and *Dctd*$^{ko/ko}$ MEFs. As seen in Figure 4-10, there was no significant difference in the response to 6-TG between the *Dctd*$^{+/+}$ and *Dctd*$^{ko/ko}$ MEFs.

I also tested the response to 6-TG in established MEFs using a colony formation assay. The results in figure 4-11 show that while a slight resistance is seen in the *Dctd*$^{ko/ko}$ cells at the lowest dose, no significant difference exists between the *Dctd*$^{ko/ko}$ and Dctd$^{+/+}$ MEFs. Thus, in both primary and established MEFs, *Dctd* does not appear to be necessary for the apoptotic response to 6-TG.

6-TG response in lymphocytes from *Dctd* $^{ko/ko}$ and *Dctd* $^{+/+}$ mice

The most dramatic increase in dCTP:dTTP pool level ratio in response to *Dctd* deficiency was previously reported for a mouse lymphoma line (Weinberg et al., 1981). To explore the possibility that *Dctd* deficiency in lymphocytes may have a more pronounced effect on dNTP pool levels, I tested the response to 6-TG in cultured lymphocytes from *Dctd*$^{+/+}$ and *Dctd*$^{ko/ko}$ mice. Spleens were harvested from *Dctd*$^{+/+}$ and *Dctd*$^{ko/ko}$ mice and lymphocytes were isolated by a Lympholyte M column as described in Materials and Methods. After stimulation with IL-2 and Concanavalin A, the cells were plated and treated with 6-TG. Again, no increase in resistance to 6-TG was seen in the lymphocytes from *Dctd* knockout mice (Figure 4-12). This suggests that *Dctd* is not necessary for the cytotoxic response to 6-TG in lymphocytes.

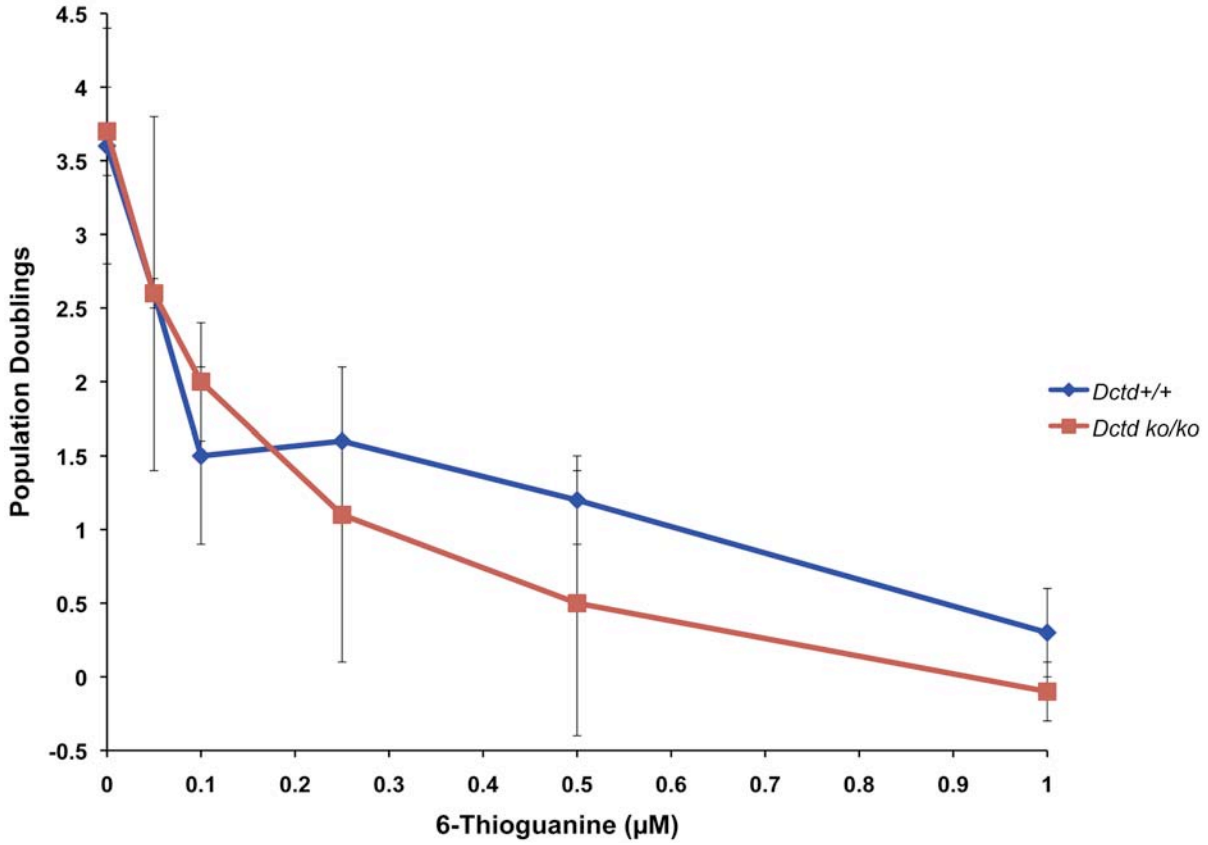


Figure 4-10. 6-TG response in *Dctd*$^{+/+}$ and *Dctd*$^{ko/ko}$ established cells. Established MEFs from *Dctd*$^{+/+}$ and *Dctd*$^{ko/ko}$ mice were plated out and exposed to 6-TG. Cells were counted at 72 hours post-drug addition.

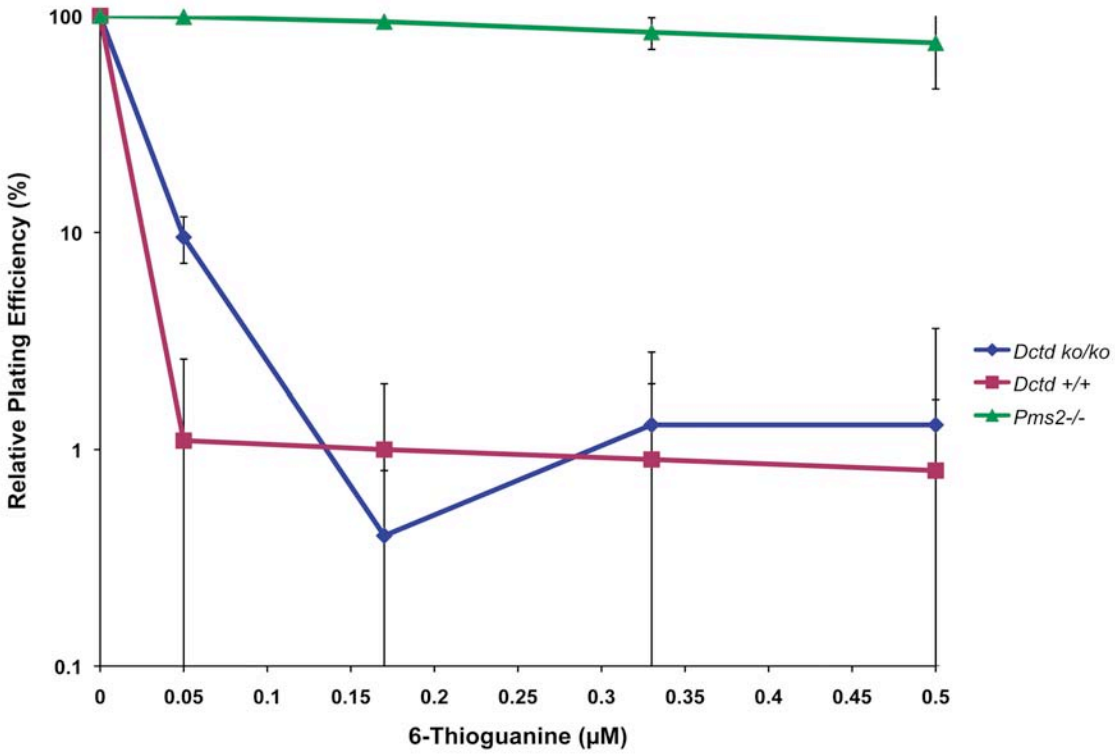


Figure 4-11 Colony Formation Assay. Established MEFs from *Dctd* $^{ko/ko}$ or *Dctd* $^{+/+}$ mice were plated out for colonies and exposed to 6-TG for 24 hours. *Pms2*$^{-/-}$ MEFs are included as a reference for a resistant phenotype.

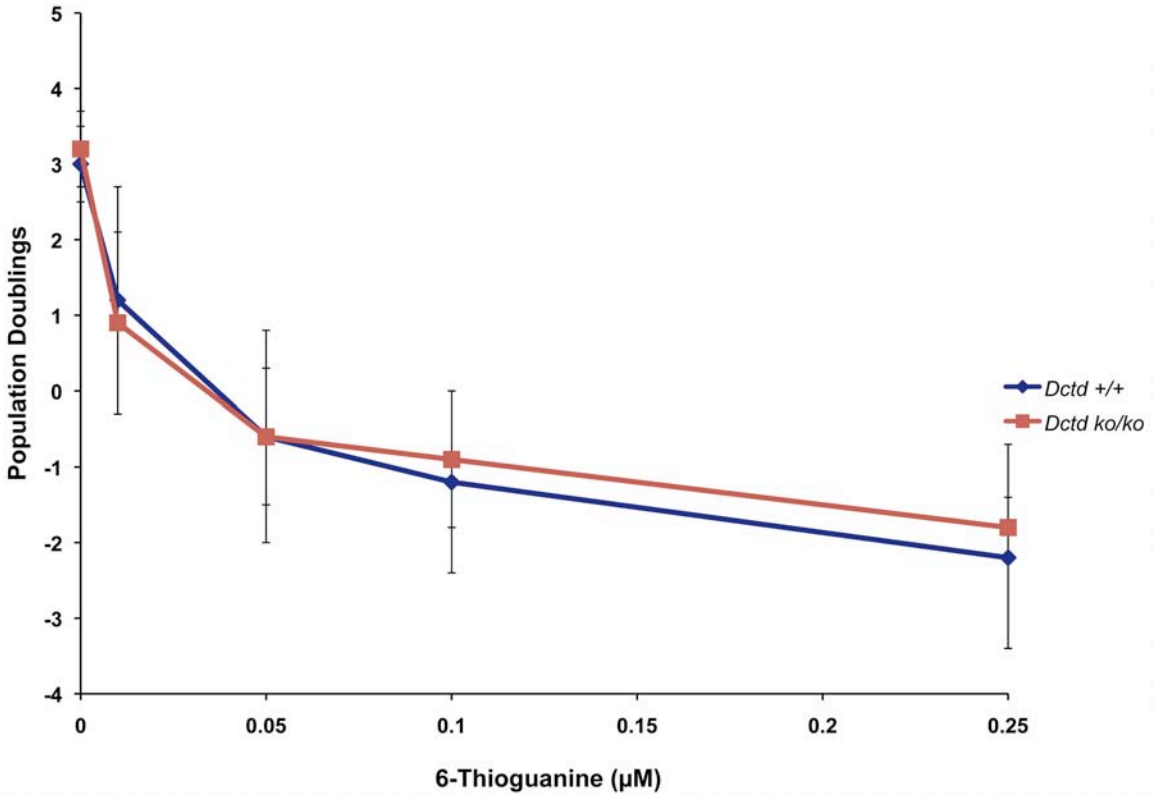


Figure 4-12. 6-TG response in *Dctd*$^{+/+}$ and *Dctd*$^{ko/ko}$ lymphocytes. Lymphocytes harvested from the spleens of *Dctd*$^{+/+}$ and *Dctd*$^{ko/ko}$ mice were activated by IL-2 and concanavalin A, plated out and exposed to 6-TG. Cells were counted at 72 hours post-drug addition.

Phenotype of *Dctd* $^{ko/ko}$ mice

Dctd (*DCD1*) deficiency has been associated with a mutator phenotype due to dTTP/dCTP pool imbalance (Kohalmi et al., 1991; Weinberg et al., 1981). Frequently, deficiency in genes that are associated with a mutator phenotype will result in an increased or accelerated cancer burden (Prolla et al., 1998). Although I observed no resistance to 6-TG in fibroblasts and lymphocytes, those represent only two cell types. If *Dctd* deficiency disrupts nucleotide pools in other cell types or tissues, the resultant mutator phenotype could manifest itself as cancer. To address this possibility, I monitored a cohort of *Dctd*$^{+/+}$ and *Dctd*$^{ko/ko}$ mice for 18 months and I observed no evidence for increased tumorigenesis in the *Dctd*$^{ko/ko}$ mice as shown by essentially identical survival to the *Dctd*$^{+/+}$ mice (Table 4-1).

dNTP pool measurements

To test whether the differing response to 6-TG observed in the HeLa and HEK293 cells and *Dctd* knockout mice is correlated with differential effects on the levels of dCTP and dTTP, cells were sent to our collaborator Dr. Chris Mathews at Oregon State University for dNTP pool measurements. The dNTP levels of primary MEFs from *Dctd*$^{+/+}$, *Dctd*$^{+/ko}$, and *Dctd*$^{ko/ko}$ mice can be seen in Table 4-2. The ratio of dCTP:dTTP is slightly elevated in the *Dctd*$^{ko/ko}$ cells, a ~2-fold increase compared to the *Dctd*$^{+/+}$cells. Therefore, the findings with *Dctd*$^{ko/ko}$ cells contrast with results from studies with mouse lymphoma, Chinese hamster ovary and Chinese hamster lung cells that demonstrated 100, 27, and 4-fold increases, respectively, in the dCTP:dTTP pool ratios in *Dctd*-deficient cells relative to non-isogenic “parent” lines (Bianchi et al., 1987; Meuth, 1981;

Weinberg et al., 1981). The modest level of pool imbalance I observed in *Dctd*$^{ko/ko}$ cells appears to be insufficient to elicit signigicant resistance to 6-TG.

The dNTP levels of the HeLa and HEK293 cells knocked down for DCTD were also measured. Ninety-six hours after transfection with either DCTD or scrambled siRNA, cells were harvested and sent for dNTP pool measurements. Surprisingly, neither the HeLa cells nor the HEK293 cells showed a significant change in the dCTP:dTTP pool ratio following knockdown of DCTD (Tables 4-3 and 4-4).

	# of mice followed	% survival at 18 months
Dctd $^{+/+}$	19	100
Dctd $^{+/ko}$	24	100
Dctd $^{ko/ko}$	18	100

Table 4-1. Survival of *Dctd* $^{+/+}$, *Dctd* $^{+/ko}$, and *Dctd* $^{ko/ko}$ mice.

dNTP (pmol/10^6 cells)

	dCTP	dTTP	dATP	dGTP
Dctd $^{+/+}$	35.2±5.6	33.0±5.4	16.7±2.0	7.4±1.1
Dctd $^{+/ko}$	49.2±5.0	37.1±5.7	18.8±1.9	7.0±1.0
Dctd $^{ko/ko}$	62.8±3.9	28.0±4.7	17.2±2.8	6.9±1.0

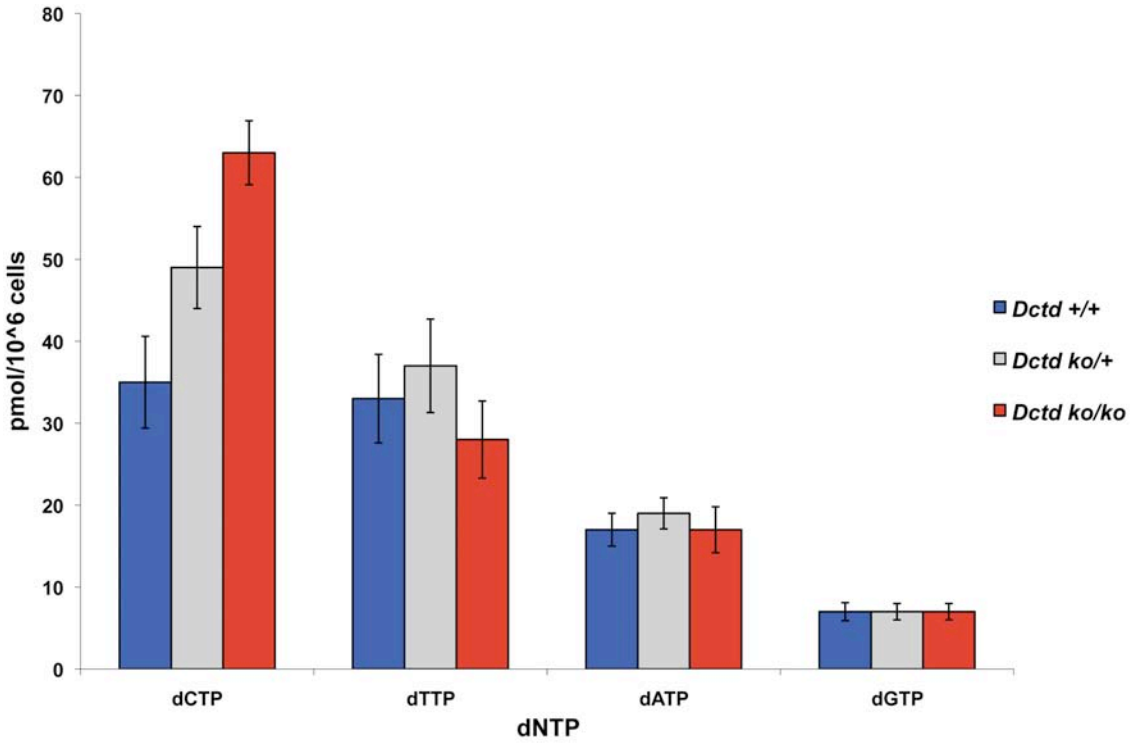


Table 4-2. Nucleotide pool levels in *Dctd* $^{+/+}$, *Dctd* $^{+/ko}$, and *Dctd* $^{ko/ko}$ mice. Duplicate measurements on each of three extractions were performed with logarithmically-growing primary cells. Results represent the mean ± standard deviation.

	dNTP (pmol/10^6 cells)			
	dCTP	**dTTP**	**dATP**	**dGTP**
HeLa DCTD	23.6±6.8	64.2±6	24.2±5.8	12.4±1.4
HeLa Scr	24.5±0.03	56±2.3	26.5±1.2	8.1±0.8

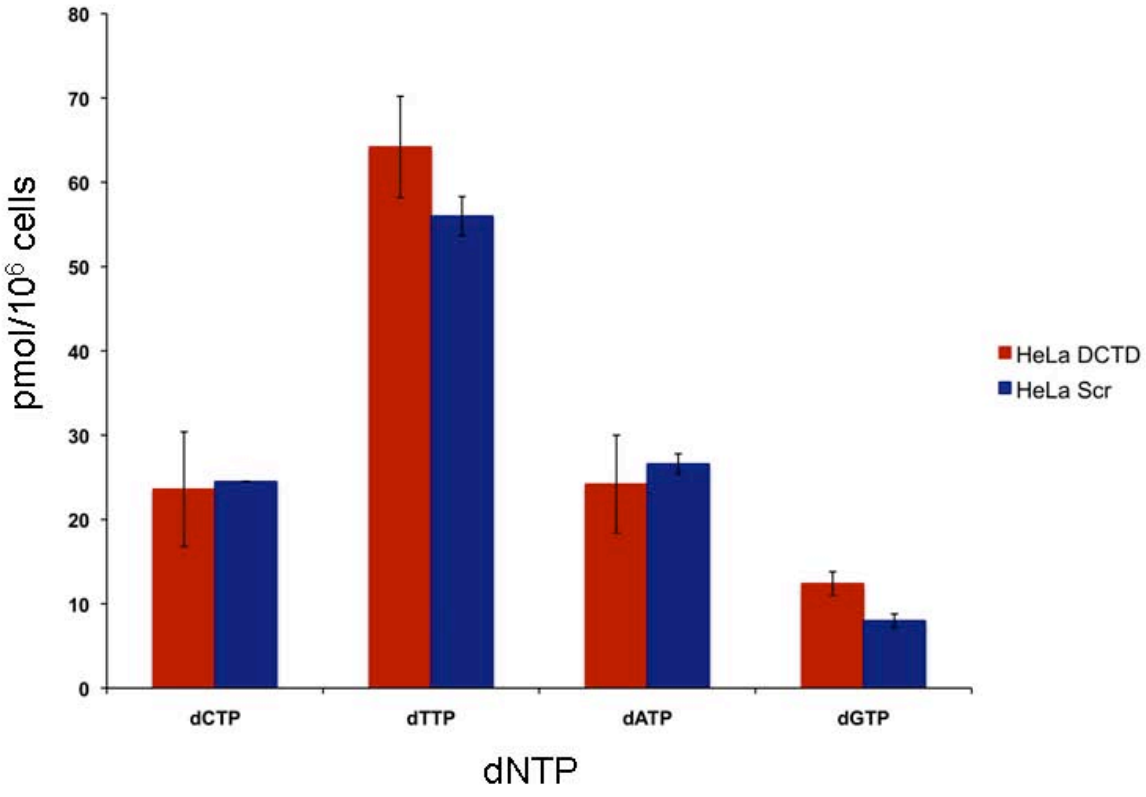


Table 4-3. Nucleotide pool levels in HeLa cells. Duplicate measurements on each of three extractions were performed with logarithmically-growing cells. Results represent the mean ± standard deviation.

	dNTP (pmol/10^6 cells)			
	dCTP	**dTTP**	**dATP**	**dGTP**
293 DCTD	19.0±2.7	84.6±4.1	30.0±1.9	14.3±1.3
293 Scr	22.2±1.7	85.3±3.6	34.1±4.5	14.6±1.3

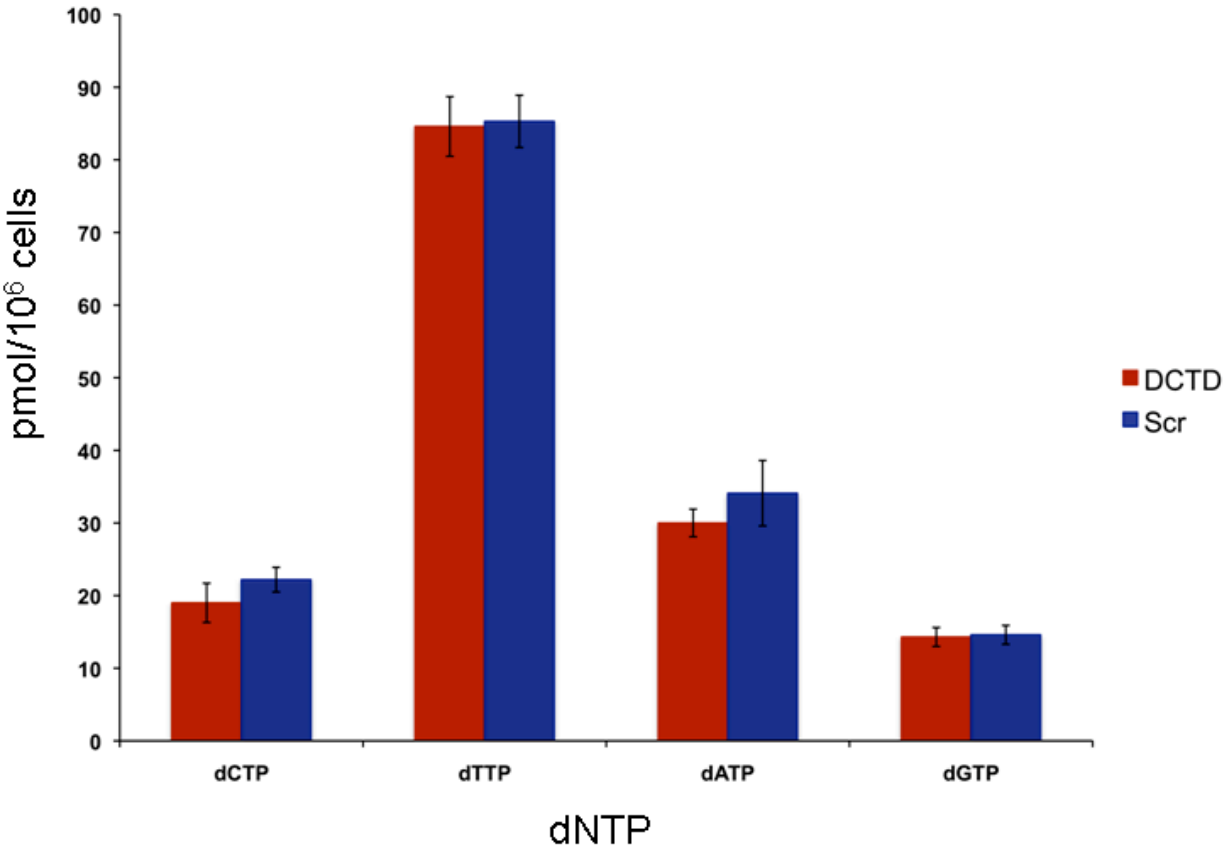


Table 4-4. Nucleotide pool levels in HEK293 cells. Duplicate measurements on each of three extractions were performed with logarithmically-growing cells. Results represent the mean ± standard deviation.

Discussion

In addition to its spellchecker role, MMR mediates the cellular response to certain DNA damaging agents, including S_N1-type methylators and 6-TG (Stojic et al., 2004a). MMR recognizes $^{O6\text{-}Me}$G:T mismatches that are generated during replication upon treatment with methylating agents and signals cell arrest and apoptosis. A fuller understanding of how this pathway is regulated has been an extensive area of research, because the S_N1-type methylator temozolomide and the methylation mimetic 6-TG are chemotherapy drugs. While a subset of MMR and checkpoint proteins are involved in this response, a fuller understanding is important. Recently, *DCD1* was shown to have a critical role in the MMR-dependent response to methylating agents in yeast (Liskay et al., 2007). Dcd1p regulates deoxypyrimidine pool levels by catalyzing the conversion of dCMP to dUMP, which are precursors for dCTP and dTTP. In cells deficient for *DCD1*, dCTP levels rise and dTTP levels fall (McIntosh and Haynes, 1984). Hence, *DCD1*-deficient cells have excess dCTP, making the polymerase more likely to place a C across from an $^{O6\text{-}Me}$G lesion following treatment with a methylating agent. In turn, fewer $^{O6\text{-}Me}$G:T mismatches occur, resulting in enhanced survival (Liskay et al., 2007).

Mammalian *Dctd* has also been shown to regulate deoxypyrimidine levels. Studies in mouse lymphoma, Chinese hamster ovary, and Chinese hamster lung cells demonstrated an increase in the dCTP:dTTP pool levels when *Dctd* is deficient (Bianchi et al., 1987; Meuth, 1981; Weinberg et al., 1981). Additionally, the Chinese hamster ovary cells displayed increased resistance to the S_N1-methylator MNNG (Meuth, 1981). However, a few factors may have confounded the interpretations in previous studies. First, the S49 mouse lymphoma line is known to contain a second mutation, in *purine*

nucleoside phosphorylase (*PNP*) (Weinberg et al., 1981), though it is not clear how a mutation in *PNP* would influence dCTP:dTTP pool levels in *Dctd*-deficient cells. Second, the Chinese hamster cell studies did not involve analysis of an isogenic pair of $Dctd^{+/+}$ and $Dctd^{-/-}$ cell lines. Consequently, additional unknown defects in other pathways affecting dNTP pools could have influenced the results. Therefore, I analyzed the effect of *Dctd* deficiency in two isogenic systems, siRNA in human cell lines and a *Dctd* knockout mouse.

Effect of *DCTD* knockdown on 6-TG resistance in human cells

Tolerance to 6-TG was measured by cell growth assays in HeLa, K562, and SUP-B15 cells. Both the HeLa and SUP-B15 cells displayed resistance to 6-TG when transfected with DCTD siRNA. In addition, the response to 6-TG was measured by colony formation assays in HeLa and HEK293 cells, with both cell lines displaying increased colony formation in the presence of 6-TG. Knockdown of DCTD in human cells resulted in cytotoxic tolerance to 6-TG in three out of the four cell lines examined.

The increased resistance to the cytotoxic response to 6-TG upon knockdown of DCTD in the HeLa, SUP-B15, and HEK293 cells is consistent with previous studies in *Dctd*-deficient rodent cells. I did not observe resistance to 6-TG in one of the human leukemia cell lines I examined, the K562 cells. However, analyzing tumor cell lines can be complicated, as some tumor cell lines accumulate genetic alterations, at times in proteins involved in the response to 6-TG (Matheson and Hall, 2003).

6-TG cytotoxic response in *Dctd* knockout mice

The *Dctd* knockout mouse, a complete null for *Dctd*, provided the cleanest system for analyzing the effect of *Dctd* deficiency on the cytotoxic response to 6-TG. However,

significant resistance to 6-TG was not observed in primary MEFs, spontaneously immortalized MEFs, or lymphocytes derived from *Dctd*$^{+/+}$ and *Dctd* $^{ko/ko}$ mice. Additionally, the mice displayed no enhanced tumorigenesis as might be expected from the increased mutation rate reported in *DCD1* and *Dctd*-deficient yeast and rodent cells (Kohalmi et al., 1991; Weinberg et al., 1981).

dNTP pool measurements in HeLa, HEK293, and *Dctd*$^{ko/ko}$ cells

The lack of resistance to 6-TG observed in the *Dctd*$^{ko/ko}$ mice is in contrast to the resistance observed in human cells with reduced *DCTD* expression. To ascertain whether resistance to 6-TG was correlated with the dCTP:dTTP pool ratio, dNTP levels were measured in primary MEFs derived from *Dctd*$^{ko/ko}$ mice as well as HeLa and HEK293 cells transfected with DCTD siRNA.

In cells from the *Dctd*$^{ko/ko}$ mice, a small increase was seen in the dCTP:dTTP nucleotide pool ratio, about a two-fold increase over *Dctd*$^{+/+}$ cells. However, this level of imbalance was smaller than previously reported for *Dctd*-deficient rodent cells. It is likely the lack of resistance to 6-TG observed in the cells from *Dctd*$^{ko/ko}$ mice is the result of the modest level of imbalance in the nucleotide pools.

Nucleotide pool levels were also measured in the HeLa and 293 cells. Unexpectedly, no imbalance was observed in the pool levels between the cells treated with DCTD siRNA and those treated with the scrambled control, for both the HeLa and 293 cells. Why the cells displayed resistance to 6-TG without nucleotide pool imbalance is unknown, but there are a few explanations for how resistance to 6-TG could be observed in the absence of nucleotide pool imbalance. First, there is evidence to suggest dCTP pools are compartmentalized within the cell (Xu et al., 1995). Within the nucleus

resides a replication precursor pool at sites of active replication; an overall general pool resides in the cytosol and rest of the nucleus. The *de novo* and salvage pathways contribute differentially to these pools (Xu et al., 1995). It is possible that repression of *DCTD* expression is affecting one pool but not the other, and therefore, its effect on overall cellular pool levels is not great enough to measure. Another possibility is that the level of siRNA-mediated repression of *DCTD* expression is likely not exact from cell to cell, creating a subpopulation of cells with severely diminished *DCTD* expression and another subpopulation of cells with normal *DCTD* expression. The cells with diminished *DCTD* expression could be responsible for the resistance observed, but when the nucleotide pools for all the cells are measured, the population with reduced *DCTD* expression is not large enough to impact the overall pool measurement. To address this possibility, deoxynucleotide pool measurements could be conducted on DCTD siRNA-treated cells that have survived treatment with 6-TG. This might enrich for a pool of cells with imbalanced dCTP:dTTP ratios.

Finally, the siRNA oligonucleotide could cause an off-target effect that could produce resistance to 6-TG through an unknown mechanism. Repeating the experiments in the HeLa and 293 cells with a second siRNA oligonucleotide would answer whether off-target effects are causing the resistance phenotype. If the second oligonucleotide caused a similar level of resistance as the first oligonucleotide, it would indicate the resistance is most likely due to reduction of DCTD and not due to an off-target effect, as it would be unlikely that both oligonucleotides would have an off-target effect leading to resistance.

The minimal pool imbalance observed in the Dctd knockout mouse could be explained by adaptation. The cells derived from the *Dctd*$^{ko/ko}$ mice have had many cell

divisions to compensate for a lack of *Dctd*. Therefore, their pool levels may not be as imbalanced as would be expected. Adaptation is one explanation for the unexpected or minimal phenotypes observed in some gene knockouts in mice (Clifton et al., 2003).

What could be causing this adaption in the mouse? Nucleotide pool levels are finely regulated by a number of different enzymes that respond to shifts in the pool levels. One possible enzyme that could be maintaining a normal level of dCTP is the salvage enzyme dCTP deaminase, which can convert dCTP to dUTP (Camiener and Smith, 1965) (Figure 4-13). If dCTP were upregulated, the excess dCTP produced in a *Dctd*-deficient cell could be turned into dUTP, which can in turn be converted to dTTP by dUTPase (el-Hajj et al., 1988), thus keeping the dCTP:dTTP pool balance in check. Additionally, when *Dctd* function is compromised, the excess dCMP that builds up can be turned into dCTP by the enzyme UMP/CMP kinase (Van Rompay et al., 2000). If CMP kinase were downregulated (Figure 4-13), the excess dCMP would remain as dCMP, leaving the dCTP levels unaltered. Finally, the majority of the dCTP used for cellular replication comes from the *de novo* pathway via ribonucleotide reductase activity on CDP. Ribonucleotide reductase (RNR) is a multi-functional enzyme that is able to reduce all four ribonucleotides, and it responds to the levels of the dNTPs in the cell to maintain a proper balance of them (Reichard, 1988). If RNR function towards CDP were diminished, less dCTP would be produced via this pathway, allowing for the excess dCTP produced by *Dctd* deficiency to comprise a majority of the dCTP in the cell.

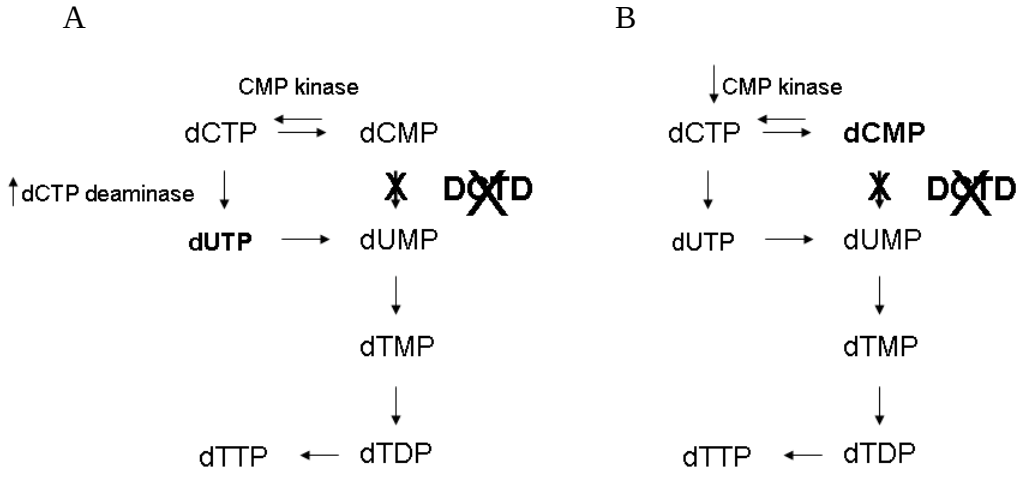


Figure 4-13. Model for adaptation to *Dctd* deficiency in the mouse. A, upregulation of dCTP deaminase could lead to the excess dCTP present in the cell to be converted to dUTP. B, downregulation of CMP kinase would lead to the excess dCMP remaining as dCMP and prevent its conversion to dCTP.

CHAPTER 5

Summary and conclusions

DNA mismatch repair is a diverse DNA repair mechanism involved in many important cellular transactions. MMR acts to correct base/base mismatches and small insertion/deletion loops that arise during replication. MMR also suppresses recombination between similar but not exact sequences and is a necessary component in the response to certain types of DNA damaging agents. In my thesis research, I investigated the role of the MutLα ATPase domains to overall MMR function. In addition, I examined the MMR-dependent response to the methylation mimetic 6-thioguanine.

Functional asymmetry in the MutLα ATPase domain

In the first part of my research, I have shown that the ATPase domain of MLH1 contributes to repair *in vivo* to a greater degree than that of PMS2. MSI analysis in mono and dinucleotide microsatellites showed an instability phenotype for cells expressing the ATP hydrolysis and Mg^{2+} binding mutants of *MLH1*, but not *PMS2*. In accordance with these results, mutations affecting ATP hydrolysis and binding of *MLH1* caused resistance to the cytotoxic effects of 6-TG, while similar mutations in *PMS2* did not. These results suggest a functional asymmetry exists in the MutLα heterodimer and are consistent with previous observations made in budding yeast, highlighting the high level of conservation of MMR between yeast and higher organisms.

As a molecular matchmaker, *E. coli* MutL acts to coordinate mismatch initiation by MutS to downstream steps, including activation of the endonuclease MutH and loading of UvrD, both of which are dependent on binding of ATP (Polosina and Cupples). Eukaryotic MutLα also contains ATPase domains necessary for functional mutational avoidance (Raschle et al., 2002; Tomer et al., 2002; Tran and Liskay, 2000).

Binding of ATP brings about a conformational change in MutLα that could recruit downstream factors such as PCNA or EXO1. Given the asymmetry observed between the contribution of the MLH1 and PMS2 ATPase domains to overall mismatch repair function, it is possible the MLH1 ATPase domain is primarily responsible for recruiting these downstream factors. Additionally, as discussed in Chapters 1 and 3, MutLα ATP hydrolysis is needed to activate the latent endonuclease function of MutLα (Kadyrov et al., 2006). However, this was only demonstrated with the double *MLH1*/*PMS2* hydrolysis mutant. It is possible the MLH1 ATPase domain is responsible for this activation. Performing assays for MutLα endonuclease activity with the ATP hydrolysis and binding mutants would shed more light on this possibility.

Asymmetry has also been observed in the order in which MutLα binds ATP. One protein binds ATP first, inducing a conformational change leading to the binding of the second protein (Sacho et al., 2008). Since MLH1 has a higher affinity for ATP, it is likely MLH1 binds ATP before PMS2 (Hall et al., 2002). If PMS2 ATP binding was dependent on MLH1 ATP binding, but not vice versa, mutations impacting MLH1 ATP binding might have a larger impact on repair than mutations affecting PMS2 ATP binding.

As discussed in Chapter 1, inactivation of MMR in humans can cause colon and many other types of cancer. The majority of mutations detected in HNPCC patients are in *MLH1*, and many of those mutations reside in the N-terminal region. On the other hand, very few mutations have been detected in *PMS2*. This could be due to the fact that *MLH3* and *PMS1* can compensate for a lack of *PMS2*. Additionally, pseudogenes do exist for *PMS2*, making sequence analysis difficult. However, given that inactivating the

ATPase domain of MLH1 affects repair efficiency, it is possible that some of the contribution to HNPCC could come from the ATPase domain of MLH1. Mutations at the ATP hydrolysis and Mg^{2+} binding residues of *MLH1* have been detected in patients meeting HNPCC criteria, but the pathology of such mutations was unknown (InSiGHT database).

While my results would suggest that the PMS2 ATPase domain is dispensable for mutation avoidance *in vivo*, a potential role for the ATPase domain of PMS2 was demonstrated by work done using yeast in our lab. When the ATP hydrolysis and binding mutants of yeast Pms1 were combined with Exo1 deletion, the mutation rate synergized (Tran et al., 2002). This indicated that the ATPase function of Pms1p was needed to recruit potential redundant exonucleases in the absence of Exo1p. It would be interesting to see if this is also observed in mammalian cells. To test this possibility, the PMS2 WT as well as the PMS2 ATP hydrolysis and binding mutations could be expressed in cells derived from *Pms2*$^{-/-}$ *Exo1*$^{-/-}$ mice. The cells expressing PMS2 WT, while still null for *Exo1*, should cause little microsatellite instability, as previously reported (Wei et al., 2003). The combination of PMS2 ATPase mutations and *Exo1*$^{-/-}$ could then be tested. If synergy were to be observed, it would strongly suggest the ATPase domain of PMS2 is needed to recruit an additional exonuclease in the absence of EXO1.

The role of DCTD in the MMR-dependent response to 6-TG

In the second part of my thesis research, I investigated the MMR-dependent response to DNA damage caused by 6-TG. It had been recently shown that in yeast, the nucleotide pool regulator *DCD1* is a necessary component of the MMR-dependent

response to methylating agents (Liskay et al., 2007). I showed than in human cells, deficiency in the nucleotide pool regulator *DCTD* increased resistance to the cytotoxic effect of 6-TG. In contrast, cells derived from a *Dctd* mouse knockout showed no significant resistance to 6-TG. Because *Dctd* regulates the levels of dCTP and dTTP in the cell and an imbalance in the ratio of dCTP:dTTP is thought to cause the resistance to methylating agents observed in *DCD1* deficient yeast (Liskay et al., 2007), I measured the nucleotide pool levels in the human and mouse cells deficient for DCTD. The mouse cells displayed a slight increase in the dCTP:dTTP pool ratio, about 2-fold increase over wild-type. Previous studies with mammalian cell lines had shown this imbalance to be much higher. One explanation for the minimal effect of *Dctd* deficiency on the nucleotide pools and thus the response to 6-TG is adaptation. The developing mouse was potentially able to adapt to the lack of *Dctd* by an unknown compensatory mechanism. As discussed in Chapter 4, a few enzymes involved in dCTP and dTTP production could have altered expression or function in the $Dctd^{ko/ko}$ mouse. Simple tests for mRNA and protein levels of the candidate enzyme dCTP deaminase may indicate whether this is occurring. However, since many enzymes involved in nucleotide pool regulation are regulated by allosteric mechanisms, enzymatic assays to determine the level of activity of dCTP deaminase in the $Dctd^{+/+}$ and $Dct^{ko/ko}$ cells may be needed.

In contrast to the mouse cells, the human cell lines HeLa and HEK293, deficient for *DCTD* expression by siRNA, showed no imbalance in their nucleotide pool levels. However, they did display resistance to the cytotoxic effects of 6-TG. The observation that the DCTD deficient cells display resistance to 6-TG without a shift in their nucleotide pool levels is unexpected but might be explained by a few factors. First, it is

possible the siRNA oligonucleotide is causing an unknown off-target effect that is responsible for the observed resistance to 6-TG. This possibility could easily be determined by utilizing a second siRNA oligonucleotide and repeating the 6-TG response assays. Additionally, the reduction of expression of *DCTD* in the cells is not 100%, and the level of DCTD expressed in each cell is most likely not equal. For example, some cells may have greatly reduced expression while others will have only moderately reduced expression. The cells with the greatly reduced expression may be responsible for the resistance to 6-TG, but the overall number of these cells in the entire population may not be large enough to impact total cellular dNTP pools. Measuring the pool levels in cells that have survived 6-TG treatment would shed light on this possibility. Finally, the reduction in *DCTD* may not affect the dCTP pools equally across the cell. dCTP pools have been shown to be functionally compartmentalized within the cell, with one compartment providing the bulk of the dCTP for replication with the other compartment providing dCTP for DNA repair (Xu et al., 1995). The salvage and *de novo* pathways are thought to contribute differentially to these compartments, with the de novo pathway contributing to the replication pool, and the salvage pathway contributing to the repair pool. It is possible reduction in *DCTD* expression is only affecting the pool responsible for mediating the 6-TG response but not affecting the overall pool levels in the cell.

Recently, the nucleotide metabolism enzyme UMP/CMP kinase (UMP/CMPK) was analyzed for its effect on nucleotide pool levels and sensitivity to deoxycytidine analogs (Liou et al.). UMP/CMPK phosphorylates UMP, CMP, and dCMP and thus the salvage and de novo pathways of pyrimidine metabolism both are thought to utilize the enzyme. Surprisingly, the authors of the study observed no alteration of nucleotide pool

levels upon reduction of UMP/CMPK expression. However, the cells with reduced UMP/CMPK did display resistance to deoxycytidine analogs. These results highlight the complexity of nucleotide pool regulation and the response to nucleoside analogs, and leads to the possibility that reduction of DCTD expression could be affecting resistance to 6-TG by a previously undescribed mechanism.

The existence of the Dctd deficient mouse does lead to the possibility for future experiments in understanding how small perturbations in deoxynucleotide pool levels can affect cellular function. One possible experiment would be to cross the *Dctd*$^{ko/ko}$ mouse with an *Mlh1*$^{ko/ko}$ mouse and then analyze MSI at A and G runs. How would a two-fold imbalance in the dCTP:dTTP ratio affect MSI at each of these types of runs? It would also be interesting to observe if the tumor incidence of the *Mlh1*$^{ko/ko}$ mice would change in a *Dctd*-deficient background.

The observation that *DCTD* in human cells may be involved in the response to S_N1-type methylators and methylation mimetics has far-reaching implications. Both 6-TG and temozolamide are chemotherapeutic agents used to treat a number of different cancers. If deficiency in DCTD were present in the patients being treated with the drugs, it is possible that their cells could become resistant to them. Further investigation into DCTD expression in patients resistant to 6-TG or temozolamide is warranted. Correlation between single nucleotide polymorphisms in *DCTD* and outcomes from treatment with 6-TG or temozolomide would also shed more light onto DCTD's role in responding to these drugs.

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Appendix: Contributions to projects and figures

ATPase domain analysis

Megan Nyugen cloned the MLH1 and PMS2 ATP hydrolysis expression constructs as well as performed the dinucleotide microsatellite instability assays on the null and WT MLH1 and PMS2 cell lines.

Sandy Dudley assisted with the mononucleotide PCRs.

DCTD deficiency analysis

The HeLa cells were a gift from Dr. Robb Moses. The K562 and SUP-B15 cells were from Dr. Bill Chang. The HEK293 cells were from Dr. Rosalie Sears.

Sandy Dudley performed the mouse handling of the *Dctd* knockout mice as well as assisted with the genotyping, fibroblast and lymphocyte preparation.