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Imbalance Of Uracil Dna Glycosylase And Activation-Induced Cytidine Deaminase Expression In Folate Depleted Human Lymphoblastoids

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IMBALANCE OF URACIL DNA GLYCOSYLASE AND ACTIVATION-INDUCED CYTIDINE DEAMINASE EXPRESSION IN FOLATE DEPLETED HUMAN LYMPHOBLASTOIDS

by

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DEDICATION

I dedicate this thesis and all of the hard work behind it to my

husband and best friend Jon Singer for being an endless source of

support and encouragement throughout these eventful few years.

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CHAPTER 1: INTRODUCTION

A. Folate Overview and Metabolism

The B-vitamin folate has long been known to play an important role in human health. Impaired folate metabolism is associated with several pathologies and developmental abnormalities, including neural tube defects (NTD) [1, 2], cardiovascular disease (CVD) [3, 4], and cancer [5-9]. Folate metabolism can be disrupted by B-vitamin deficiencies, folate antagonist medications, and penetrant genetic mutations and polymorphisms [10-13]. However, the risk associated with these disruptions, as well as interactions with other environmental risk factors, have yet to be fully elucidated.

Folic acid is the synthetic monoglutamated form of folate found only in fortified foods and supplements, whereas natural food folates are a mixture of the reduced forms of the vitamin with a variable number of glutamate residues. Natural folates in foods are labile and prone to oxidative damage when compared to synthetic folic acid, which is considerably more stable and has significantly higher bioavailability. Mixed dietary intake of fortified and natural food sources of folate are regularly reported in dietary folate equivalents (DFE) to account for differences in the absorption of naturally occurring food folate and the more bioavailable synthetic folic acid.

Folate metabolism involves the reduction of folic acid by dihydrofolate reductase to form tetrahydrofolate (THF), the active form of folate and cofactor in single-carbon enzymatic reactions. The ability of folate cofactors to accept or donate one-carbon units at the N-5 and/or N-10 positions without changing their nature is essential for several important biochemical processes including purine and thymidylate synthesis, remethylation, and S-adenosyl methionine (SAM) formation. (Figure 1.1).
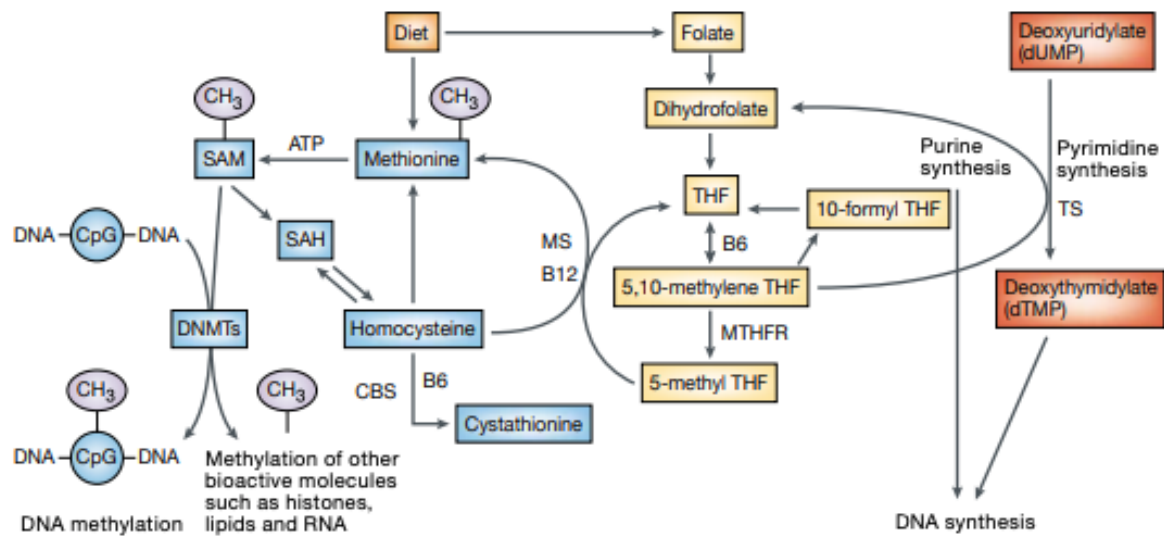
Figure 1.1 Overview of Folate Metabolism [14]. THF derived methyl groups are transferred in the synthesis of dTMP, purines, methionine and subsequently SAM.
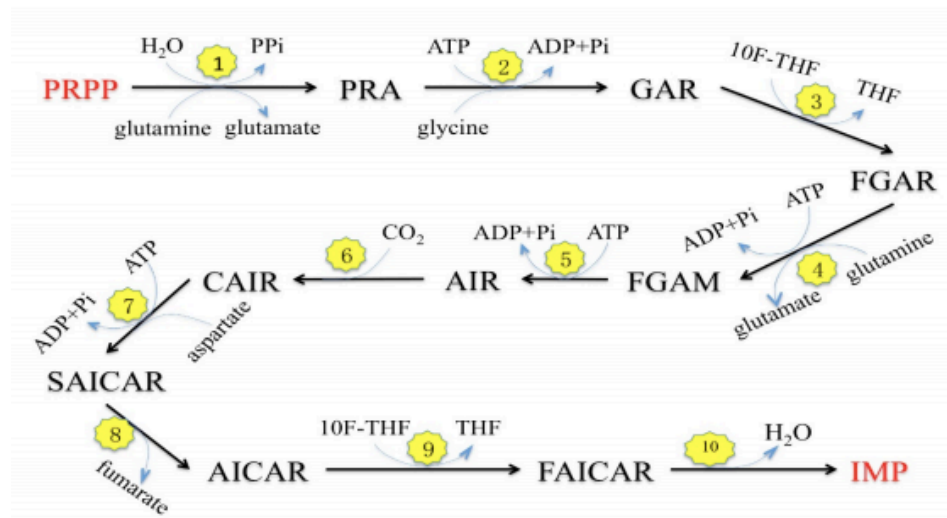
Figure 1.2 *De Novo* Synthesis of Purines [15]. Formyl group is transferred to form purine ring at **step 3** via GAR (glycinamide ribonucleotide) and **step 9** via AICAR (5-aminoimidazole-4-carboxamide ribonucleotide) to form IMP (inosine monophosphate), the adenine and guanine nucleotide precursor.
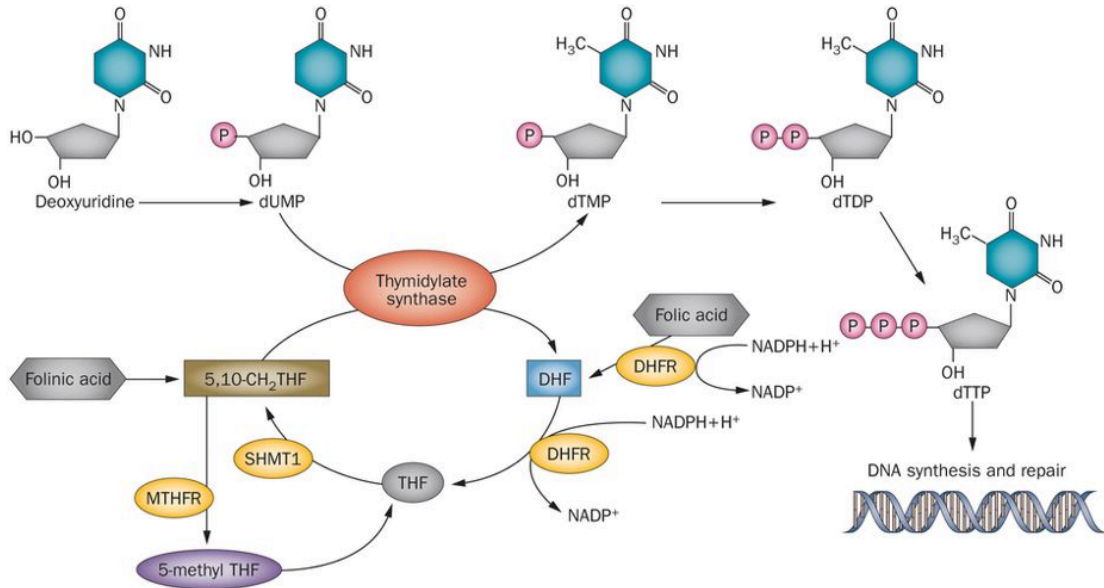
Figure 1.3 Folate dependent conversion of dUMP to dTMP [16]. Thymidylate synthase catalyzes the methyl transfer from 5,10, methylene THF to dTMP.

De novo synthesis of the adenine and guanine require two folate-dependent transformylases; GAR and AICAR, which facilitate the transfer of the formyl group from 10-formyl THF to form the purine ring at C-2 and C-8 (Figure 1.2). Folate is also required as a coenzyme for the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) in a reaction catalyzed by thymidylate synthase (TS) (Figure 1.3). Due to significantly increased proliferation in transformed cells, antifolate drugs that limit the source of one-carbon moieties, specifically, 10-formyl-THF in de novo purine biosynthesis and 5,10-methylene-THF in dUMP to dTMP conversion are effective chemotherapeutics since they reduce the nucleotide pool available for replication.

The remethylation of homocysteine to methionine is catalyzed by methionine synthase (MS) in a 5-methyl-THF, zinc and B12-dependent reaction (Figure 1.1). Disruptions in

methionine metabolism not only lead to accumulation of homocysteine, which is a risk factor for CVD and possibly Alzheimer's disease, but may also diminish the availability of other folate cofactors for nucleotide synthesis due to a "5-methyl-THF trap [17]." Additionally, most cellular processes in which a compound acquires a methyl group use SAM as the methyl donor. The labile methyl group that enables SAM to serve that function is derived from its precursor, methionine, which in turn obtains that moiety from 5-methylTHF. Due to the role of SAM-dependent methylation in epigenetic regulation, inadequate levels of SAM have been linked to abnormalities in DNA methylation patterns.

B. Folate Status and Pathologies of Deficiency in Humans

In the United States, the current RDA for folate in men and non-pregnant women over 14 years is 400 µg/d DFE. This level is intended to meet the nutrient requirement for approximately 98% of the population, and is based on the average serum RBC folate, an index of tissue stores, and long-term status [18, 19]. Folate requirements are higher during life-stages in which there is increased cell division, such as during fetal development and lactation, during which the RDA increases to 600 ug/d and 500 ug/d, respectively.

Although most people consume adequate amounts of folate, due in part to the US fortification program discussed below, the National Health and Nutrition Examination Survey (NHANES) reports that certain groups, including women of childbearing age and non-Hispanic black women, have higher levels of insufficient folate intakes. Even when intakes of folic acid from dietary supplements are included, 19% of female adolescents aged 14 to 18 years and 17% of women aged 19 to 30 years do not meet the estimated average requirement [20]. Similarly, 23% of non-Hispanic black women have inadequate

total intakes, compared with 13% of non-Hispanic white women. In addition, certain populations are known to be at elevated risk for clinical folate deficiency due to inadequate absorption or impaired metabolism, this includes chronic alcohol abusers, patients with malabsorptive disorders and sickle cell disease, those requiring renal dialysis, and those on clinical antifolate therapy for treatment of cancer or autoimmune disorders.

Folate is required for adequate growth and repair by virtue of its functions in nucleotide biosynthesis, which impacts many different systems, some of which are described below. Megaloblastic anemia resulting from folate deficiency, which can occur at serum folate levels between 2-6 ng/mL, is characterized by hypercellular bone marrow with large erythroblasts and uncondensed chromatin, leading to elevated mean cell volume. Additionally, there is increased cell death of these abnormal megaloblasts prior to maturation, leading to pancytopenia and anemia. These features have been directly attributed to impaired DNA synthesis and methylation in the folate deficient state in hematopoietic cells. *In vitro* studies of folate deficiency in erythroblasts indicate apoptosis occurs in S phase suggesting a deficiency of available nucleotides [21]. The trigger for bone marrow apoptosis is unknown, although DNA damage from double strand breaks (DSB) in folate deficiency may play a role [22]. Nonetheless, it is apparent that purine and thymidine synthesis from one-carbon metabolism is important in preventing megaloblastic cell death.

Folate status has also been linked to vascular and neurological disease in epidemiological studies [23-26]. This link is supported by multiple plausible mechanisms including hyperhomocysteinemia from incomplete B12-dependent remethylation of homocysteine to methionine, impaired DNA replication leading to damage of the

endothelium [27], decreased SAM production resulting in hypomethylation, as well as an age related decline in THF transport across the blood-brain barrier [28, 29]. Though, the role of folate in the pathogenesis of vascular and neurological disease remains controversial, and clinical trials suggest that vitamin supplementation appears beneficial only in those with baseline intake well below the EAR [30, 31].

The most severe manifestation of disrupted folate metabolism is the occurrence of birth defects that interfere with the development and closure of the neural tube. The prevalence of NTDs in the US is approximately 0.8 per 1,000 births [32]. The association between maternal folate status and the risk of NTDs is not fully understood; however, compelling evidence from clinical trials demonstrated that maternal intake of folic acid significantly reduced the occurrence and recurrence of NTDs by 44-71% [32-35].

In 1998, due to mounting evidence of the protective effect of maternal folic acid supplementation in the periconceptional period, the US FDA required cereal and grain products to be folic acid fortified. Currently, grain products are fortified at 140 μg of folic acid per 100g of flour. Several studies from population-based state surveillance systems have reported a 19-32% reduction in the overall birth prevalence rate of NTDs [31, 32] as a result of the fortification program. An additional outcome of fortification was an increase in the overall folic acid intake for all US populations. The median blood folate concentration in the US population increased approximately 3-fold from 5.5 ng/mL pre-fortification, to 14.1 ng/mL in the subsequent 1999-2000 NHANES survey period.

C. Folate and Carcinogenesis

<u>Folate and Cancer Risk</u>

Folate metabolism is crucial for many important biochemical processes that support cell proliferation and epigenetic regulation. Unsurprisingly, insufficient folate availability has been causally linked with many types of cancer. Over the past 40 years a large body of epidemiological research has suggested that raised intakes or concentrations of folate protect against several major human malignancies including colorectal, pancreas, prostate, breast, cervical, and hematopoietic cancers [36, 37]. However, the use of folate antagonists, starting in the 1950's, as effective chemotherapy agents demonstrate the duality of intracellular folate status, and recent studies have highlighted the association between elevated folate levels and an increased risk of some cancers [38]. These studies suggest a complex interplay between folate intake, existing genetic polymorphisms in folate metabolism, and the stage and site of cell transformation. This is especially relevant in relation to existing fortification programs where the daily upper limit (UL) of folate in DFE can easily be surpassed with supplementation. [39-41]. Although the specific molecular basis for the association between folate and cancer is unclear, there are two highly researched pathways that have been hypothesized; global genomic hypomethylation, and dNTP deficiency leading to increased uracil misincorporation into DNA. The rationale for these two carcinogenic pathways is outlined in Figure 1.4.

Inadequate levels of one-carbon nutrients such as folate are linked with DNA hypomethylation through decreased SAM production. Aberrations of DNA methylation are common in cancer and are hypothesized to be involved in its development. However, a review completed by Crider et. al. in 2012 indicates that the majority of studies on folate

and global hypomethylation have only examined a limited number of genetic loci and/or a small number of samples and to-date, and the available research does not support a linear relationship or dose response between folic acid and global or site-specific DNA methylation level [8].
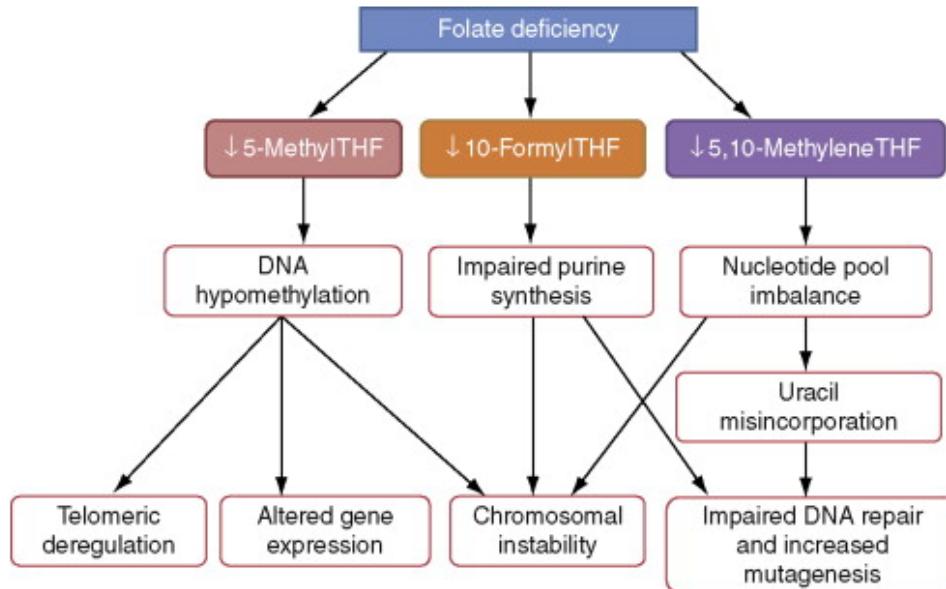
Figure 1.4 Potential Mechanisms by which Folate Modifies Cancer Risk [42].

Uracil Misincorporation

A crucial step in the biosynthesis of deoxythymidine triphosphate (dTTP) is the methylation of dUMP to dTMP, which is catalyzed by TS in a folate-dependent reaction (Figure 1.3). Perturbations in this cycle can have a profound effect on the cellular dUTP/dTTP ratio [43]. During the G_1 to the S phase of the cell cycle, TS concentration is increased at multiple levels of regulation to ensure adequate levels of dTTP for incorporation into replicating DNA. However, a reduction of THF precursors, mainly through insufficient folate availability, concomitantly reduces dTTP synthesis and

increases dUTP accumulation. Since replicative DNA polymerases have similar affinity for both deoxynucleotides, incorporation of dUTP is largely dependent on the relative cellular pool sizes of dTTP and dUTP [44, 45]. During S phase, detection of misincorporated uracil at the replication fork will signal for repair, primarily by the base excision repair (BER) pathway. If the uracil lesions are extensive, the cell cycle may be arrested until the uracil can be processed by BER, or apoptosis may ultimately occur.

D. Base Excision Repair of Uracil Lesions

Excision of misincorporated uracil from the genome is completed by the BER pathway (Figure 1.5). This process is initiated by damage-specific glycosylase cleavage of the N-glycosylic bond to excise the uracil base. In nuclear DNA, UNG2 is the uracil DNA glycosylase predominately responsible for this step [46, 47]. Uracil is quickly identified and removed from post-replicative DNA via the long patch process involving PCNA. However, genomic uracil that escapes the replication fork is repaired primarily via the short-patch route. Of the three additional uracil DNA glycosylases present in humans, only SMUG1 has a role in excising uracil from single strand DNA (ssDNA), though this may be limited to excision of deaminated cytosines [48]. Luhnsdorf et. al. found that processing of U:A mismatches resulting from uracil misincorporation may be different from that of U:G pairs resulting from cytosine deamination [49]. Likewise, TDG prefers U:G mismatch substrates, exclusively in double-stranded DNA (dsDNA), while MBD4 seems to function at specific loci in double-stranded DNA [50].

After UNG2 excisional action in BER, the resulting abasic (AP) site is cleaved by the endonuclease, APE1. In dsDNA BER then typically proceeds via the short patch route with excision of the 5'-deoxyribose-5-phosphate residue (5'dRP) and insertion of the

appropriate base paired nucleotide to the 3' end of the gap through the coordinated action

of polymerase β (POLβ). A DNA ligase then functions to seal the remaining nick.
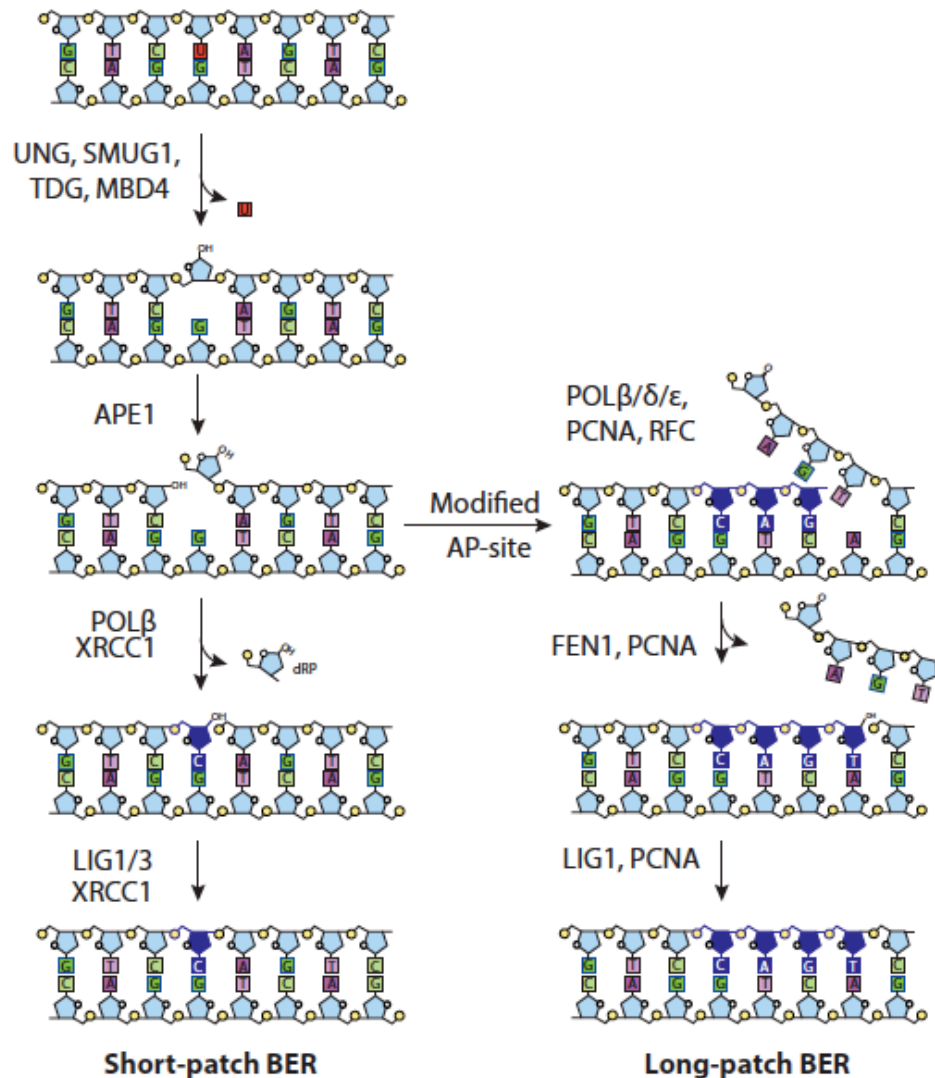
Figure 1.5 Outline of the Base Excision Repair Pathway [46]. The short-patch pathway, shown on the left is responsible for repair of genomic uracil. On the right, the long-patch pathway processes uracil at the replication fork. Both processes are initiated primarily by the Uracil DNA glycosylase UNG.

Incorporation of dUMP through one round of replication in dsDNA containing 30%

thymine has been estimated in the range of 2-10 x 10^7 based on the dUTP/dTTP ratio

observed in human tumor cell lines. However, the genomic level of uracil is 4-5-fold lower,

suggesting that a large number of these uracils are rapidly removed by post-replicative BER [46, 47, 51]. The consequences of misincorporated dUMP that persists in DNA are still relatively unknown, though there is evidence that it may have deleterious effects on gene expression, and genomic stability through incomplete repair of AP sites or errors in gap-filling in the BER process [52, 53].

<u>Effect of Folate Deficiency on BER</u>

While folate deficiency is known to increase DNA strand breaks, somatic mutations, and chromosomal aberrations, the mutagenic response to DNA-damaging agents such as ethyl nitrosourea, ethyl methanesulfonate, and hydrogen peroxide is also greater when folate is deficient. This phenotypic response closely resembles the effects of inadequate or fragmented DNA repair activity. These findings point to a folate deficiency-induced inability to process the types of DNA damage repaired by the BER pathway. In support of this hypothesis, Unnikrishnan et. al. found that folate depletion prevents BER induction in murine liver tissue by blocking a region in the CRE element of POLβ, the rate limiting enzyme in BER [54]. Since BER is a damage-inducible pathway, this study suggests that folate depletion may prevent this upregulation at the level of transcription. Given the role of folate in SAM production, the methylation pattern of the CpG-rich regions sites was investigated, but no methylation differences in the POLβ promoter between the folate adequate and folate deficient tissues were found, making hypomethylation an unlikely contributor. Since DNA polymerases require sufficient dNTP levels for optimal functioning, it is possible that folate deficiency, due to its role in purine synthesis and dTTP formation, may contribute to the regulatory effect on POLβ through reduced dNTP availability.

UNG2-mediated induction of BER begins a series of enzymatic reactions that induces a transient break in the phosphodiester backbone that will persist until the appropriate nucleotide is inserted by POLβ. Figure 1.6 demonstrates how the removal of uracil by UNG2 and APE1 creates a 3'-OH lesion that persists until POLβ is available to insert a new nucleotide, and the repair can be completed. Accumulation of repair intermediates due to insufficient inducibility of POLβ in folate deficiency are a likely source of the strand breaks and genomic instability generally seen in this condition. Concerningly, there is evidence that UNG activity is actually induced in folate deficient liver tissue, without a corresponding upregulation of subsequent BER enzymes [55]. Without a sufficient POLβ, increased BER initiation by UNG2 may increase the amount of unprocessed repair intermediates significantly. Thus, accumulation of DNA single-strand breaks associated with folate deficiency may be due to an imbalance of repair proteins. Single strand breaks due to attempted BER can be converted to a DSB if the lesions are clustered in opposing strands in the DNA. DSBs, in which both strands of the double helix are severed, is particularly hazardous to the cell because they can lead to gene rearrangements. Therefore, folate deficiency may contribute to accumulation of unstable repair intermediates, and consequent DSBs by two mechanisms: a) an imbalance in dUMP:dTMP ratio resulting in increased uracil incorporation and stimulation of BER through upregulation of the uracil DNA glycosylase, and b) by inhibiting the rate limiting step in the repair process.
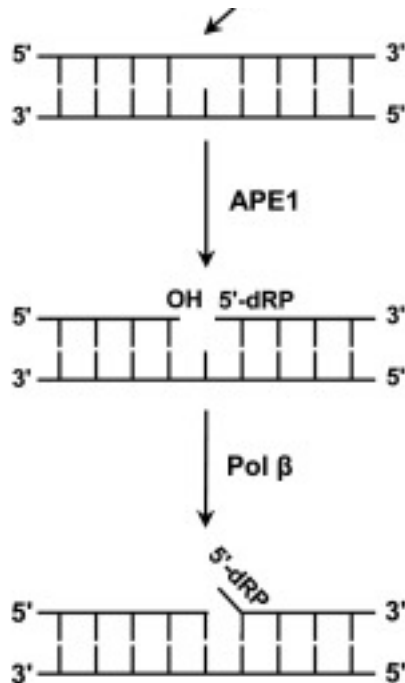
Figure 1.6 Base Excision Repair Intermediate [56]. Following the action of a DNA glycosylase, APE1 hydrolyzes the phosphate bond 5′ to the AP site and generates a single strand break with a 3′-OH end group on one strand and a 5′ terminal 2′-deoxyribose phosphate residue (5′-dRP) residue on the other strand.

E. Tissue Specific Inducibility of BER

Misincorporation of uracil as a result of folate deficiency has been reported in studies in lymphocytes and it has become a *de facto* assumption that accumulation of uracil in DNA occurs ubiquitously when folate is depleted [6, 57]. Subsequent reports of uracil accumulation have involved manipulations including a knockdown model of *Shmt [58]*, *Ung*-null yeast [59], additional methyl depletion [60], aging [61], and methotrexate exposure [62]. However, using an uracil DNA glycosylase-coupled aldehydic slot blot assay developed by Nakamura et. al. [63] and adapted by Cabelof et. al. [64], unpublished results from our lab indicate that there is no accumulation of uracil in liver tissue from animals depleted of folate. Though there is a reported increase in uracil excision activity in these animals [55]. In support of this finding, no uracil accumulation was reported in rat

colonocytes [57], and in mouse brain, folate deficiency also had no impact on uracil accumulation [65]. To evaluate whether different cell types have a distinct response to folate deficiency, our lab cultured transformed liver cells derived from BALB/c mice (BNL.C2) and transformed mouse embryonic fibroblasts, Tag 92 (MEF) cells in folate-free media in a yet-to-be published study. The BNL.C2 cells exhibited inducibility of UDG similar to previous findings of a clastogenic phenotype of folate deficiency in murine liver tissue [55], but no accumulation of uracil. However, in MEFs we found a 40% increase in the accumulation of uracil in genomic DNA but observed no upregulation of UNG. This is despite equivalent responses at the level of folate uptake, retention and metabolism. These results seem to correspond to a tissue specific UNG response to folate deficiency whereas fibroblasts lack inducibility of UNG, resulting in more accumulation of uracil in the DNA, yet liver cells continue to upregulate UNG in folate deficient states. In the latter scenario, these cells types are subject to the mutagenic repair intermediates associated with BER imbalance in folate deficiency. In the case of phenotypic uracil accumulation, as discussed, misincorporated dUMP may increase risk of genomic instability. More pertinent however is uracil that enters the DNA by deamination of cytosine, which will result in a U:G mismatch that, if left unprocessed, can produce a highly carcinogenic C to T transition mutation as shown in Figure 1.7.
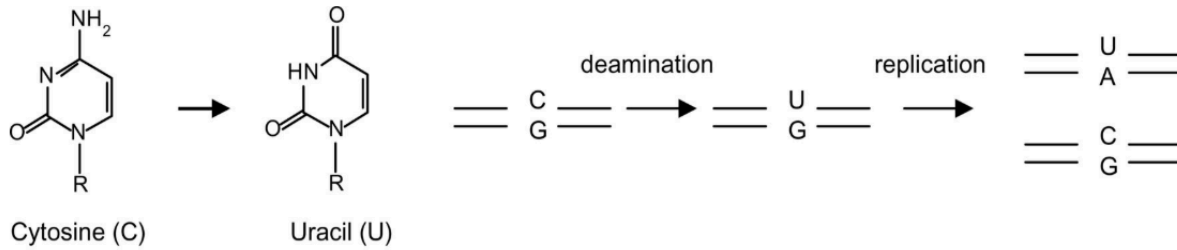
Figure 1.7 Transition Mutation Resulting from Cytosine Deamination [66]. Loss of ammonia group during hydrolytic cytosine deamination resulting in uracil. If uracil strand is replicated, it will result in U:A and eventually T:A transition mutation in place of C:G pair.

F. AID and the Adaptive Immune Response

In addition to misincorporated dUMP and spontaneous deamination of DNA cytosine, a third source of uracil in DNA has been identified as an essential intermediate in both adaptive and innate immunity. The targeted action of activation-induced cytidine deaminase (AID) introduces uracils into the B-cell genome, primarily in the immunoglobulin (Ig) loci in order to initiate the process of efficient antibody development [67]. Likewise, APOBEC A3 enzymes, which are highly expressed in immune cells, restrict viral replication by deaminating cytosines. This action results in direct mutation and degradation of the viral DNA by UNG2 (or SMUG1) and APE. The deamination of cytosine by AID and APOBEC enzymes has attracted interest not only due to their importation role in the adaptive and innate immune systems, respectively, but also due to their suspected role in human oncogenesis. Sequencing of human cancer genomes has identified mutational signatures in many cancer types attributed to the AID/APOBEC family of deaminases, indicating a significant contribution by genomic uracil in the mutations found in many cancer types [68].

Target action in IG genes

Activated B cells have developed complex mechanisms to combat pathogenic bacteria, viruses, and parasites. Somatic hypermutation (SHM) and class switch recombination (CSR) are antigen-dependent diversification processes which depend on AID and subsequent error-prone trans-lesion (TLS) repair to produce high affinity antibodies. These molecules can recognize and bind to any foreign molecules, known as antigens, and thereby neutralize potential disease-causing agents. In both of these processes, uracil is introduced into the B-cell genome at the immunoglobulin (Ig) loci by the canonical action of AID [67]. Notably, UNG2 is the glycosylase used almost exclusively during repair of AID-induced lesions in both SHM and CSR.

Millions of B lymphocytes are generated in the bone marrow every day as well as in the liver during fetal development. Each of these cells express a unique variant of membrane-bound Ig antibody proteins thanks to V(D)J rearrangement of the somatic Ig genes. Antibody molecules consist of a constant effector region, and a variable antigen-binding region. In naive B lymphocytes the variable region is acted on by the enzymes recombinase activating gene 1 & 2 to induce double strand breaks (DSB). These DSBs stimulate gene rearrangement by non-homologous end joining in the V, D, and J domains of the Ig gene. This process is non-antigen dependent and creates an immense diversity of B-cell receptors in these naive cells.

Once matured, B-cells are released into the blood and migrate to the secondary lymphoid organs, primarily the spleen and lymph nodes. When antigens are presented by dendritic cells, the activated B-cells form germinal centers where they undergo clonal expansion, AID-dependent SHM and CSR, selection by affinity maturation, and

differentiation into memory B cells or plasma cells. AID is highly expressed in activated B-cells, prompting both SHM and CSR to take place in the germinal centers.

<u>Somatic Hypermutation</u>

During SHM, single nucleotide substitutions increase the diversity at the IgV loci by error prone repair of AID-induced uracils in the DNA. There are three main pathways by which mutations are generated in SHM. The first is by direct replication of AID generated uracil. Identical to the process outlined in Figure 1.7, uracil is then read as deoxythymidine by replicative DNA polymerases, resulting in a cytosine to thymine transition mutation [67]. The second source of mutations involves the action of UNG2 to excise uracil, leaving an abasic site. This site may be acted on by error-prone trans-lesion TLS polymerases, resulting in a non-template addition of any base across from the AP site. In the third pathway, U:G mismatches are acted on by a non-canonical MutSα-dependent mismatch repair (MMR) process, which may interact with BER to further induce mutagenesis during antibody diversification [69].

<u>Class Switch Recombination</u>

In CSR, DSBs are initiated by AID activity within specific sequences known as switch regions on the Ig gene. The molecular mechanisms that generate DSBs during CSR are similar to SHM. The two main pathways for uracil processing in these switch regions are both UNG2-dependent. The first pathway relies on the proximity of uracil excisions followed by AP endonuclease (APE1) action to generate DSBs. In the second pathway, UNG2/APE1 and components of the MMR pathway cooperate to produce DSBs. A simplified overview of UNG2-initiated error-prone repair process utilized by SHM and CSR is outlined in figure 1.8. The coordinated action of AID in SHM and CSR grants

the B-cell pool the potential to generate an unlimited repertoire of highly specific antibodies, with each cell expressing Ig on its surface with a unique antigen-binding specificity.

Ig diversification by SHM and CSR in B-cells

IgM — Low affinity

Ig gene (S-region)   Ig gene (V-region)

| G C | | C |
| C G | | G |

Enzymatic deamination by AID

| G U | | U |
| U G | | G |

UNG2   UNG2

| G | | |
| G | | G |

APE1   TLS POL

| G | | * |
| G | | * |

Error prone

CSR   SHM

IgG   High affinity
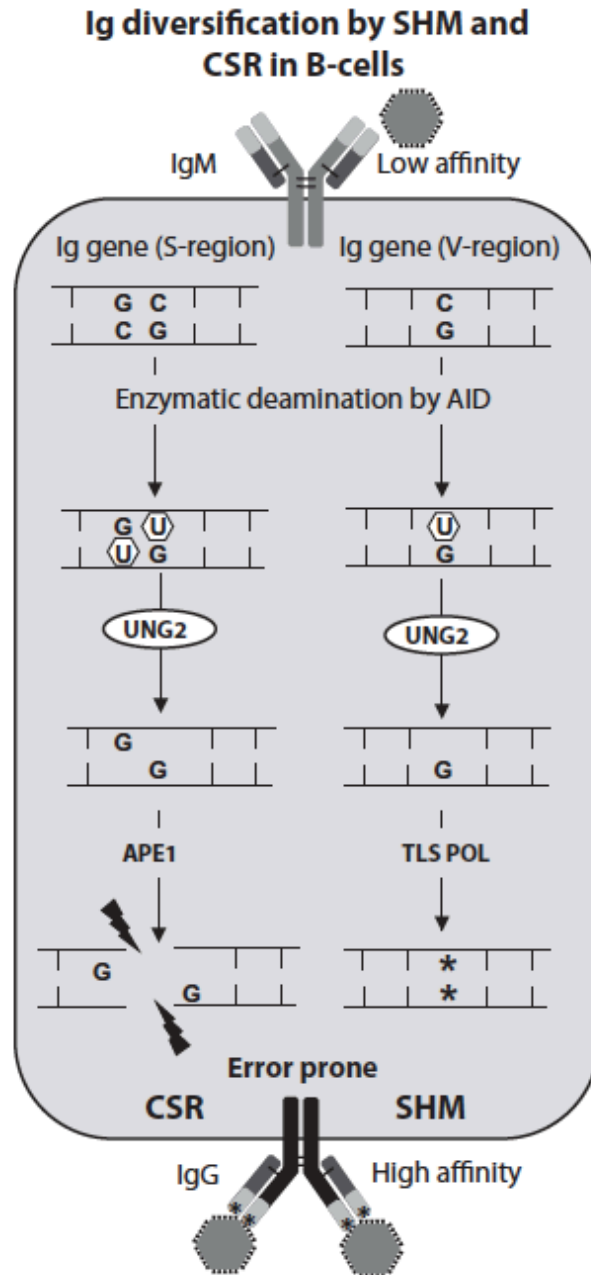
Figure 1.8 Ig Diversification by CSR and SHM [70]. Simplified model of processing of genomic uracil during Ig diversification in activated B-cells. UNG2 is the glycosylase that processes AID-induced uracil at Ig switch (S) and variable (V) regions to promote CSR and SHM.

Non-target AID action

Although AID and APOBEC A3 DNA deaminases are required for both the first line innate and second line adaptive immune systems to protect us from infection and disease, the deaminating action of these enzymes in non-target sites can cause mutations that may lead to cancer. Specifically, evidence has been growing in support of a lymphomagenic effect of AID and other APOBEC enzymes action in mature B cells. These actions may take place on the Ig loci, possibly as a result of dysregulated CSR or SHM, as well as outside of the Ig gene, contributing to genome-wide mutations. Studies in mice have indicated that the off-target action of AID contributes to somatic mutations, DNA breaks, and chromosomal translocations [71]. Additionally, AID was shown to be required for lymphomagenesis in mouse models of B-cell lymphoma driven by the ectopic expression of oncogenes [72]. Interestingly, UNG$^{-/-}$ mice develop B-cell hyperplasia and lymphomas at higher frequencies than normal mice, suggesting that B cell maturation in the absence of UNG promotes oncogenic transformation.

CHAPTER 2: HYPOTHESIS AND SPECIFIC AIMS

Evidence suggests that in some tissues, such as fibroblasts, the initiation of the BER pathway is disrupted when folate is depleted. In MEF cells, downregulation of UNG2 results in increased genomic uracil content (unpublished results). It has been established that uracil accumulates in folate deficient lymphocytes, though enzymatic regulation of BER in these cells has not been reported [73]. Since folate deficiency is known to increase incorporation of dUMP into the genome in place of dTMP [6], insufficient UNG2 in this condition would limit repair of these uracil lesions, possibly affecting gene expression at the site of incorporation and reducing proliferation due to overwhelmed repair requirements at the replication fork.

Decreased dNTP availability has been reported in folate deficient cells and thought to be responsible for reduced replication, cell cycle arrest, and possibly decreased activity of polymerases such as POLβ in some cell types [74]. Therefore, in addition to overwhelmed repair requirements, nucleotide deficiency due to reduced folate availability may also contribute to significantly increased cellular doubling time. This is particularly relevant in highly proliferative B-lymphoblastoids which are sensitive to the effects of nucleotide deficiency. In addition, UNG2 expression is cell cycle regulated, with the highest mRNA levels just prior to and during S phase. As such, cell cycle interference may contribute to a tissue specific effect in these conditions. Therefore, we hypothesized that moderate folate deficiency would significantly reduce proliferation in B-lymphoblastoids *in vitro*.

Finally, given the role of UNG2 in AID-initiated repair, and the possible consequences of off-target AID action, we were interested as to whether folate deficiency

may disrupt repair of uracils resulting from genomic cytosine deamination in the context of adaptive immunity. Since BER is a damage-inducible pathway, UNG2 is known to be tightly correlated with AID expression and activity [75]. In AID-expressing B-cells we would expect to see an upregulation of BER through UNG2 and POLβ expression. However, in folate deficiency we have evidence to suggest that UNG2 will be downregulated in lymphoblast cells. Thus, we seek to establish the relationship between AID expression and BER inducibility in folate deficient B-lymphoblastoids.

To investigate our hypothesis that **tissue specific downregulation of BER in folate deficient B-lymphoblastoids will result in a functional imbalance between AID and uracil repair capacity**, we tested the following Specific Aims:

Specific Aim 1: Confirm the effect of low folate media on intracellular folate concentration, proliferation, and dNTP level in B-lymphoblastoid cells *in vitro.*

Specific Aim 2: Establish whether there is a tissue specific effect of folate deficiency on BER inducibility of UNG2 and POLβ expression in B-lymphoblastoid cells.

Specific Aim 3: Determine if folate deficiency has an effect on the balance of UNG2 and AID expression in lymphoblastoid cells *in vitro*.

CHAPTER 3: MATERIALS AND METHODS

A. Cell Culture and Proliferation Study

Transformed human lymphoblastoid cells were obtained from Coriell Cell Repositories (Camden, NJ) and cultured in RPMI 1640 medium (Invitrogen) with standard folic acid concentration of 12 nM, supplemented with 15% dialyzed fetal bovine serum (Invitrogen), 1% antibiotics (penicillin/streptavidin), 0.5% L-glutamine (Glibco), and 1% glutaMAX (ThermoFisher). Folate deficiency was induced in experimental cells by culturing in folate free RPMI 1640 medium and adding folic acid (Sigma) for a final concentration of 12 nM. Every 3-4 days the cells were spun down, sampled for expression, folate concentration, and proliferation analysis, and resuspended at 0.5 x 10^6 cells/ml in fresh medium plus 10% used medium. At days 3, 6, and 11 of culture, live cells were counted with a hemocytometer, recorded per 8 ml, and the doubling time was calculated.

B. Lactobacillus casei Folate Assay Optimization

Depletion of folate concentration in the experimental lymphoblastoid cells was confirmed using the microbiological method as described by Horne and Patterson, and optimized for measuring relative folate concentrations of cultured cells with the methods as follows. Folate standard and depleted lymphoblastoid cells were harvested at days 6, 10, 14, 18 and 21 at a standardized concentration of 10^6 cells/ml, washed in PBS, and stored at -80°C. Within 72 hours prior to assay, folinic acid standard solution along with 50 ml of homogenization buffer and 30 ml of extraction buffer was prepared and stored at 4°C however, beta-mercaptoethanol was withheld from solutions until immediately prior to sample preparation. A serial dilution of 6 mM folinic acid calcium salt solution (Sigma) was prepared to a final solution with a concentration of 2 fmol/ul folinic acid. Light

exposure to dilution solutions was minimized with a low lighting and aluminum foil wrapped tubes to minimize vitamin degradation. Homogenization buffer (0.25 M sucrose, 10 mM HEPES, 10 mM ascorbic acid, 10 mM beta-mercaptoethanol) was prepared with the first three components plus approximately 30ml diH2O and heated to dissolve completely. Upon return to ambient temperature, 5 M NaOH was added in 500 ul increments until pH reached 7.4 as measured by calibrated benchtop pH meter. Additional diH2O was added to bring solution to 50ml. Extraction Buffer (10% w/v ascorbic acid, 0.25 M HEPES, 0.25 M CHES, 1 M beta-mercatoethanol) was prepared with the first three components plus approximately 10 ml ddH2O, pH was brought to 7.85 using procedure described above, and diH2O was added to bring final volume to 30ml.

Bacterial inoculum was prepared by applying a sterile loop to a previously prepared L. casei glycerol stock, (one vial lyophilized Lactobacillus rhamnosis (ATCC 7469), single strength folic acid casei medium-prepared as described in the package insert, glycerol), subcultured in 5ml single strength medium, and grown for at least 18 hours at 37°C. The final assay culture was prepared by dilution of the subculture in single strength folic acid casei medium to an optical density of 0.01 via spectrophotometer.

After 21 days in culture, harvested lymphoblastoid cells were thawed and excess PBS was removed, beta-mercaptoethanol was added to buffers, and homogenization buffer was applied at 3x volume of packed cells. The cells were homogenized to release folates, centrifuged at 14,000 rpm for 30 minutes at 4°C, and supernatant transferred to new tube. The extraction buffer was applied at 0.2x volume of supernatant and heated to 100°C for 5 minutes to completely remove folates from their binding protein, then centrifuged at

12,000 rpm for 5 minutes. Supernatant was distributed into 20 ul aliquots and stored at -80°C or assayed immediately.

Immediately prior to the assay, a master mix of single strength folic acid medium and working buffer (sodium ascorbate, di H2O, potassium phosphate buffer, filtered through 0.22 um filter) was prepared in a ratio of 150:8 ul. A 96-well microtiter plate was set-up in low light with duplicate columns of folinic acid standard and blank intrinsic controls ranging from 0 ul to 60 ul of 2 fmol/ul folinic acid standard solution, with diH2O added to bring well volume up to 72 ul. 158 ul master mix was added to all wells using a multichannel pipette, and additional 62 ul single strength folic acid medium was added to sample wells. 10 ul of cell sample preparation was added to samples wells in triplicate. Finally, 20 ul of prepared L. Casei culture was added to all wells except blanks to bring total volume to 250 ul/well. The plate was then covered and wrapped in Parafilm and aluminum foil to prevent light oxidation and incubated at 37°C for 18 hours. The plate was then read at 600nm in BioRad plate reader and standard curve was plotted for comparative analysis. The results were analyzed using the t-test.

C. Reverse Transcriptase Qualitative PCR Analysis

The mRNA levels of POLβ, UNG2, and AID was quantified via RT-qPCR. Total RNA was extracted from lymphoblastoid cells cultured in folate adequate and folate deficient media using the RNeasy Kit (Qiagen, Valencia, CA). cDNA was synthesized from 2 ug RNA using random hexamer primers and purified using QIAquick PCR purification kit (Qiagen). Real time RT-PCR was conducted on a LightCycler 480 instrument (Roche, Indianapolis, IN). PCR reactions contained 2 ug cDNA, 4 nM MgCl2, 0.4 mM each of sense and antisense primers and 2ml FastStart LightCycler 480 SYBR Green I Master

(Roche). External standards were prepared by cloning cDNA amplicons into Topoll vectors, sequenced for confirmation, and used to create 10-log standard curves. All transcripts were normalized to housekeeping gene B2M for analysis. Data is expressed as mean ± standard error of the mean (SEM) from at least three independent experiments and is considered statistically significant at p-value <0.05.

D. Liquid Chromatography with Tandem Mass Spectroscopy

Intracellular deoxyribonucleotide triphosphate levels of folate depleted and folate adequate lymphoblasts were analyzed by the Pharmacology Core Lab, Karmanos Cancer Institute. Cell samples were prepared according to the Core Lab standard procedures and run on a Waters LC-MS/MS system controlled by MassLynx software.

CHAPTER 4: RESULTS/FIGURES

<u>Intracellular Folate Concentration and Doubling Time</u>

B-lymphoblastoids were cultured at a standard media concentration of 3000 nM folate and at 12 nM folate to induce deficiency. Our experimental folate level was chosen based on previous experiments in lymphocytes evaluating proliferation and DNA uracil incorporation [73, 76]. This was done to allow some inference into the accumulation of uracil in these cells, and to measure the effects of folate deficiency at a level consistent with mild deficiency *in vivo*. Due to reduced proliferation in folate deficient cells, 12 nM folate is the minimum folate concentration we found feasible for experimental cell volumes without nucleoside supplementation. Cellular folate deficiency was confirmed by modified L Casei microbiological folate assay (Figure 4.1). Ten days of 12 nM culture medium was sufficient to deplete cellular folate levels in lymphoblasts by 93% ($p < 0.000001$). The cells were depleted by 98% by day 21 ($p < 0.000001$). Cell doubling time at day 3, 6, and 11 was calculated to elucidate the effect of relative folate deficiency on cell proliferation over time. Unsurprisingly, folate deficient B-lymphoblastoids had significantly decreased proliferation relative to cells cultured at 3000 nM folate (Figure 4.2). At day 6 in culture, we observed a trend toward increased doubling time ($p > 0.05$), however this effect was significantly amplified by time at folate deficiency. By day 11, the proliferation of cells cultured at 12 nM folate was 20% that of control cells ($p < 0.05$), suggesting significant number of cells were cell cycle arrested and/or apoptotic.

Figure 4.1 Relative cell folate concentration
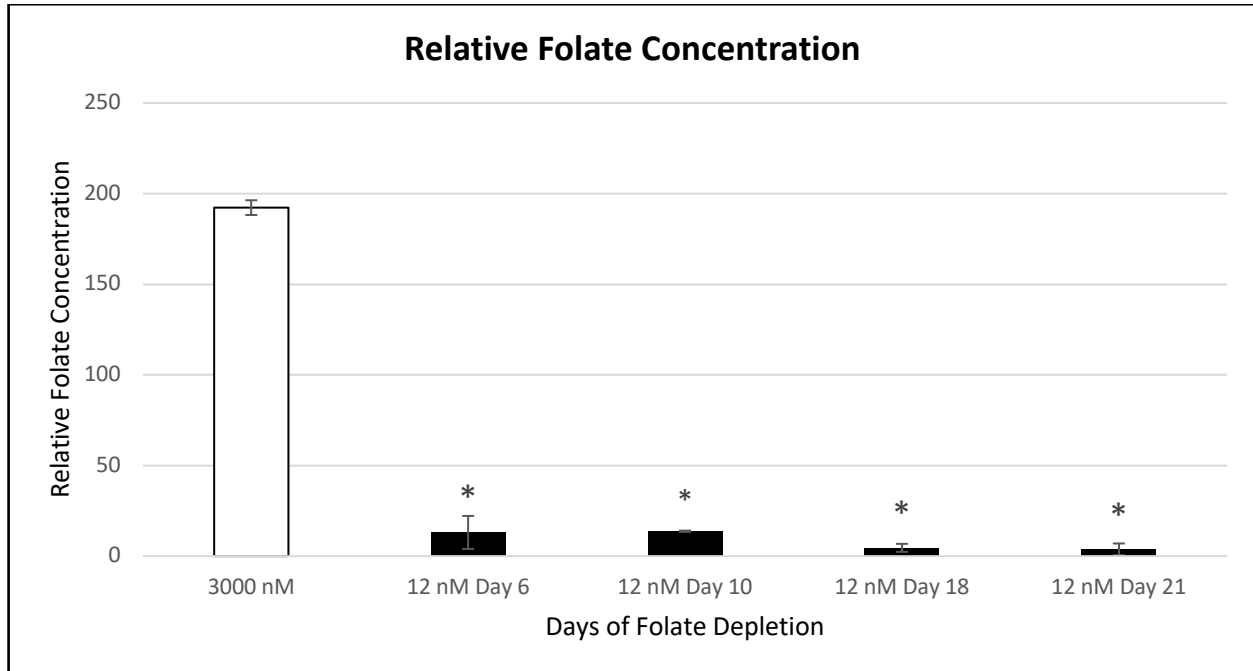
Figure 4.1: Relative cell folate concentration measured by L. casei microbiological assay. Lymphoblastoid cells were cultured at 3000 nM folate and 12 nM folate for 21 days. Relative folate concentration was measured by microbiological assay approximately every 4 days starting at day 10. Experiments were run in triplicate and results are shown as mean for each assay. Folate concentration and SD fell below measurable threshold at day 14.

Figure 4.2 B-lymphoblastoid Doubling Time
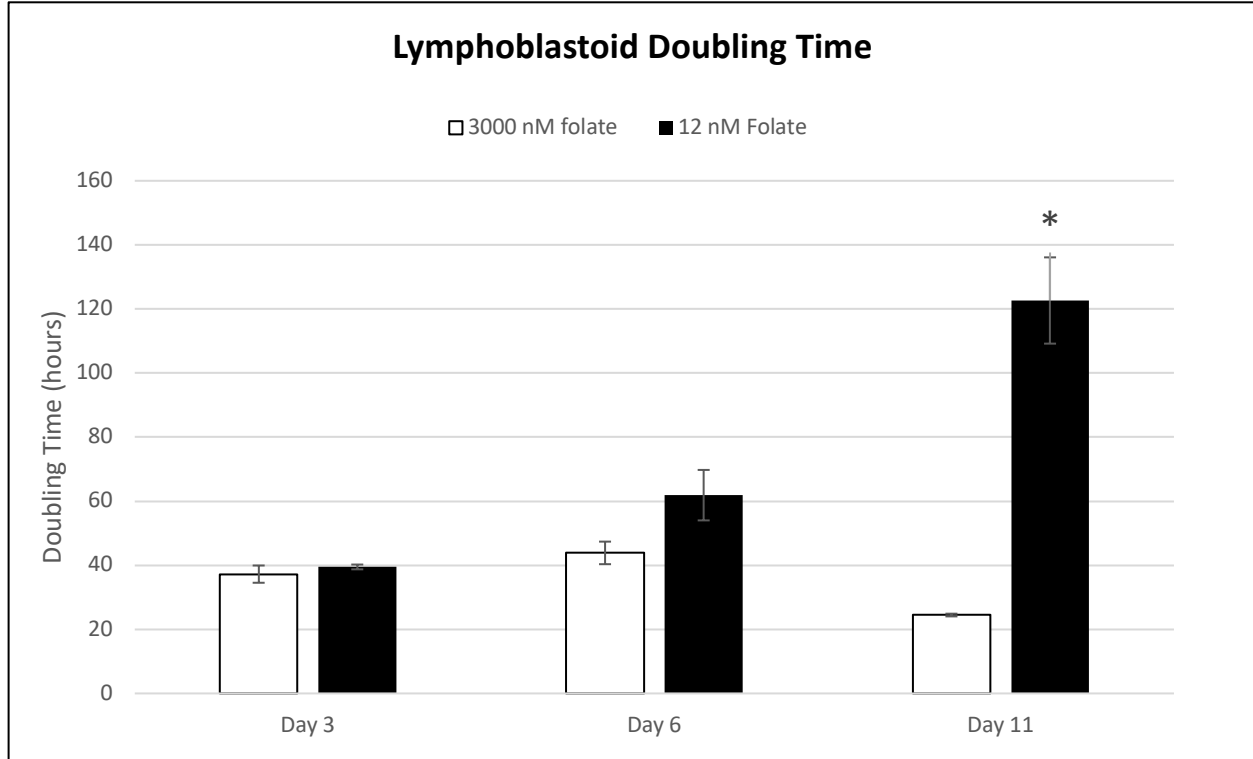
Figure 4.2: Lymphoblastoid Doubling Time. Cellular culture doubling time was calculated at days 3, 6 and 11 of culture at 3000 nM and 12 nM folate. Live cells were counted with hemocytometer, recorded per 8ml and reseeded with 10^6 cells at each passage.

Deoxyribonucleotide Triphosphate Levels

Disruption of the cell cycle as a result of folate deficiency has been attributed to replication inhibition triggered by insufficient deoxyribonucleotide (dRN) levels [77]. We confirmed this condition in B-lymphoblastoids by assessing the available dNTP pool by LC-MS/MS. Folate depletion decreased measured dNTP levels among all four dNTPs by an average of 40%. Individually, dATP decreased 38% in folate depleted vs. folate adequate cells, 56% in dCTP, 45% in dGTP, and 20% in dTTP. However, as the values were obtained from a single experiment in limited folate depleted cells, P-values could not be calculated for these results.

Figure 4.3 dNTP Levels in Folate Deficient B-Lymphoblastoids
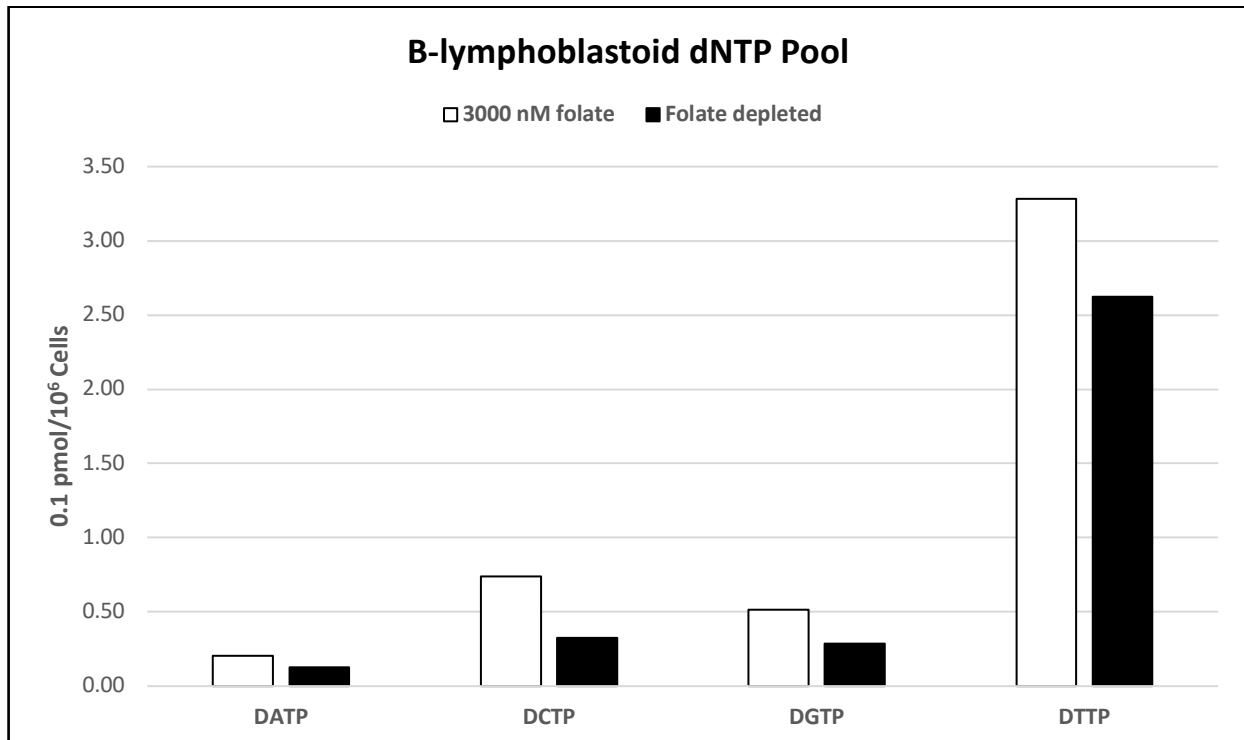
Figure 4.3 Deoxyribonucleotide Triphosphate Levels in Folate Deficient B-Lymphoblastoids. Cells were cultured for 10 days in folate adequate and folate depleted conditions. Nucleotides were isolated and quantified by LC-MS/MS.

Expression of BER Enzymes

Transcriptional regulation of the BER enzyme coding genes *UNG2* and *POL*β are known to be influenced by folate deficiency in some cell types. Disruptions in BER inducibility in B-cells may affect AID-mediated uracil repair. Therefore, we measured the expression of these enzymes in B-lymphoblastoids. RT-PCR was performed every 3-4 days for three weeks from total RNA extracted from folate deficient and folate adequate cells. Results are expressed relative to *B2m* (beta2-microglobulin), which is documented to be a stable reference of expression in hematopoietic cells [78, 79]. UNG2 mRNA levels in the folate deficient cells were decreased by 16% after 3 days of culture ($p<0.05$), these levels also generally decreased over subsequent days; 6 ($p<0.05$), 14 (p <0.01), 17 ($p<0.01$), and 21 ($p<0.01$), though there was a deviation from this trend at day 10 (Figure 4.3). By day 21 in folate deficiency, there was a 44% decrease in *UNG2* expression in B-lymphoblastoids. There has been evidence of a lack of *POL*β inducibility in folate deficiency in some cell types [54], and inadequate POLβ in liver tissue is a likely source of unstable repair intermediates and subsequent DNA strand breaks in folate deficient cells [55]. This is supported by the POLβ mRNA levels measured by our lab (Figure 4.4). We found no upregulation of *POL*β in the folate deficient B-lymphoblastoids and there is a trend toward decreased mRNA levels, and a significant reduction observed at days 10 and 17 ($p<0.05$).

Figure 4.3 UNG2 Expression in Folate Deficient Lymphoblastoids Relative to B2M

Figure 4.3: Relative UNG2 Expression in Folate Deficient Lymphoblastoids. RNA was isolated from folate deficient lymphoblastoids every 3-4 days for 3 weeks. UNG2 gene expression was determined by RT-PCR. Experiments were run in triplicate and normalized to B2M. Data is presented as mean ±SEM and significance of $P<0.05$ and $P<0.01$ is denoted by *, and ** respectively.

Figure 4.4 POLβ Expression in Folate Deficient Lymphoblastoids Relative to B2M
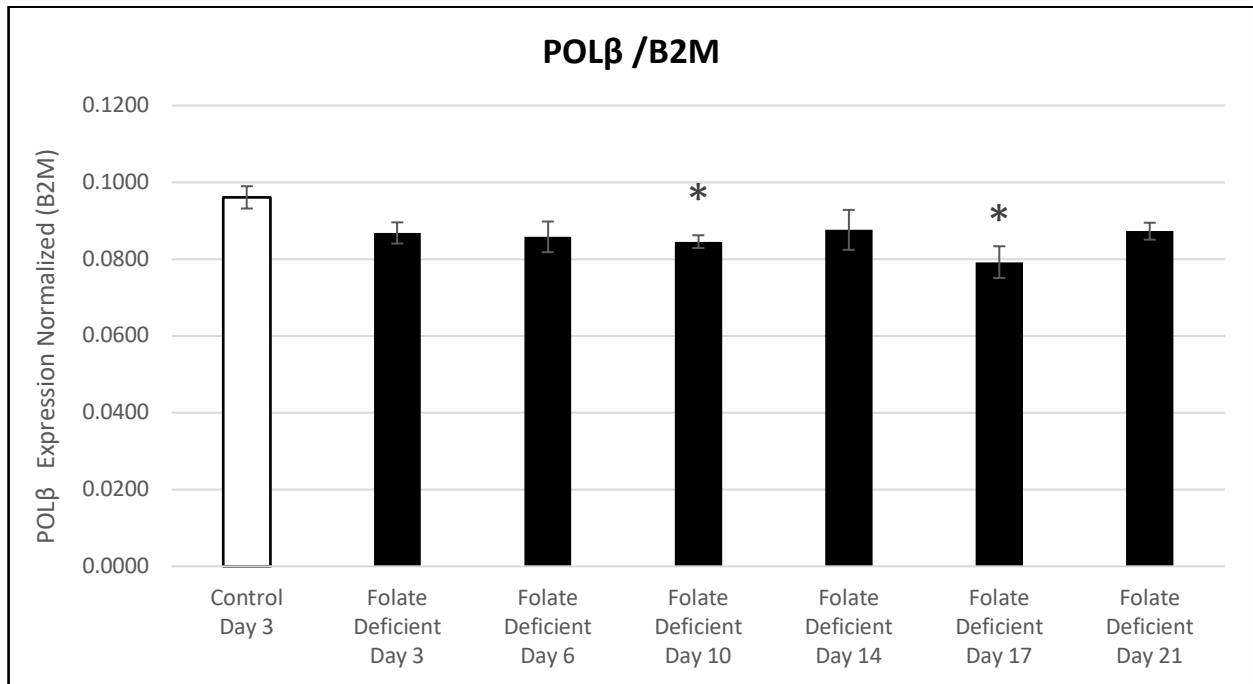
Figure 4.4: POLβ Expression in Folate Deficient Lymphoblastoids. RNA was isolated from folate deficient lymphoblastoids every 3-4 days for 3 weeks. POLβ gene expression was determined by RT-PCR. Experiments were run in triplicate and normalized to B2M. Data is presented as mean ±SEM and * denotes significance at $P<0.05$.

AID Expression

APOBEC-family cytosine deaminase AID interacts functionally with UNG2 in adaptive immunity and may also introduce uracil into non-target genes [80, 81]. The expression of *AID* in folate deficient B-cells, as well as the interaction between *AID* and BER transcript regulation in this condition has not been previously reported. In B-lymphoblastoids cultured in 12 nM folate, we found that AID mRNA levels were decreased by 34% and 30% at days 3 ($p<0.0005$) and 6 ($p<0.001$) respectively, compared to folate adequate cells. Interestingly, at day 10 of folate deficiency *AID* expression increased by 25% ($p<0.001$) and remained upregulated at days 17 ($p<0.005$) and 21 ($p<0.01$). Taken together, these findings suggest an inverse relationship between *UNG2* and *AID* gene expression in folate depleted B-lymphoblastoids.
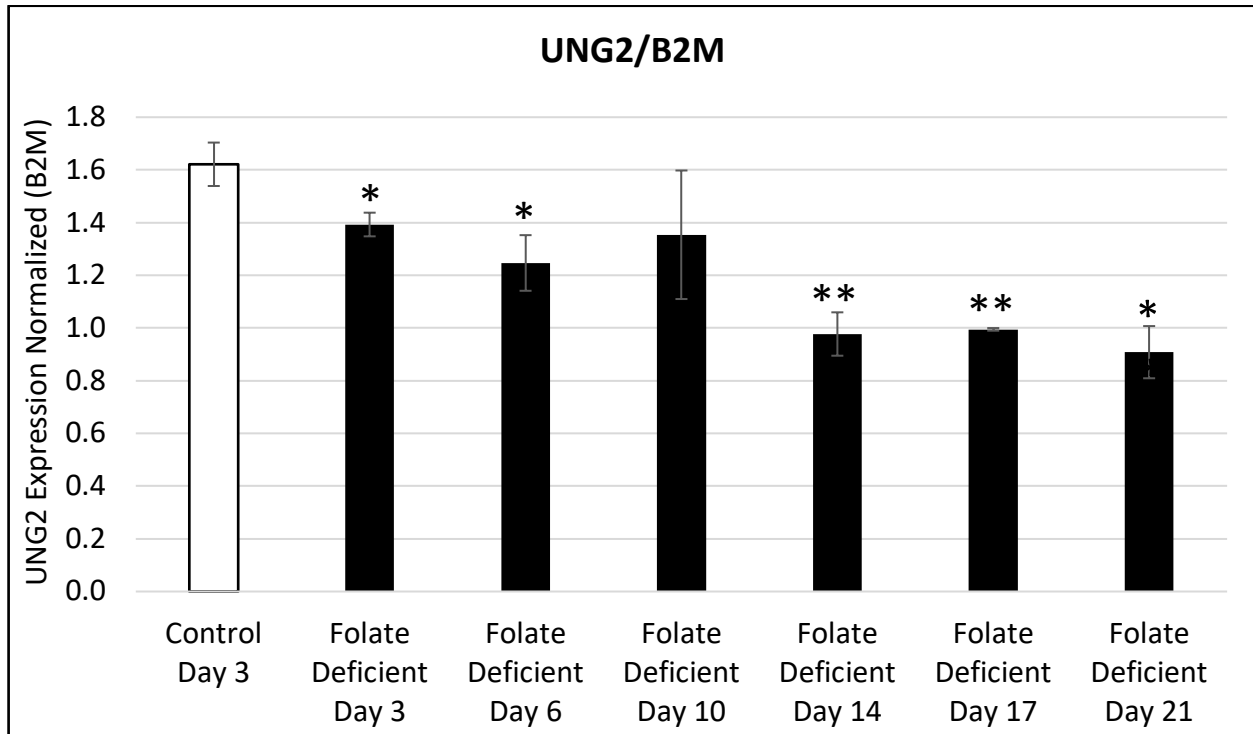
Figure 4.5 AID Expression in Folate Deficient Lymphoblastoids Relative to B2M
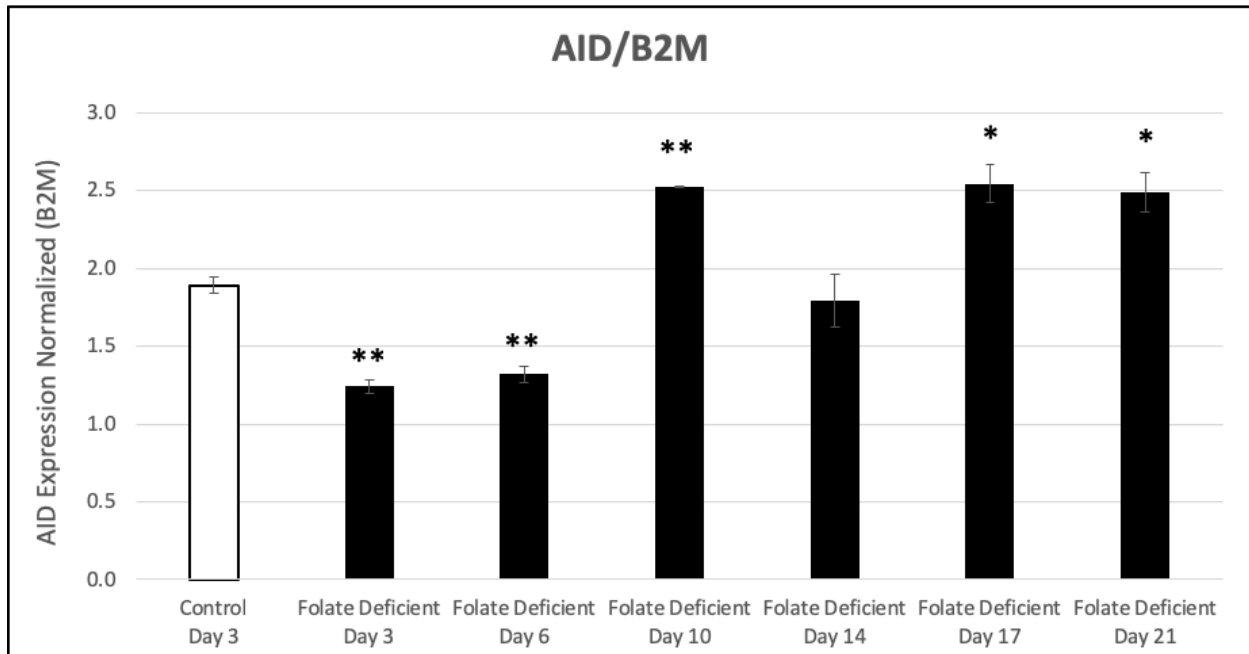
Figure 4.5: AID Expression in Folate Deficient Lymphoblastoids. RNA was isolated from folate deficient lymphoblastoids every 3-4 days for 3 weeks. AID gene expression was determined by RT-PCR. Experiments were run in triplicate and normalized to B2M. Data is presented as mean ±SEM and significance of $P<0.01$ and $P<0.001$ is denoted by * and ** respectively.

CHAPTER 5: DISCUSSION

Folate deficiency has been tied to reduced dNPT availability, stalled replication, BER dysregulation and DNA strand breaks [6, 54, 61, 82]. In the context of AID activity in activated B-cells, a disruption of BER may have significant implications for the processing of genomic uracil. However, we have found that the consequences of DNA repair imbalance in folate deficiency is not consistent among tissue types. Due to a reported increase in uracil accumulation in lymphocytes, we expected that BER inducibility was decreased in these cell types. In support of our hypothesis, we observe a significant step-wise decrease in UNG2 that follows the length of time in folate deficient media. In addition, upregulation of POLβ is blocked by folate deficiency, further limiting the ability of AID-induced uracils to be repaired in these cells. Thus, we establish an inverse relationship between AID expression and BER inducibility in folate deficient hematopoietic cells, increasing the risk for DNA strand breaks and oncogenic mutations resulting from unrepaired AID-induced genomic uracil.

Proliferation and dNTP Availability

We observed a reduction of dNTP levels in B-lymphobastoids which confirms folate depletion in these cells and provides further evidence that the availability of all four dNTPs are reduced by folate depletion *in vitro* (Figure 4.3). Phenotypic depletion of dNTPs due to a reduction in dRN precursors results in a global inhibition of DNA replication and fork stalling [74, 77]. As expected, proliferation in these cells was decreased in parallel with duration in folate deficiency (Figure 4.2), indicating significant growth arrest within 11 days in 12 nM folate media. Generally, cell cycle arrest is a marker of induction of cell cycle checkpoints, DNA damage and initiation of repair mechanisms,

though we cannot distinguish the stage of arrest in these cells without cell cycle analysis. When flow cytometry was completed in lymphocytes grown for 10 days at 12 nM folate, Mashiyama et. al. reported that approximately 45% of the cells were arrested in S phase, and another 45% in G0/1 [73]. This provides good evidence that lymphocytes grown at this level of folate are blocked in S-phase, likely in association with reduced dNTP levels. Additionally, prior research by Courtemanche et. al. indicates that there is a dose-dependent induction of apoptosis in folate deficiency, with significant cell death only demonstrated in cells cultured in 6 nM folate and below [82], well below our experimental cells.

There is evidence that the cell proliferation rate directly impacts the uracil content of DNA in folate deficient cells [83]. In the same publication, Mashiyama et. al. found that when proliferation was drastically reduced in lymphocytes by culturing in 0 nM folate medium for 10 days, uracil in DNA measured by GC-MS was not significantly different from the uracil content in cells at standard folate concentrations. However, when the media folate concentration was increased to 12 nM, DNA uracil content increased 6.5-fold [73]. This suggests that moderately folate deficient lymphocytes, such as those cultured at 12 nM folate have more uracil content than those in severe folate depletion. Whether this is a consequence of increased dUMP misincorporation, cytosine deaminase activity, decreased repair capacity, or a combination of all three remains unknown. Nonetheless, S phase block in folate deficiency may serve to preserve dNTPs for DNA damage repair, or alternatively prevent DNA synthesis under suboptimal conditions. Since we know that the proliferation rate is quickly restored in arrested cells once folate deficient cells are provided with folate or dNTP precursors [73], the severity of folate

deficiency may be an important non-dose dependent modifier of DNA damage. As cells in moderate folate deficiency favor arrest over the apoptosis observed in severe folate depletion [73, 76], repair of uracil accumulation in moderate deficiency may be sensitive to a lag in repair capacity once folate, and consequently replication is restored. This would imply that frequent fluctuations in folate availability may be detrimental vis-à-vis insufficient DNA repair of genomic uracil, though this effect has yet to be confirmed.

Tissue Specific BER Response

Folate deficiency disrupts DNA repair through imbalance in the BER pathway [54, 55]. This has been demonstrated by a normally induced UNG2 response to DNA damage, without a corresponding upregulation of POLβ in murine liver tissue and BNL.C2 cells [54]. Correspondingly, there is no accumulation of uracil in these cells types due to the action of UNG2 [unpublished results] however, there is an increase in mutagenic repair intermediates, specifically 3'-OH containing lesions [55]. In contrast, we have also found that damage-induced upregulation of UNG2 is inhibited in folate deficient MEFs, and that uracil does accumulate in the genome of these cells [unpublished results]. Additionally, when *Ung*-null MEFs were depleted of folate there was a 3-fold increase in detected DNA uracil levels [unpublished results], establishing tissue specific UNG2 regulation as a primary factor in determining the fate of uracil in DNA.

Here we show that *UNG* expression is decreased in folate deficient B-lymphoblastoids, and that the expression decreases over time in this condition. This is consistent with the evidence that DNA uracil does in fact accumulate in folate deficient conditions in lymphocytes [73]. We also find no upregulation of POLβ in these cells, suggesting a complete loss of the BER inducibility response.

There is precedent for tissue specificity in the BER pathway. Karahalil et. al. found that UNG activity differed drastically among solid tissues with heart tissues having the highest activity of the tissues measured, and muscle the lowest, though no hematopoietic cells were measured in this study [84]. It is reasonable to speculate that highly proliferative progenitor cells such as MEFs and B-lymphoblastoids place preservation of the genome at a higher priority than fully differentiated cells, and thus limit potentially mutagenic repair activities to a greater extent. However, in terms of uracil processing it remains unclear which tissue specific phenotype of folate deficiency is favorable. Continued upregulation of UNG2 without the corresponding POLβ response results in an accumulation of repair intermediates, as seen in liver tissue, is almost certainly detrimental, and likely responsible for the increase in DNA strand breaks and chromosomal aberrations characteristic of folate deficiency in the literature [37, 55, 61]. However, depending on the source of the uracil, genomic uracil that escapes repair prior to replication may result in an oncogenic somatic mutation. Misincorporation of uracil, as occurs as a result of folate deficiency-induced rise in dUMP:dTMP ratio, results in a U:A mismatch that is not miscoding, though could result in mutations at abasic sites or during gap-filling in BER [55]. However, deamination of cytosine in DNA results in a C to T transition mutation since the copying of uracil during replication will invariably result in the generation of a U:A pair, and eventually at T:A pair, in the place of the original C:G pair (Figure 1.7). This type of transition mutation is the most common mutation found in human cancer [68, 81], and may be more likely to occur in cells that phenotypically accumulate uracil in response to folate deficiency, such as lymphocytes.

Imbalance of *AID* and *UNG2* Expression in Folate Deficiency

AID converts cytosine to uracil in *Ig* genes to create antibody diversity through SHM and CSR. AID is also strongly connected to cancer in animals, and many human B cell lymphomas constitutively express *AID* [72, 75, 81]. In both the error prone AID-induced uracil repair pathway in *Ig* genes, and the non-error prone BER repair of deaminated cytosine genome-wide, UNG2 is the primary DNA glycosylate responsible for base excision. Shalhout et. al. has established that *UNG2* gene expression parallels *AID* expression following B-cell stimulation in WT mouse splenocytes, and that the upregulation of UNG2 was 2.5-fold higher in WT cells than in *AID* $^{-/-}$ cells [75]. This finding confirms a regulatory balance between UNG2 and AID. Folate deficiency is known to modulate UNG2 expression, although this effect is tissue-dependent, as we have established. Lymphocytes accumulate uracil in their DNA when folate is deficient [73], possibly due to inhibition of BER inducibility. Since AID is known to actively generate DNA uracils in B-cells, we measured the relative expression of *UNG2* and *AID* in our experimental cells (Figures 4.3, 4.5). Here, in contrast to the direct correlation between UNG2 and AID in WT cells, we find an inverse association between the relative expression of *UNG2* and *AID* after 10 days of folate deficiency in B-lymphoblastoids.

The balance between uracil creation by AID-mediated cytosine deamination and uracil elimination by BER or MMR mechanisms is tightly controlled in normal stimulated B-lymphocytes. The resulting homeostasis maintains low genomic uracil content in typical conditions. This has been confirmed in stimulated WT B-cells, since uracil only accumulates when *UNG2* is inactivated [75]. This is true throughout the whole genome and is not specific to the *Ig* loci [75]. It is known that AID acts on non-Ig target genes,

possibly to a large extent [80]. This off-target action resulting in deaminated cytosines is repaired by short-patch BER, with the excision primarily completed by UNG2. In folate deficiency we find that UNG2 is downregulated, presumably disturbing the balance of uracil creation and excision. The downregulation of AID that we observed in the first six days of folate deficiency may be protective in light of the concurrent downregulation of UNG2. However, by day ten, UNG2 continues to decrease while AID is upregulated. Previous work from our lab has demonstrated a tight correlation between levels of UDG protein and UDG activity [55], suggesting that the inverse relationship between UNG2 and AID in the folate deficient state may result in significant uracil formation and accumulation in these cells. The parallel kinetics of UNG2 gene expression and nuclear uracil excision activity was also confirmed by Shalhout et. al [75]. As discussed, deamination of cytosine by AID will result in a U:G mismatch, and may result in a C to T transition mutation if left unprocessed by UNG2 prior to replication (Figure 1.7). Transitions of this kind are by far the most prevalent mutations found in human cancer [68].

A recent study by Alvarez-Prado et. al using machine learning of high-through-put sequencing of >1500 genomic regions from germinal center B-cells has found that 2.3% of the genome or ~500 genes are likely targets of AID mutations, and that both the BER and MMR pathways contribute to reducing mutations at off target AID sites [80]. Additionally, chromosomal translocations resulting from faulty repair of uracil lesions are a frequent source of lymphomagenesis. These translocations frequently occur between proto-oncogenes such as Bcl-2 (B-cell lymphoma-2) and c-Myc and the IG locus, as well as at off-target sites [80, 85]. Though it is outside the scope of this paper, inducibility of

error prone BER at the *Ig* locus may also be dysregulated by folate deficiency during SHM and CSR due to their reliance on UNG2, resulting in unknown effects from AID action. In addition, off-target deamination by other ABOBEC family enzymes active in the innate immune system provide additional uracil substrate for UNG2-initiated BER. These lesions result in C to T transition mutations if left unprocessed in a manner similar to AID, although the extent to which non-AID APOBEC enzymes introduce uracils into off target loci is undetermined.

So far, epidemiological evidence linking folate deficiency in humans to risk of B-cell lymphomas has been equivocal, and very few cohort studies have been completed on this topic [86]. However, in a population-based study of 1,304 Connecticut women, Koutros et. al. found lower risks of diffuse large B-cell lymphoma (OR = 0.54) and Non-Hodgkins's lymphoma overall, and marginal zone lymphoma (OR = 0.23) in the highest vs. lowest quartile of folate intake reported by food frequency questionnaires [87]. Additionally, while rates of overt folate deficiency in the US are low, the level of depletion modeled in our experiment is consistent with only moderate sub-clinical folate deficiency *in vivo,* which undoubtedly comprises a larger share of the population. However, it is important to keep in mind that incongruities in direct comparison may exist due to differences in the uptake of specific folate forms and known genetic SNPs affecting folate metabolism.

Interestingly, in both murine and human lymphoma cell lines, Shalhout et. al. observed high rates of accumulated uracil at AID preferred sequences (AID hotspots) despite UNG2 and AID activity similar to non-cancer cell lines [75]. They propose that during maturation some B-cells may lose coordination between uracil creation and

elimination, creating a "DNA repair crisis." However, if the excisional activity eventually catches up with uracil lesions to stabilize the cell, a new steady state condition may occur at a uracil level that is much higher than typical cells.

Building upon these studies by Shalhout et. al. on UNG2 and AID, here we introduce a nutritional modifier that directly impacts uracil excision coordination with AID. Further research is needed to explore the relationship between UNG2 and AID in folate deficiency, including downstream effects of uracil accumulation in AID expressing B-cells. We also observed a significant reduction in intracellular folate concentration, proliferation and dNTP levels in B-cells at moderately depleted folate media. Given the complex association between folate status and carcinogenesis, it is possible that modest fluctuations in folate availability confer yet unknown influence on the cell cycle and DNA repair/AID interaction. In conclusion, these findings provide preliminary evidence of a nutritionally-induced mechanism by which the AID/BER functional interaction may become destabilized and increase risk for the AID hotspot oncogenic mutations described in the literature [72, 80, 81]. Specifically, dysregulation between AID and UNG2 in B-lymphoblastoids increases the risk transition mutations resulting from unrepaired AID-induced genomic uracil.

CHAPTER 6: CONCLUSION

B-lymphoblasts deprived of folate at physiologic levels have a corresponding decrease intracellular folate and dNTP levels, and 80% decrease in proliferation by day 10 *in vitro*. The nuclear uracil DNA glycosylase UNG2 is downregulated in this condition, limiting DNA repair capacity. Regulation of UNG2 in folate deficiency is likely a tissue-specific response in lymphocytes and other normally highly proliferative cells, possibly in an attempt to limit mutagenic repair intermediates resulting from insufficient POLβ activity in BER however, at the expense of increased DNA uracil content. Expression of AID in cultured EBV-transformed B-lymphoblasts has been documented to correlate well with genomic cytosine deaminase activity [75]. In normal activated human lymphocytes, UNG2 and AID are functionally linked, and their expression and activity are highly synchronized. When exposed to folate deficient media AID mRNA is decreased initially, but by day 10 levels are upregulated. These modulations are significant, though weak. This response is inversely correlated with both UNG2 expression and cell proliferation. It remains unknown whether this discoordination between UNG2 and AID in folate deficiency results in an accumulation of genomic uracil in these slowly proliferating cells, or if this imbalance occurs *in vivo*, specifically at AID mutational hotspot sequences. Additionally, if AID activity continues during cell cycle arrest, as is suggested here, supplementation of folate or nucleosides to rescue cells from S phase block, may have yet to be determined consequences associated with a lag in the repair of uracil lesions in newly replicated cells. Given growing evidence of off-target AID action playing a role in oncogenic transformation, the role of folate deficiency in the dysregulation between BER and AID warrants additional investigation.

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ABSTRACT

IMBALANCE OF URACIL DNA GLYCOSYLASE AND ACTIVATION-INDUCED CYTIDINE DEAMINASE EXPRESSION IN FOLATE DEPLETED HUMAN LYMPHOBLASTOIDS

by

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Background: The DNA base excision repair (BER) pathway is responsible for processing of genomic uracil lesions however, in some tissue types the excisional and gap-filling steps performed by UNG2 and POLβ, respectively, are impaired by folate deficiency in human and murine models *in vitro*. Genomic uracil damage can be acquired by inadequate conversion of uracil to thymine nucleotide precursors resulting from insufficient folate cofactors, or through activation induced cytosine deaminase (AID) activity during antibody diversification in B-cells in the context of adaptive immunity. The immunoglobulin (Ig) diversification methods in B-cells depend on the coordinated interaction between AID and UNG2, and as such, their analogous transcriptional regulation has been established. However, AID-induced uracil lesions at non-Ig loci have been found to be associated with lymphomagenesis, suggesting a functional BER deficit. Therefore, the objective of this research is to establish the tissue specific effect of folate deficiency on BER enzymes in B-lymphoblastoids, and to examine the functional

coordination between AID expression and BER inducibility during moderate folate deficiency in B-cells *in vitro*. **Methods:** Human B-lymphoblastoid cells were cultured in 12nM and standard folate media concentration for 21 days. Intracellular folate concentration measured by microbiological assay, doubling time, and genetic expression of *UNG, POLβ*, and *AID* analyzed by RT-qPCR was completed with culture samples taken every 3-4 days. **Results:** Within 10 days in moderate folate deficient media, intracellular folate concentration was decreased by 93%, and cell proliferation was reduced by 80%. Expression of *UNG* was significantly downregulated by 16% at day 3 of folate deficiency, and by 44% at day 21. AID mRNA levels were significantly increased by 25% by day 10, demonstrating an inverse relationship between UNG2 and AID gene expression. **Conclusion:** Tissue specific downregulation of BER in folate deficient B-lymphoblastoids results in a functional imbalance between AID and uracil repair capacity, possibly increasing the risk of oncogenic transition mutations resulting from unrepaired AID-induced genomic uracil.

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