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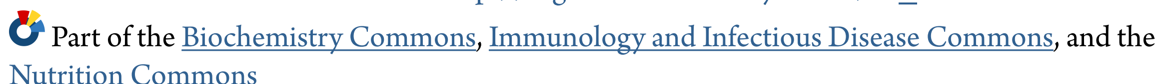
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THE IMPACT OF DOWN SYNDROME AND FOLATE DEPLETION ON GENOMIC STABILIZING PATHWAYS OF LYMPHOBLASTOID CELLS

by

KHADIJAH ALNABBAT

THESIS

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

2014

MAJOR: NUTRITION AND FOOD SCIENCE

APPROVED BY:

____

Advisor Date

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DEDICATION

I would love to dedicate this thesis to my loving and supporting Family

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Diane Cabelof; you have been a great support and mentor. I would like to thank my Committee member for their cooperation, Dr. Ahmad Heydari and Dr. Kequan Zhou. I would like to thank Meijung York for her support, and my Lab members: Aqilah, Ghada, Kasia, Monicha, and Denise for their help and being lovely partners.

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CHAPTER 1: INTRODUCTION

The emerging field of studying epigenetics via nutrition –mediated perspective has been enriching our knowledge and expanding our understanding of how to maintain our health and how to modify diets to improve our health. The relationship between nutrition and epigenetics becomes particularly important when considered within the context of individuals with genetic disorders, such as individual with Down syndrome. Understanding the role of nutrition plays in Down syndrome could help in the development and implementation of strategies that help overcome the negative consequences phenotypes of Down syndrome. Conserving genome integrity is crucial for cells to survive, and thus understanding how genetic defects induce damage to genomic DNA and impair subsequent repair of this damage is important. Evidence accumulated points to increased DNA damage and mutation accumulation associated with a decline in DNA repair capacity, Base Excision Repair (BER) in particular. Many experiments have demonstrated that elevated levels of chromosome 21-linked gene products (HSA21) are correlated to the increase level of DNA damage and decrease level of BER in DS. Thus, the successful clinical management of DS resides in understanding the metabolic imbalance provoked by overexpression of genes on chromosome 21.

A. Down syndrome (DS):

Trisomy 21,DS, is a complex metabolic and genetic disorder that stems from the failure of chromosome 21(HSA21) to segregate normally during meiosis [1]. The origin of the extra copy of HSA21 has been related to the maternal chromosome in 93% of cases, and to the paternal chromosome in 7% of cases [6]. DS is the most common genetic cause of intellectual disability and is characterized by accelerated aging [2], and has an incidence of 1~1/700 live birth [3]. Individuals born with DS exhibit phenotypes with varying severity of cognitive impairment, craniofacial dysmorphology, heart defects [3], and immunologic disorders, such celiac disease and diabetes [1]. It is assumed that the excess products derived from overexpression of multiple genes located on HSA21 is the underlying factor for the abnormality in the nervous, endocrine, and immune system which are features of DS [3,4]. However, recent studies suggest that certain tissues and gene product are more sensitive than others to gene dosage effects. Sod1, the gene that encodes Superoxide dismutase 1, is one example of a gene that is well documented to be overexpressed in DS [5]; there is a significant increase in Sod1 activity between 1.4 and 1.8 times higher in trisomic cells than normal lymphocyte cells [5]. ATP-binding cassette, subfamily G, member 1(ABCG1) was found to be 2.67 fold overexpressed in adult DS brain tissue [55], whereas it was only 1.25 overexpressed in DS lymphocyte cells [56]

B. DNA damage; Genome Instability:

Preserving and maintain genome integrity is crucial for survival of cells. The integrity and identity of the genome is challenged by the incorporation of aberrant molecules unrelated to genomic DNA such as ribonucleoside triphosphate (rNTPs) in plase of deoxyribonucleoside triphosphate (dNTPs) during replication [7], or an altered base like uracil. This misincorporation of the aberrant molecules prompts DNA repair machineries to fix the damage and restore genome integrity; otherwise, the lesions formed convert to mutation disrupting cells survival. Evidence indicates a linkage between aging and nuclear DNA lesions; premature aging can be a result of DNA damage accumulation with age associated with DNA repair defects [57]. Various aging biomarkers are seen in DS, including oxidative stress, mutation accumulation and reduced DNA repair [58].

B.1 Uracil:

Uracil is a natural base present in RNA structure; however, it may arise in DNA via two distinctive pathways. First pathway is through deamination of cytosine, and second pathway through misincorporation of deoxyuridine 5'-triphosphate nucleotide (dUTP) in place of thymidine 5'-triphosphate (dTTP) during DNA replication. The incorporation of dUTP into DNA during replication phase has been estimated to be up to 10^4 uracil residues in human genome per day [8]. This incorporation of uracil creates DNA lesions, and such lesions are cleared out through high fidelity DNA repair mechanism and thus preventing

genome injury [8]. Deoxyuridine 5'- triphospatase nucleotide hydrolase (dUTPase) regulates the dUTP/dTTP ratio. dUTP is synthesized in eukaryotic cells from the phosphorylation of dUDP , which in turn arise from either UDP undergoing the action of ribonucleoside diphosphate reductase (rNDP) or from the phosphorylation of dUMP by nucleoside diphosphate kinase (NDPK) , an essential intermediate for the synthesis of the intracellular dTTP pool and thus is a permanent source of dUTP, as shown in **Figure 1.1** [9].

B.2 Ribonucleotide misincorporation:

Rribonucleotides (rNTPs) have been shown to be misincorporated in yeast DNA during replication at a high rate, demonstrating that replicative polymerases do not exclude rNTP absolutely [34]. Okazaki fragments in RNA primers or RNA/DNA hybrid are another source of rNTP, which arise when nascent RNAs base pair with their template to prime DNA synthesis from the discontinuous strand [34].

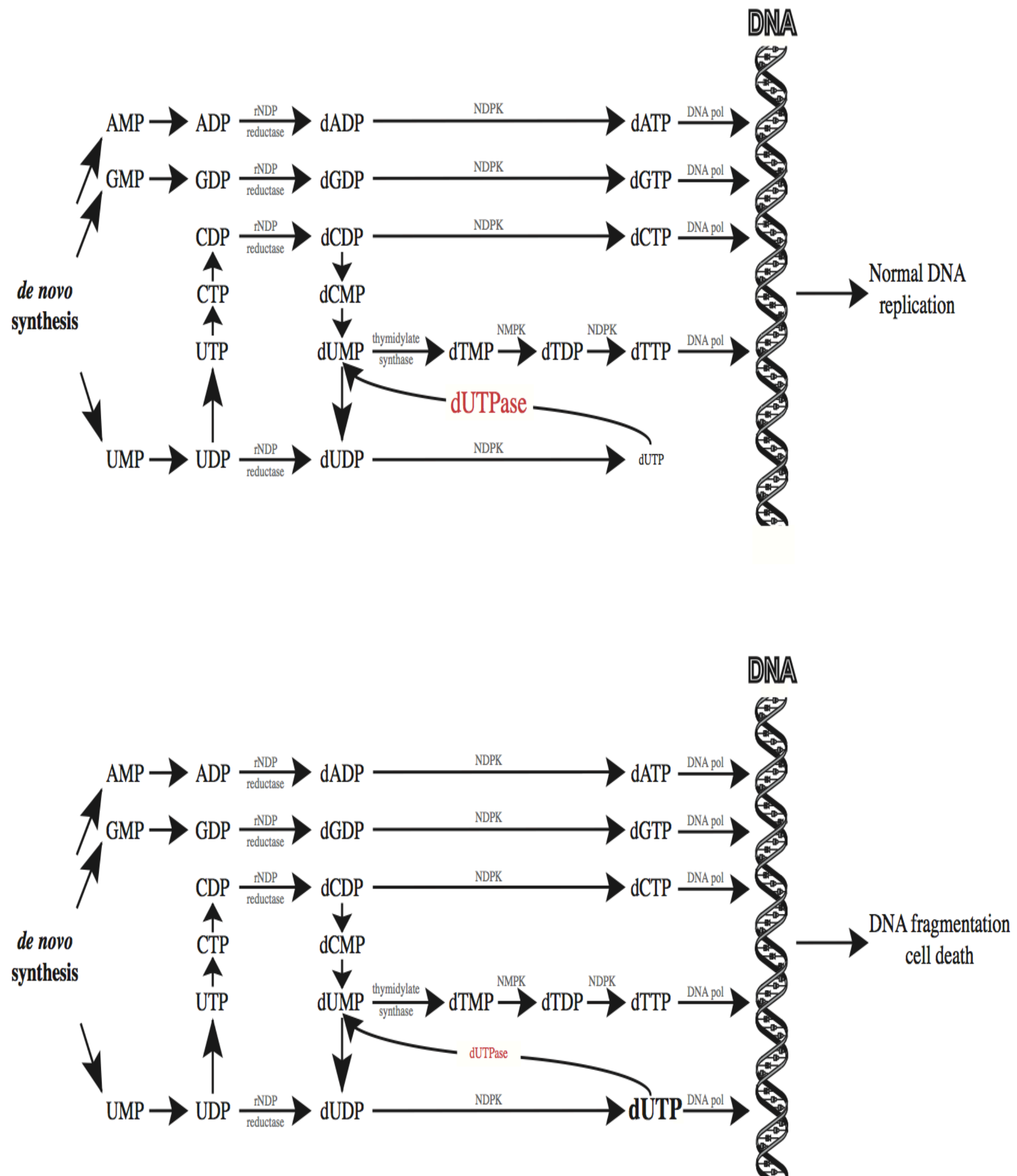


Figure 1. 1 De novo synthesis of Deoxynucleotides

C. MicroRNA -155:

MicroRNAs (miRNAs) are class of small noncoding RNAs, 19-24 nucleotides, produced from genes encoding RNAs with a hairpin secondary structure [10,11]. These hairpin RNAs are then processed to produce mature single stranded miRNAs that become incorporated into many different protein complexes. These miRNAs bind to a complementary sequence located in the 3'-translated regions (3' UTRs) of messenger RNAs (mRNAs), and can regulate the expression of large group of genes at post- transcriptional level via various mechanism such as cleavage, degradation, cell cycle control, translational inhibition, and mRNA transport [10].

miR-155 is a product of MIRN155 gene in humans or Bic in mice, and is located on chromosome 21 [10,11,12]. Strong evidence indicates that miR-155 plays an essential role in biological process including hematopoiesis, inflammation, and immune modulation [11], and its expression is induced in activated leukocytes and germinal center of B cells [10]. Deregulation of miR-155 has been linked to different kind of cancer, cardiovascular diseases and viral infection [11]. It was found that miR-155 expression is highly increased in activated B and T cells, and found to be required for lymphocyte development and generation of B-cell receptors (BCRs) and T-cell receptors (TCRs) activation in vivo [11]. Mice deficient in miR-155 experience deregulation of expression of hundreds of mRNAs, some of which are direct targets of miR-155 leading to vast abnormalities in the germinal center reaction and antibody responses *in vivo* [10]. miR-155 has been shown to target over 60 different genes in B- cells. Recently,

AID gene, which is restricted to activated B cells, has been identified, as an important target of miR-155 *in vivo*, and solid evidence points that miRNA-155 as a negative regulator of AID [11]. Hence, the significant role in controlling the germinal center reaction of miR-155 in B-cells found to be at least in part related to its role in suppression AID expression [13]. Also, expression of AID gene was found to be 1.6 fold increased in miR-155 deficient b-cells [14]. People with Down syndrome have aberrant miR-155 expression due to the extra copy of chromosome 21 [15]. miR-155 was found to be triplicated and over expressed in Ts65Dn mouse model of down syndrome [2,15].

D. AID; Role in Somatic Hyper Mutation:

In response to foreign pathogens, B cells induce diversity of the anti bodies by mutating their genome through Somatic hyper mutation (SMH) [16]. This mechanism is tightly regulated by activation induced cydtidene deaminase (AID). AID promotes diversity by converting cytosine to uracil within the immunoglobulin loci [17], producing U:A mismatch, which is non-mutagenic, or U:G mispair, which is mutagenic. This deoxyuracil residue is mutagenic if paired with deoxyguanosine since it mimics thymidine during DNA replication, and will promote C>T mutagenesis. Although U:A mispairs are not mutagenic, they are followed by the removal of uracil base by Uracil DNA glycosylase (UNG2), which generates a transient DNA strand break. Therefore, a critical intermediate in this process is the appearance of strand breaks and the loss of DNA integrity. Almost 60% of mutation accumulated *in vivo* in mice and human are in A:T bases, and

are not caused by the direction of AID **Figure 1.3** [18]. Once AID has altered dC to dU in DNA, uracil is either replicated or removed by UNG to create an abasic site, which can be converted into a single strand break. This single-stranded break recruits error prone polymerases for further repair. Whereas replication generates only transition mutation, UNG-dependent pathway can generate transition and transversion mutation **Figure 1.2** [19].

The results from (Poltoratsky et al, 2007) showed that down-regulation of the normal BER gap-filling DNA polymerase, Polβ, in the somatic hypermutation (SHM) proficient B cell lines (BL2 cells). This is consistent with the hypothesis that normal error-free BER must be silenced to make way for an error-prone BER process that may be required during SHM [23].

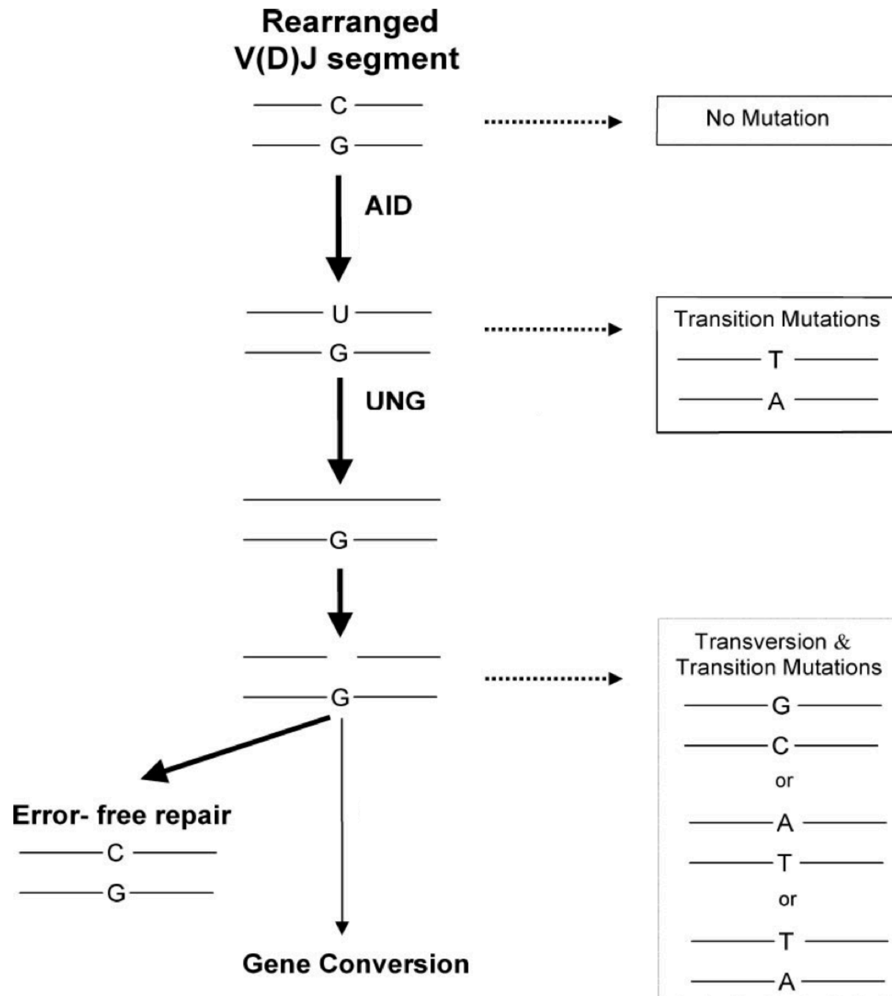


Figure 1. 2 Mutation generated by AID activity

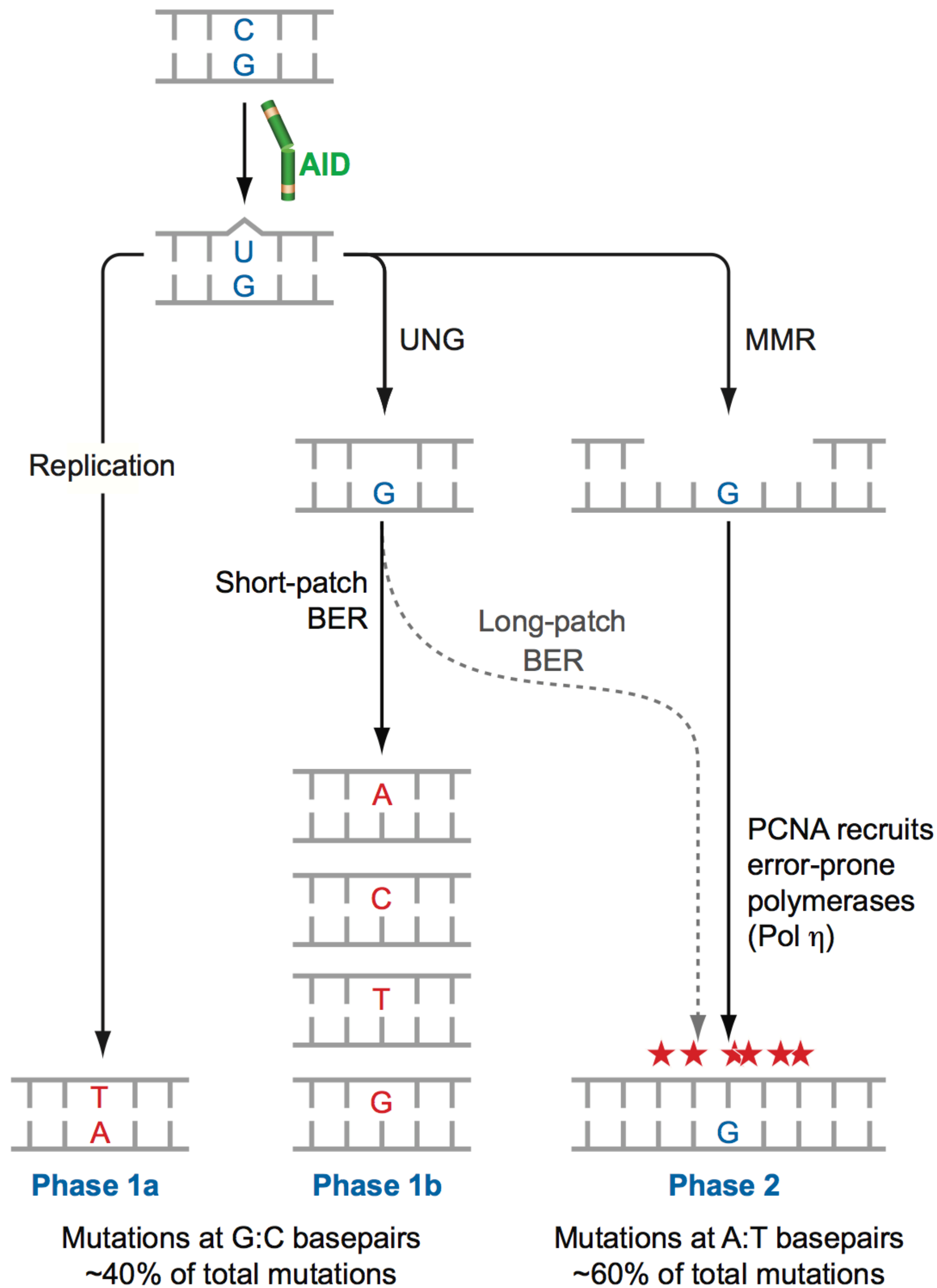


Figure 1. 3 A model for Somatic Hypermutation (SHM)

(Peled et al.) [18]

E. Uracil -DNA Glycosylase:

The fact that multiple uracil-DNA glycosylase (UDG) enzymes exist across vast variety of species (bacteria, archaea, and eukaryotes) [20], and the fact that are conserved in all types of human cells [21] support the significance of removing uracil from the genome. The UNG gene encodes human UDG protein; this gene produces two different isoforms, mitochondrial UNG1 and nuclear UNG2, due to the alternative promoter usage and splicing of this gene [21]. UNG2 is the most dominant form in the cells illustrating more than 90% of the total activity of this enzyme [22]. Nuclear UDG activity is cell cycle- dependent and increases during S-phase [21]. Mice lacking UNG2 have an increased level of uracil in their DNA, and UNG2 enzyme was shown to be the principle uracil-DNA glycosylase responsible for the excision of uracil and the formation of U:G lesions in mouse [20]. UDG is able specifically to process the mutagenic U:G lesions, resulting from spontaneous deamination of cytosine, which specifically relates to immunoglobulin diversification during SHM and CSR [20]. The important role of UDG in eliminating uracil from DNA arises from the fact that DNA polymerases are poor discriminators between dTTP and dUTP, a precursor of dTTP in *de novo* biosynthesis pathway, so that dUMP will be misincorporated into the newly synthesized DNA in corresponding to the increasing dUTP pool. The resultant U:A pair in the daughter DNA is neither mutagenic nor cytotoxic, yet it has deleterious effects including changing the affinity of DNA-binding factor for its target sequence [24]. Additionally, excision of the U from the U:A mispair

will generate a transient DNA break. UDG catalyzes the excision of uracil and initiates DNA base Excision repair (BER) [25]. Once uracil is excised by UDG, the abasic site is efficiently converted to a transient single strand break by apurinic/apyrimidinc endonuclease (APE1). This abasic site can be repaired further by error-prone polymerases such as DNA polymerase κ, η, and ι , or by error –free polymerases such as DNA polymerase β (POL β) [18].

F. Base Excision Repairer (BER):

BER is a DNA repair mechanism, by which damaged bases are excised by DNA glycosylase, followed by several subsequent steps to repair the lesion [18].

Error –prone repair is essential feature in SHM and CSR, restricted to the non-replicative phases, whereas error-free repair is crucial for DNA replication during the S phase of the cell cycle. Error free BER is commonly initiated by UNG2 leading to an abasic site (AP-site) that is Further cleaved on its 5' side by an apurinic/apyrimidinic (AP)-endunuclease (APE), leaving a free 3'-OH end a 5'-deoxyribose phosphate (dRP) group. The dRP group is then incised on its 3' side via the lyase activity (dRpase) of the DNA polymerase β (Pol β) for short patch repair pathway whereas a short oligonucleotide is cleaved by flap endonuclease 1 (FEN1) for the long patch repair pathway. The resulting gap, finally, is filled by pol β/ or pol δ/ε and sealed by DNA Ligase III or I [18,27].

Since the damage generated by AID creates single base G:U mismatches, and since short-patch BER generates single abasic site mismatches, enzymes that recognizes larger mismatches, such as Msh3, have little effect on SHM [18].

G. Folate Deficiency; One Carbon Metabolism Pathway:

Adequate intake of Folate is crucial for cell division and homeostasis due to the vital role of folate coenzymes in nucleic acid synthesis, methionine regeneration, oxidation and reduction of one-carbon units required for normal metabolism and regulation [28]. Folate deficiency has been shown to impair DNA excision repair and mismatch repair [26,28,29], and elevates homocystenie levels, which in turn increase oxidative stress [30].

Tetrahydrofolate (THF) is the naturally occurring reduced form of folate [31]. Folate is metabolized via one carbon metabolism pathways, which collectively include purine and pyrimidine synthesis, formation of the primary methylating agent, S-adenosylmethionine (SAM) [28]; the amino acids methionine, serine, glycine, and histidine are, also, metabolized through folate-dependent reactions [31].

The main function of folate coenzymes is to accept or donate one-carbon units in key metabolic pathways **(Figure 1.4)**, and the central folate acceptor molecule is THF. The conversion of THF to 5,10-methylene-THF is the first essential step that drives the cycle in which the one carbon unit from serine, as a major carbon source, is transferred to THF via serine hydroxymethyltransferase (SHMT) to form 5,10- methylene-THF and glycine. A portion of 5,10- methylene-THF is oxidized by methylene tetrahydrofolate reductase (MTHFR) to form 5-methyl-THF. The N-5-methyl group of 5-methyl-THF can only be transferred to homocystenie for regeneration/remethylation of methionine. MTHFR plays a key

role in one-carbon metabolism by directing the methylation step of homocystenie [1,32]

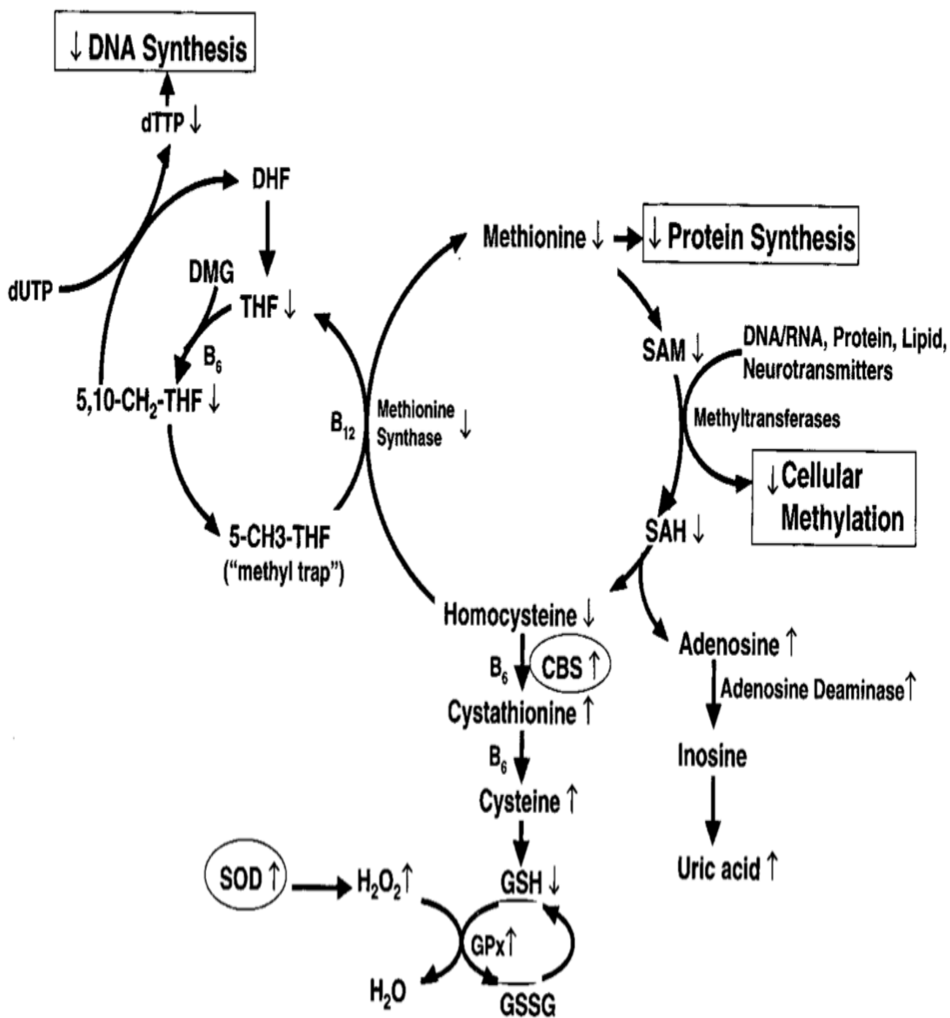


Figure 1. 4 Folate one-carbom metabolism

Overexpression of CBS gene, located on chromosome 21, leads to increase transculturation pathway, which in turn indirectly deprives methionine synthase reaction of homocysteine leading to the accumulation of 5-MTHF. This exponential conversion of homocystenie to cysteine and reduction level of methionine synthase activity thus creates the well established “methyl trap” [1]. As the increase of transsulfuration activity of converting homocysteine to cysteine requires B6 as cofactor, the bioavailability of B6 as co factor in conversion THF to 5,10-methylene-THF is compromised leading to the accumulation of THF, which is the well-established “Folate trap”, functional folate deficiency [1,32], and directing the folate coenzymes away from the cycle that produce purine and pyrimidine for DNA synthesis [33].

Folate deficiency, as source of endogenous DNA damage, causes an imbalance in the thymidine/uracil ratio leading to uracil being incorporated into DNA during synthesis. The repair of two nerby opposing lesions can cause DNA double strand breaks (DSBs), which are considered the most lethal DNA lesion [28].

H. RNaseH2; Ribonucleotide Excision Repair:

The presence of a single reactive hydroxyl group at the 2’ position of ribose sugar of a ribonucleotide (rNTP) makes rNTPs susceptible to spontaneous hydrolysis [35]. Thus, the incorporation of rNTP in genomic DNA increases its susceptibility to breakage. Even though replicative DNA polymerases have

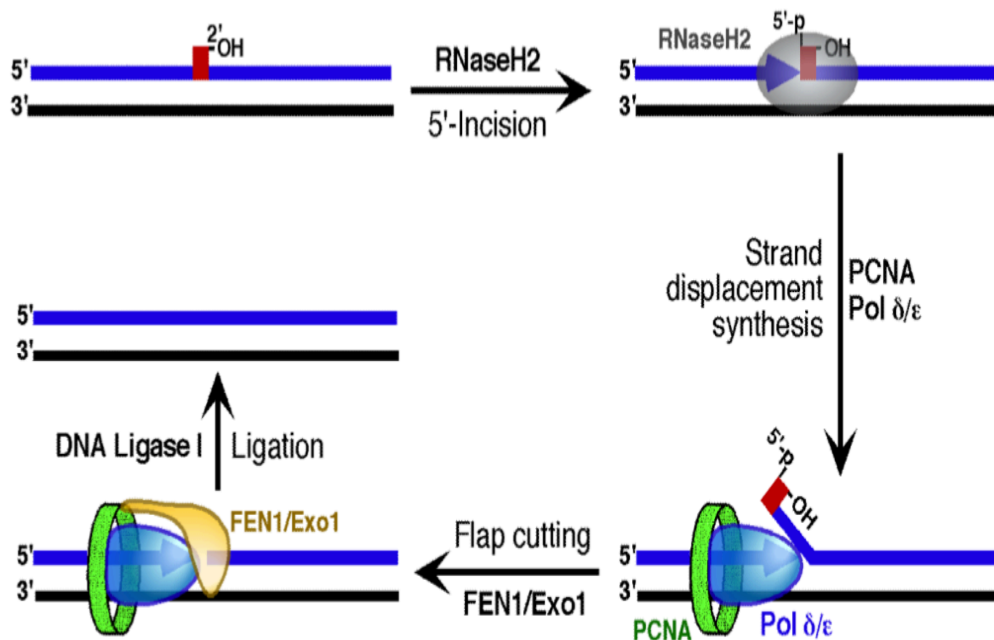
extraordinary ability to distinguish and favor deoxyribonucleotide (dNTP) selection over rNTP election, DNA polymerases can and do incorporate rNTP into DNA [38]. Under conditions where rNTP:dNTP imbalance exists in the cell, this misinsertion of rNTPs by polymerases is accelerated. Such incorporation of rNTP in DNA is expected to trigger removal and repair machinery catalyzed by Ribonuclease H2 (RNaseH2) [34]. Other sources of ribonucleotide in DNA are Okazaki fragment of RNA primers, which ascend from base pairing with nascent RNA transcript with their template [35].

RNaseH2 is a member of a family of endonucleases that cleave RNA moiety in RNA: DNA hybrids, following the reconstruction of double strand molecule [36]. Inactivation of RNaseH2 leads to accumulation of RNA/DNA hybrids that in turn activates innate immune response [37]. RNaseH2 is a complex of three subunits: RNaseH2A, RNaseH2B, and RNaseH2C; RNaseH2A contains the catalytic center, whereas RNaseH2B and RNaseH2C are more likely to interact with other proteins [38].

Mutation in RNaseH2 results in Aicardi-Goutières syndrome (AGS), which is a pediatric neuroinflammatory disorder of intrauterine viral infection [35], and has immunological similarities to autoimmune disease systemic lupus erythematous [38].

RNaseH2 recognizes single ribonucleotide in DNA, suggesting its role in removing and repairing single ribonucleotides embedded within double strand DNA [35,39]. In absence of RNaseH2, misincorporated ribonucleotides are processed by topoisomerase I (topo I), which is responsible for increasing

mutation rate in part [39]. An estimate rate of at least 1 rNTP incorporated every 7,600 nt in RNaseH2 ^{null} cells, corresponding to ~ 1,300,00 lesions per cells [38]



(Sparks et al.)[41]

Figure 1. 5 A model of Ribonucleotide Excision Repair (RER)

Ribonucleotide Excision repair (RER) is most efficient when rNTP is incised by RNaseH2 and excised by flap endonuclease FEN1 generating a single nucleotide gap that is further processed with strand displacement synthesis carried out by DNA polymerase δ, PCNA clamp, its loader RFC, and completed by DNA ligase I **(Figure 1.5)** [40, 41].

CHAPTER 2: HYPOTHESIS AND SPECIFIC AIMS

Overexpression of genes coding specific enzymes in Down syndrome (DS) as a result of trisomy 21 affects multiple interacting metabolic pathways that leads to cellular dysfunction and contributes to the unique pathogenesis and dysmorphic characteristics of the neurological, immunological, and biochemical abnormalities in DS [4,42]. Individuals with DS exhibit DNA damage accumulation, mutation accumulation and chromosomal sensitivity to mutagens [2]. In addition, Evidence accumulated indicates the inherent defect of DNA repair and BER capacity in DS [2,43]. Several studies point to the low level of DNA polymeraseβ and BER capacity associated with low level of thymidine incorporation in DNA. The integrity of the genome is challenged by the incorporation of anomalous substrates such the incorporation of ribonucleotides (rNTPs) in place of deoxyribonucleotides (dNTPs) during replication or the incorporation of uracil in DNA by spontaneous deamination of cytosine during antibody diversification induced by activity of AID gene [44,46], which is a direct target for miR-155 [10,45], Moreover, Folate depletion facilitates uracil and ribonucleotide misincorporation in DNA due to the significant role of Folate in one carbon metabolism pathway. This integration of aberrant molecules is the most frequent DNA lesions triggering different repairing mechanisms to preserve genome integrity. Furthermore, high levels of cystathionine β-synthase (CBS) alters homocystine metabolism compromising the Folate –dependent resynthesis of methionine [1,42]. The decreased availability of homocystine, which is associated with DS, promotes the well –established “Folate Trap” as a functional

Folate deficiency [1]. The fact that miR-155, and CBS genes, located on chromosome 21(HSA21) are over expressed in people with DS [1,42] leads us to investigate the effect of the excessive products of these two genes and folate depletion on DNA lesions formation and subsequent repair pathways in Lymphoblastoid cells.

We developed a model to test our hypothesis that **a mechanism derived by the high dosage of MIR155 and CBS genes present on chromosome 21 could be the underlying mechanism for reducing BER activity and thymidine incorporation in human Down syndrome lymphocytes.** This hypothesis is tested in the following Specific Aims:

Specific Aim 1: we will test whether over expression of miR-155 is associated with reduced AID gene expression in DS B-lymphocytes.

Specific Aim 2: We will test whether folate depletion reduces dNTP levels and induces RNaseH2 expression in normal B-lymphocytes.

Specific Aim 3: We will test whether gene-dosage overexpression of CBS gene is associated with RNaseH2 expression in DS B-lymphocytes.

CHAPTER 3: MATERIALS AND METHODS

A. Cell Culture:

Epstein Barr Virus (EBV) – transformed human Lymphoblastoid cell lines (LCLs) obtained from Coriell Cell Repsitories. Two types were used in this project, down syndrome (DS) and non –down syndrome (NDS). The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 growth medium containing 15% non- dialyzed Fetal Bovine Serum (FBS), 0.5% glutamine, and 1% antibiotic. The cells were grown at 37ºC and 5% CO_2 atmosphere. The NDS cells were also grown under different conditions; Folate added (FA) and Folate depleted (FD) using RPMI1640 media deficient in Folic acid at 37ºC and 5% CO_2 atmosphere. The cells were cultured for 3 passages and grown to 75 ~80% confluency before each passage.

B. RNA Isolation/ cDNA Synthesis:

Once the cells had been harvested, the total RNA was isolated using Trizol® Reagent protocols. Starting with 2µg of isolated RNA, cDNA was synthesized using random hexamer primer and reverse transcriptase. The newly synthesized cDNA was then purified using PureLink® PCR Purification Kit.

C. MicroRNA Assay:

Having been isolated and purified, RNA was reverse transcribed by stem-loop primer using TaqMan micro RNA assay®. Then, performing RT-PCR amplification. Data were analyzed by Δ CT method and presented as fold change.

D. Quantitative Rt-PCR analysis:

The expressions of the genes of interest were quantified via Real-time polymerase chain reaction using LightCycler®480 instrument (Roche, Indianapolis, IN), Light Cycler® 480 SYBR Green I Master, the resultant cDNAs, and gene- specific primers. The data were normalized to the to geometric mean of the house keeping genes: GAPDH and HPRT1 for accurate normalization and optimization [47]. All RT-PCR products were then run on 1.5 % agarose gel electrophoresis for further verification.

E. Gene cloning:

Genes were cloned using TOPO® TA Cloning® Kit, and GeneJET Plasmid Miniprep Kit or Wizard® *P*lus Midipreps DNA Purification System for plasmid isolation and purification. The sequences of the genes were confirmed via the sequencing core at Wayne state university, Applied Genomic Technology Center (AGTC), Detroit, MI.

F. Folate microbiology Assay:

Lactobacillus Casei microbiological assay were used to detect the Folate status and to confirm depletion of Folate in FD cells. *L. casei* bacteria were grown overnight in growth media supplemented with folic acid. After harvesting bacteria, the plate was setup by adding 18 µl of working buffer, which is 3.2 g sodium ascorbate + 1 M potassium phosphate buffer PH 6.1), 150µl of the single strength folic acid casei medium, the sample (1µl), then we add distilled water to adjust the total volume to 180ul. Finally, we add 20µl diluted L. casei inoculums to each well. The plate is then covered with polystyrene cover and aluminum foil,

and incubated at 37˚C for about 21hrs, and read at 595nm in the model Genios basic of TECAN-GENios plus plate reader with the software TECAN megellan v6.00. The results were analyzed using the t-test ($p< 0.05$). [48]

G. LC-MC/MC Analysis:

Intracellular nucleoside triphosphate and deoxynucleoside triphosphate was determined via Karmanos Cancer Institute, Pharmacology Core Laboratory, Detroit, MI 48120, using LC-MS/MS method SOP for preparing cell pellete samples.

H. Statistical Analysis:

Data are presented as Mean ± SEM and analyzed using (unpaired) t-test. Values of $p < 0.05$ were considered significant

CHAPTER 4: RESULTS/ FIGURES

Figure 4. 1 Relative SOD1 gene expression across LCLs (DS and NDS)

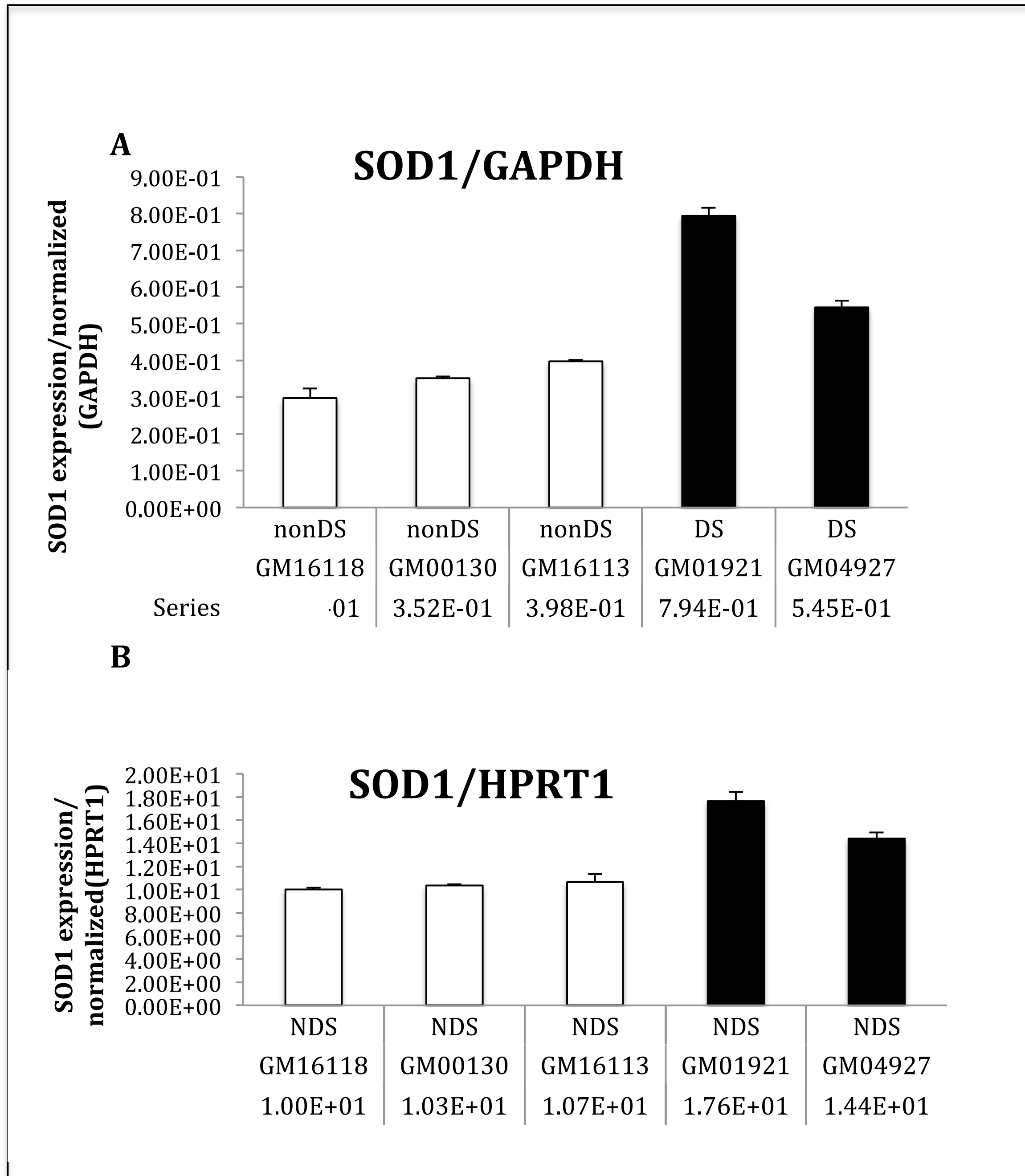
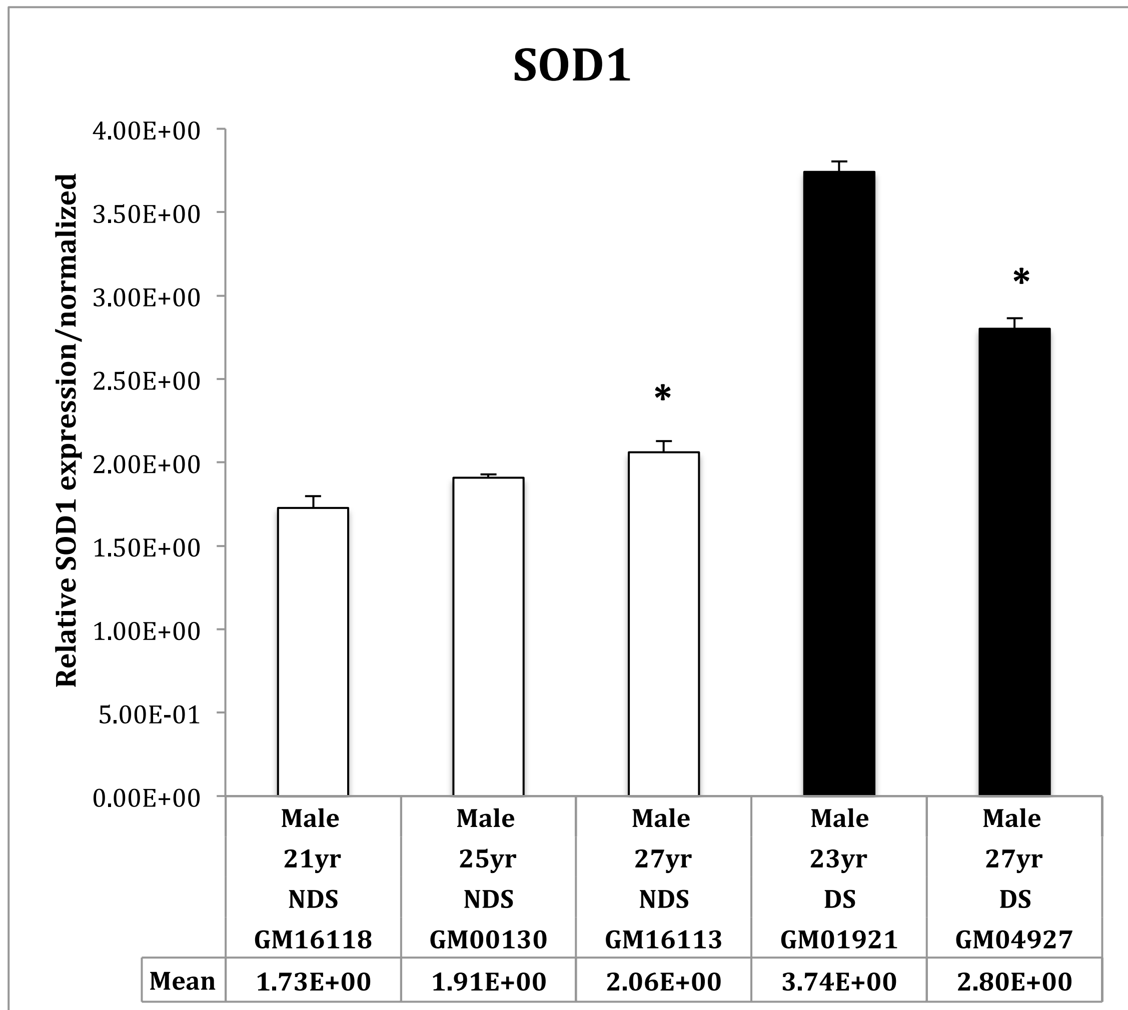


Figure 4.1: Relative SOD1 expression across LCLs (DS and NDS). RNAs were isolated from cells grown for 3 passages. SOD1 gene expression was determined by RT-PCR. Data were normalized to GAPDH (**A**), and to HPRT1 (**B**). Data were triplicated and presented as mean ±SEM.

Figure 4. 2 Selection of appropriate LCLs (DS and NDS) for the experiment.



	Male 21yr NDS GM16118	Male 25yr NDS GM00130	Male 27yr NDS GM16113	Male 23yr DS GM01921	Male 27yr DS GM04927
Mean	1.73E+00	1.91E+00	2.06E+00	3.74E+00	2.80E+00

Figure 4.2 Selection of appropriate LCLs (DS and NDS) for the experiment. SOD1 gene used as control gene is up regulated in trisomy 21. RNAs were isolated from cells grown for 3 passages. SOD1 gene expression was determined by RT-PCR. Data were normalized to the geometric mean of Housekeeping genes (HPRT1 and GAPDH) as described in the methods. Data were triplicated and presented as mean ±SEM. Cells are age matched, labeled with (*), were selected as models for our experiment.

Figure 4. 3 Elevated SOD1 gene expression in DS cells

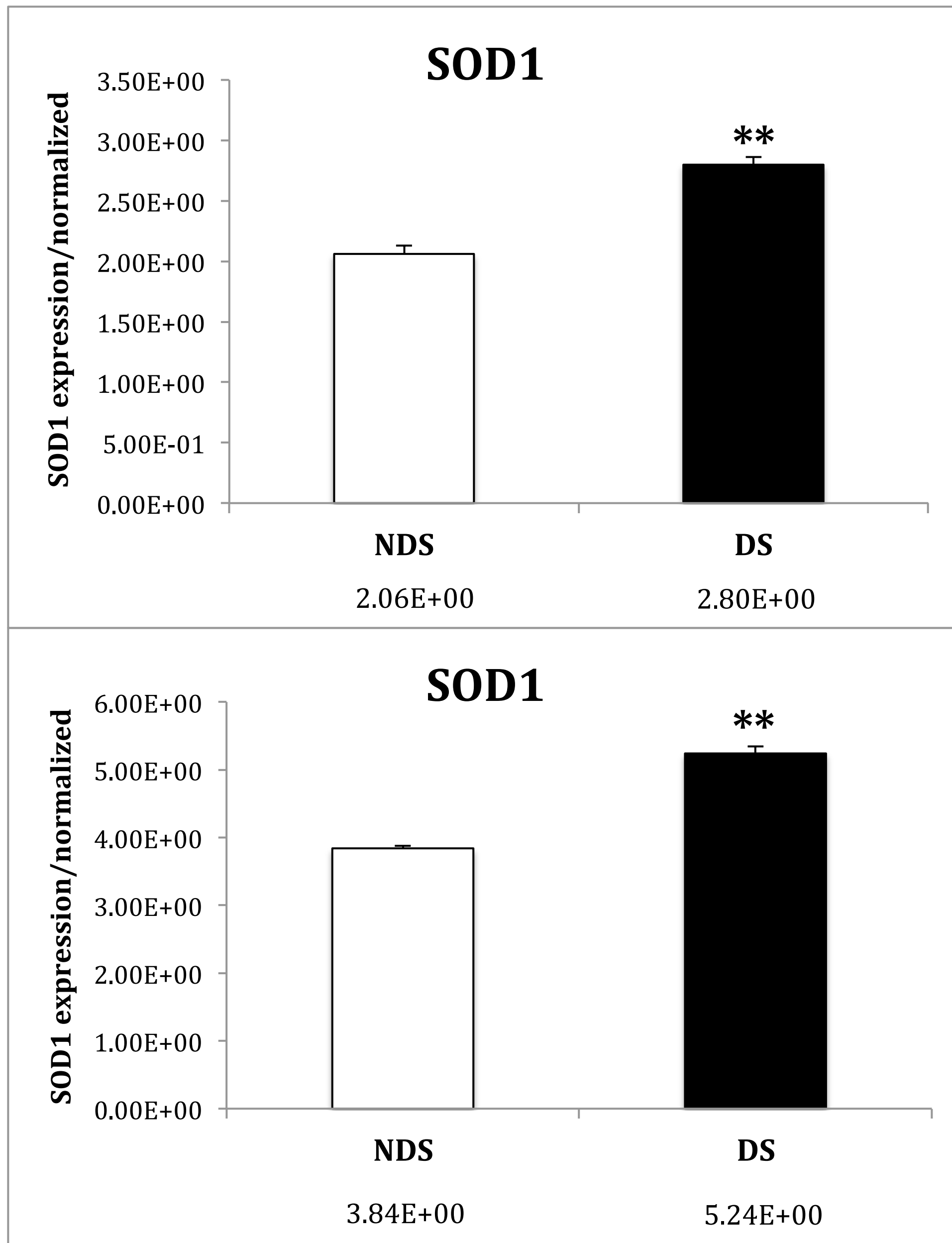


Figure 4.3: Elevated SOD1 gene expression in DS cells. RNAs were collected from 2 sets (A,B) of DS and NDS cells grown for 3 passages. Gene expression was determined by RT- qPCR . Data were normalized to the geometric mean of HPRT1 and GAPDH. Data were triplicated and presented as mean ± SEM. Statistical significant was determined via T-test. **NDS** is non-DS cell line (GM16113), **DS** is DS cell line (GM04927) .**A.** $p< 0.001$, SOD1 was elevated 1.4 Fold in DS. **B**. ***p*< 0.0001, Sod1 was elevated 1.4 fold in DS.

Figure 4. 4 MIR155 expression in DS and NDS cells

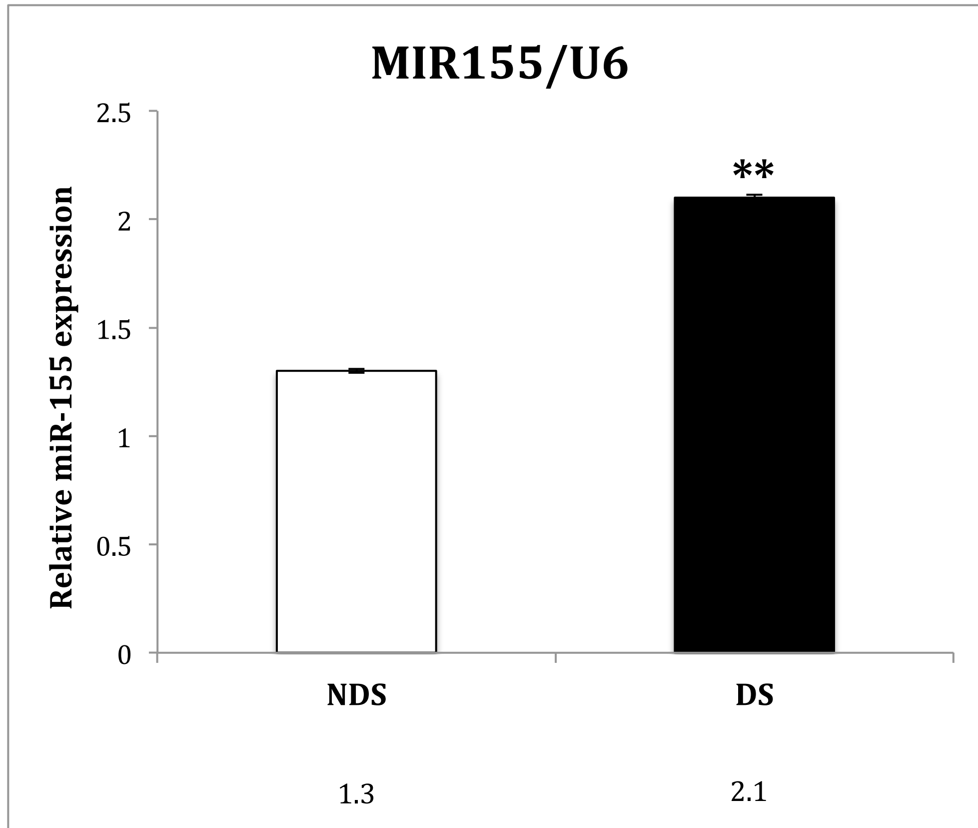


Figure 4.4: MIR155 expression in DS and NDS cells. MIR155 expression is highly expressed in DS cells. RNAs were isolated from cells grown for 3 passages. Mir-155 was transcribed as we describe in methods. MIR155 expression was determined by RT-qPCR analysis. Data were normalized to U6 gene. Data were triplicated and presented, as mean ± SEM. Statistical significant was determined via T-test. ***p*< 0.0001.

Figure 4. 5 Relative AID gene expression in DS cells

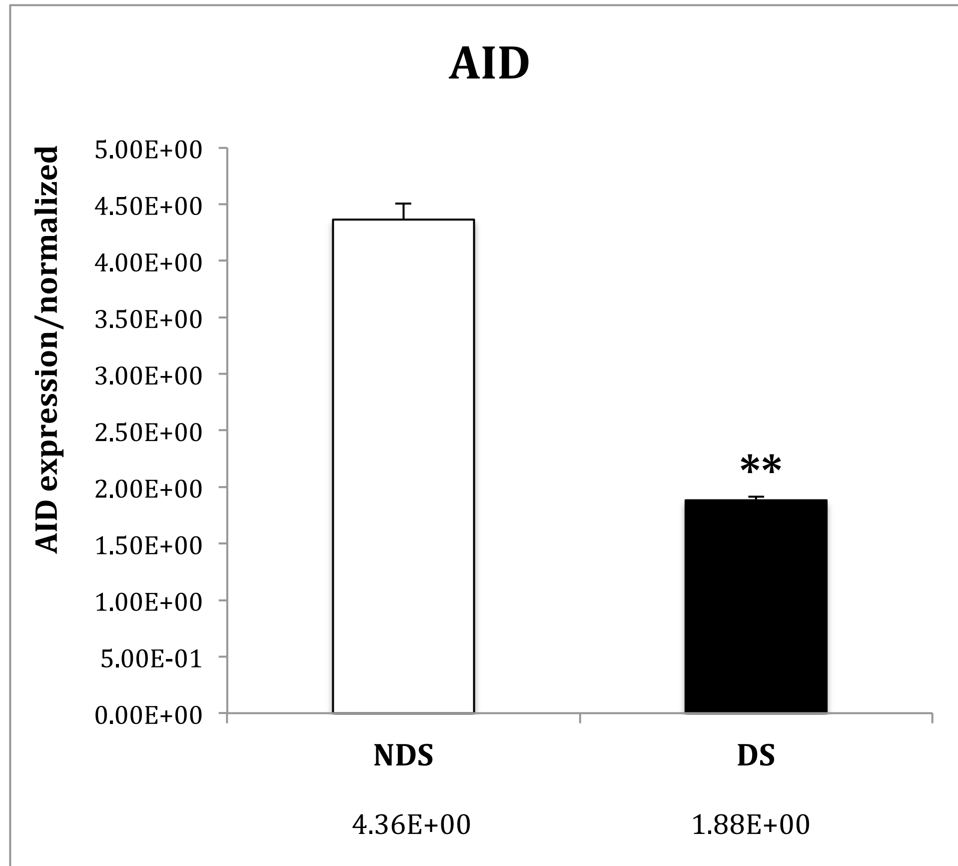


Figure 4.5: **Relative AID gene expression in DS cell**. AID gene was almost 50% depressed in DS cells. RNAs were isolated from cells grown for 3 passages. AID expression was determined by RT-qPCR analysis. Data were normalized to the geometric mean of HPRT1 and GAPDH. Data were triplicated and presented, as mean ± SEM. Statistical significant was determined via T-test. $p < 0.0001$. AID relative expression was depressed 0.43 fold in DS cells comparing to NDS.

Figure 4. 6 Relative UNG2 gene expression in DS cells.

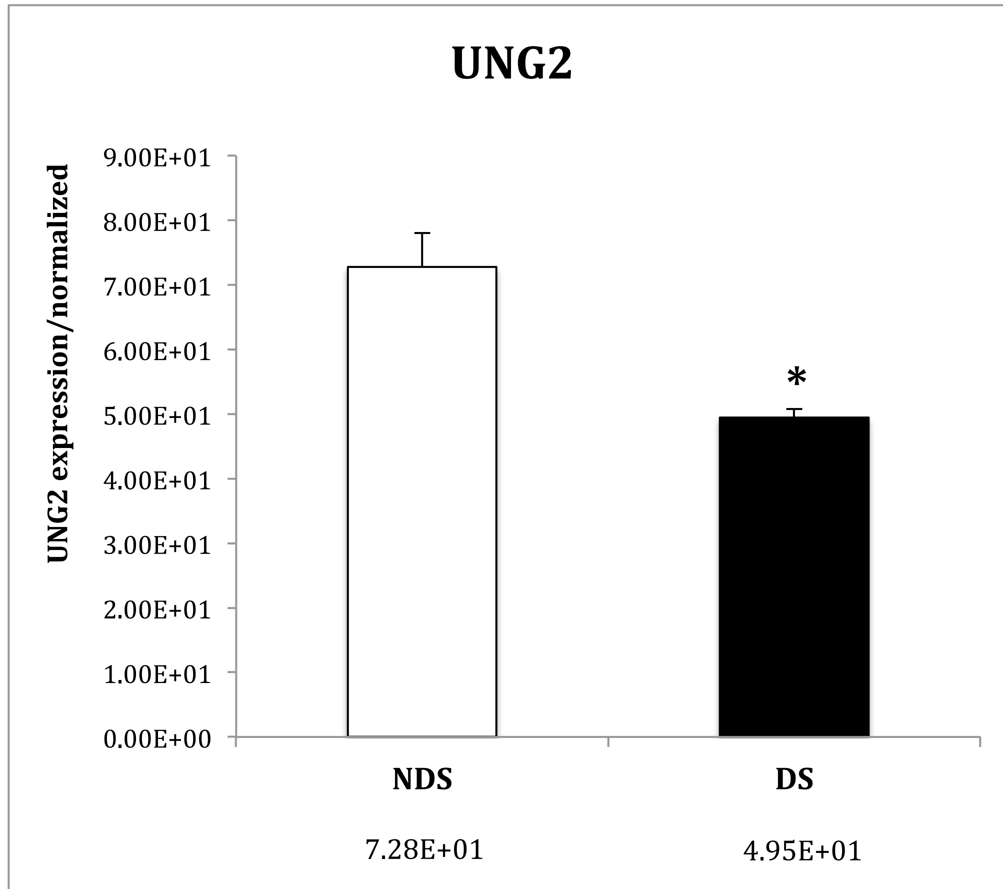


Figure 4.6: Relative UNG2 gene expression in DS cells. UNG2 was more than 50% depressed in DS cells. RNAs were isolated from cells grown for 3 passages. UNG2 expression was determined by RT-qPCR analysis. Data were normalized to the geometric mean of HPRT1 and GAPDH. Data were triplicated and presented, as mean ± SEM. Statistical significant was determined via T-test. $*p < 0.01$. UNG2 relative expression was depressed 0.68 fold in DS cells comparing to NDS.

Figure 4. 7 Relative expression of POLB gene in DS and NDS cells.

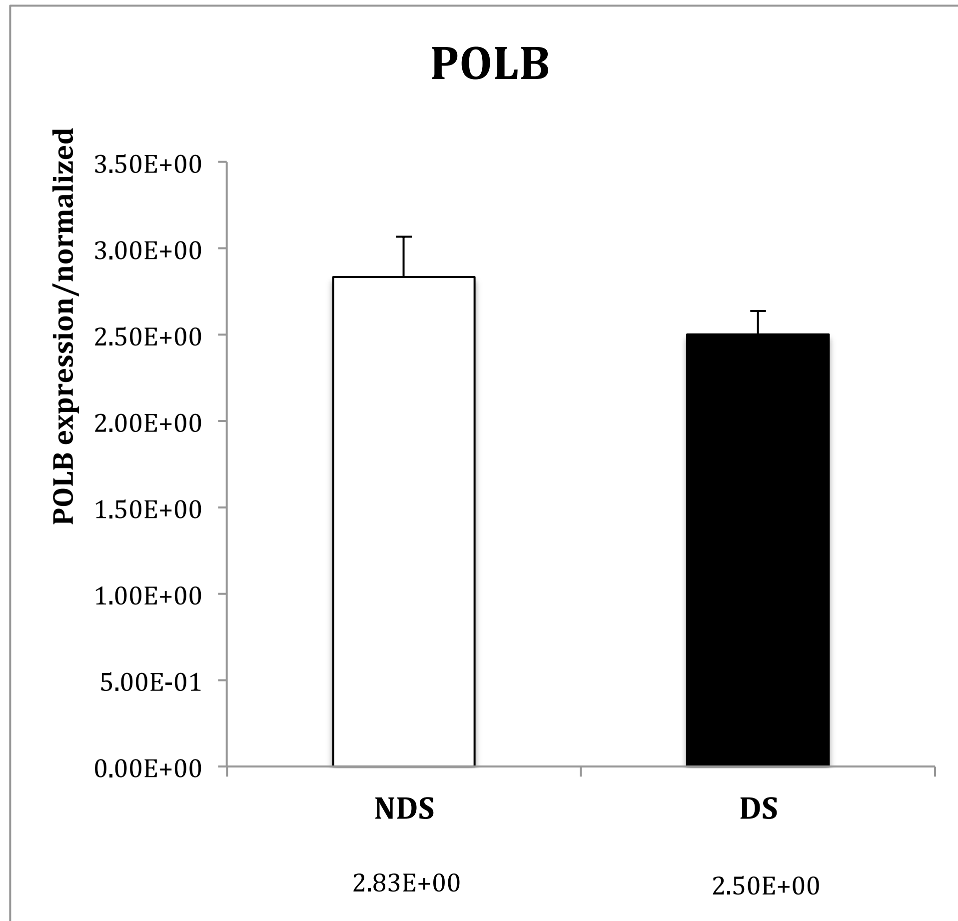


Figure 4.7: Relative expression of POLβ gene in DS and NDS cells. RNAs were isolated from cells grown for 3 passages. POLβ expression was determined by RT-qPCR analysis. Data were normalized to the geometric mean of HPRT1 and GAPDH. Data were triplicated and presented, as mean ± SEM. Statistical significant was determined via T-test. POLβ relative expression was down regulated 0.88 fold in DS cells comparing to NDS, yet does not show significant difference, T-test = 0.2959.

Figure 4. 8 Overexpression of CBS gene in DS cells

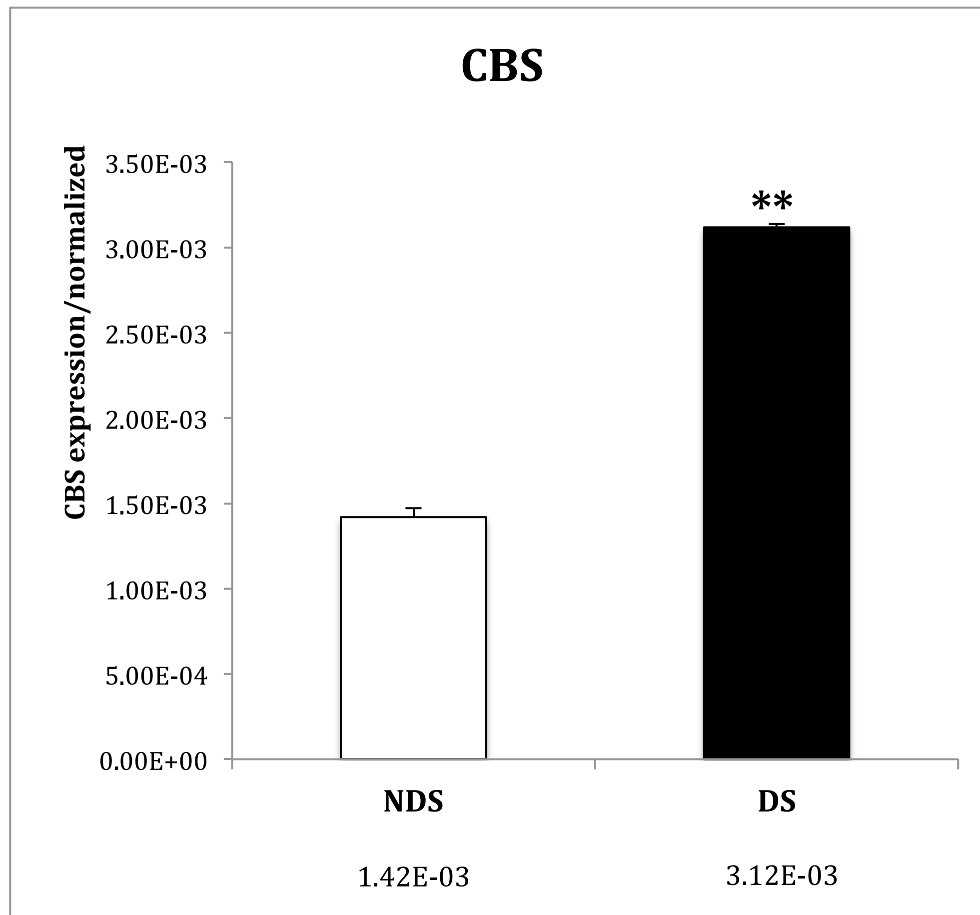


Figure 4.8: Overexpression of CBS gene in DS cells. RNAs were isolated from cells grown for 3 passages. CBS expression was determined by RT-qPCR analysis. Data were normalized to the geometric mean of HPRT1 and GAPDH. Data were triplicated and presented, as mean ± SEM. Statistical significant was determined via T-test. ***p*< 0.0001. CBS relative expression was elevated 2.2 fold in DS cells comparing to NDS.

Figure 4. 9 Relative Folate level in response to Folate depletion in normal LCLs.

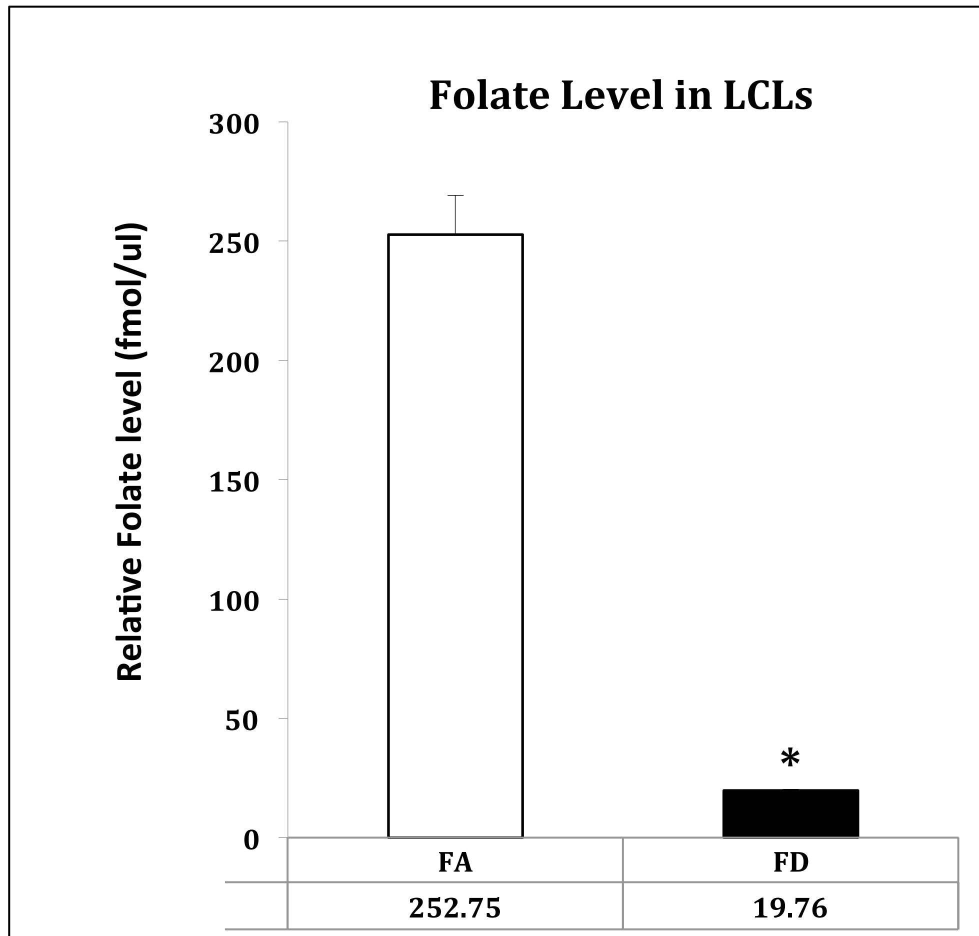


Figure 4.9: Relative Folate levels in response to Folate Depletion in normal LCLs. Cells were grown in absence and presence of Folate Absence of Folic acid in media used for FD cells deplete folate cellular level unlike FA. Folate level in normal B- cells was measured using the microbiological assay as described in methods. Data were triplicated and presented, as mean ± SEM. Statistical significant was determined via T-test (**p* value < 0.01.)

Figure 4. 10 Relatve dNTP levels in response to Folate depletion in normal LCLs.

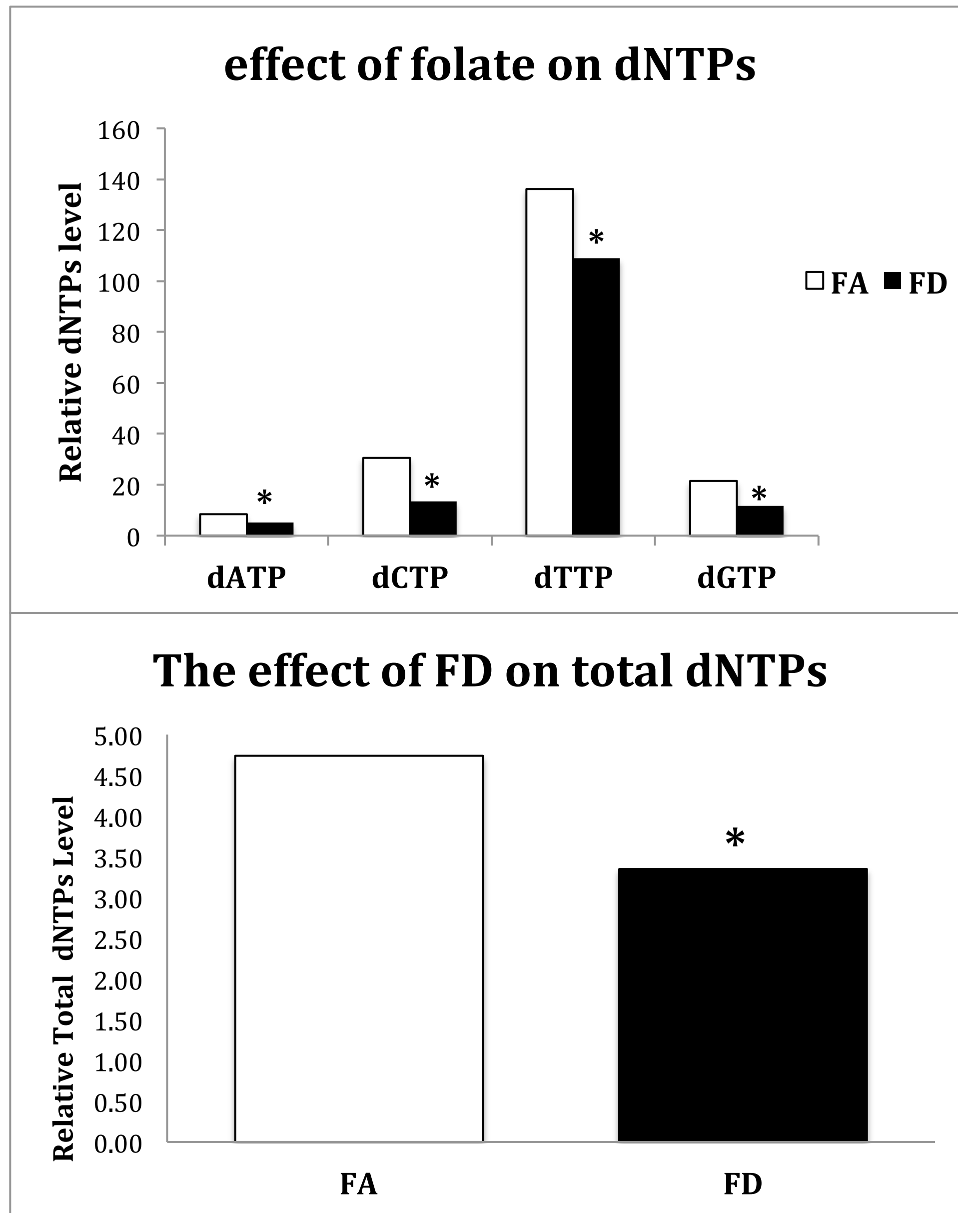


Figure 4.10: Relative dNTP levels in response to Folate Depletion in normal LCLs. Folate depletion reduced total dNTPs level. Cells were grown in absence and presence of Folate. Intercellular deoxyribonucleotides were measured by LC-MS/MS Analysis as described in methods. *p<0.05.

Figure 4. 11 Relative AID gene expression in response to Folate depletion in Normal LCLs.

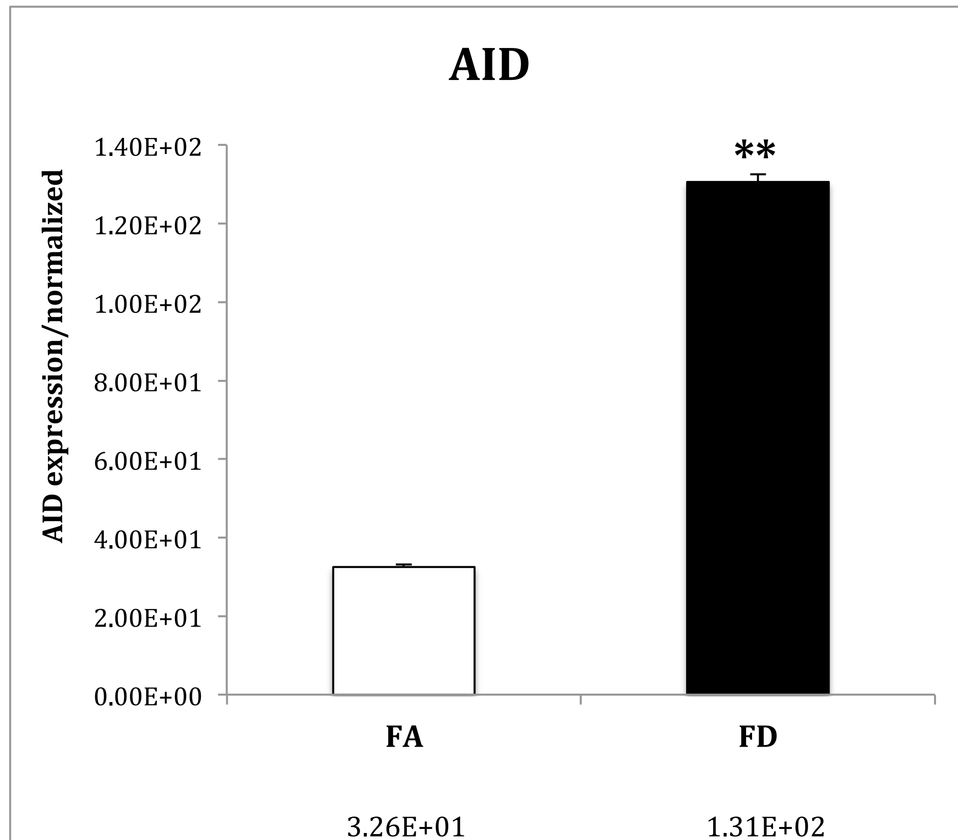


Figure 4.11: Relative AID gene expression in response to Folate Depletion in normal LCLs. Folate depletion induces AID expression. Cells were grown in absence and presence of Folate. RNAs were isolated from cells grown for 3 passages. AID expression was determined by RT-qPCR analysis. Data were normalized to the geometric mean of HPRT1 and GAPDH. Data were replicated and presented, as mean ± SEM. Statistical significant was determined via T-test. ** $p < 0.0001$. AID relative expression was elevated 4.00 fold in FD cells comparing to control FA.

Figure 4. 12 Relative UNG2 expression in response to folate depletion in normal LCLs.

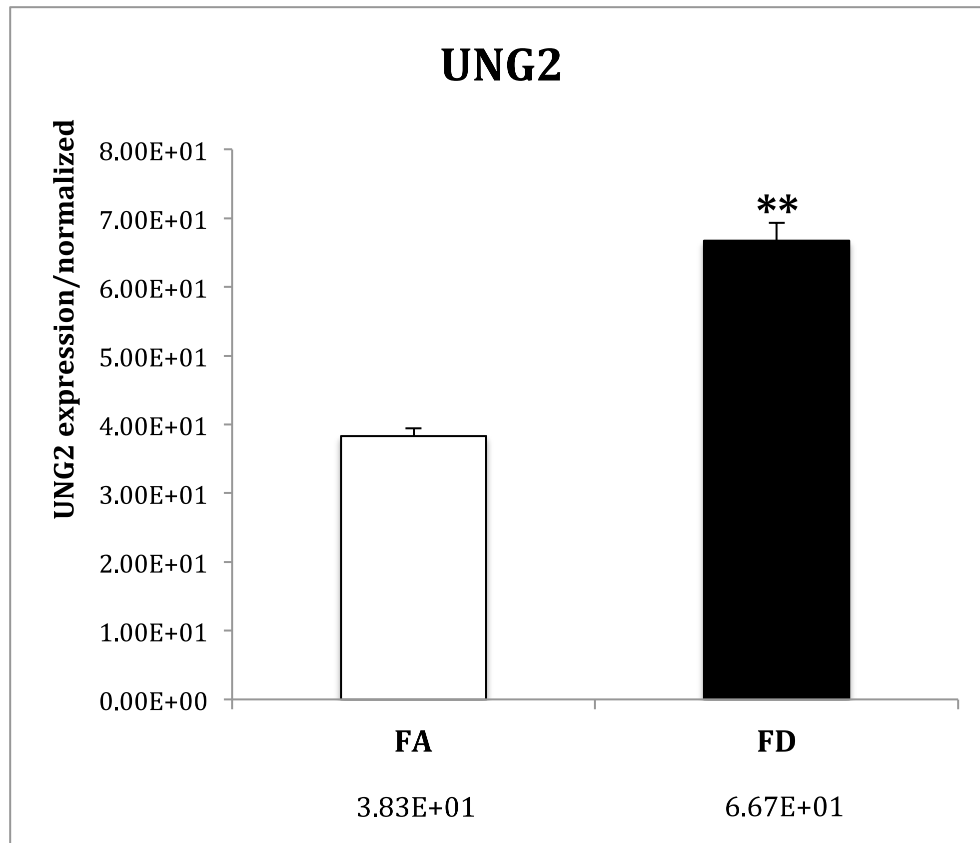


Figure 4.12:Relative UNG2 expression in response to folate depletion in normal LCLs. Folate depletion induces UNG2 expression. Cells were grown in absence and presence of Folate. RNAs were isolated from cells grown for 3 passages. UNG2 expression was determined by RT-qPCR analysis. Data were normalized to the geometric mean of HPRT1 and GAPDH. Data were replicated and presented, as mean ± SEM. Statistical significant was determined via T-test. ***p*< 0.0001. UNG2 relative expression was elevated 1.74 fold in FD cells comparing to control FA.

Figure 4. 13 Relative POLB expression in response to Folate depletion in normal LCLs

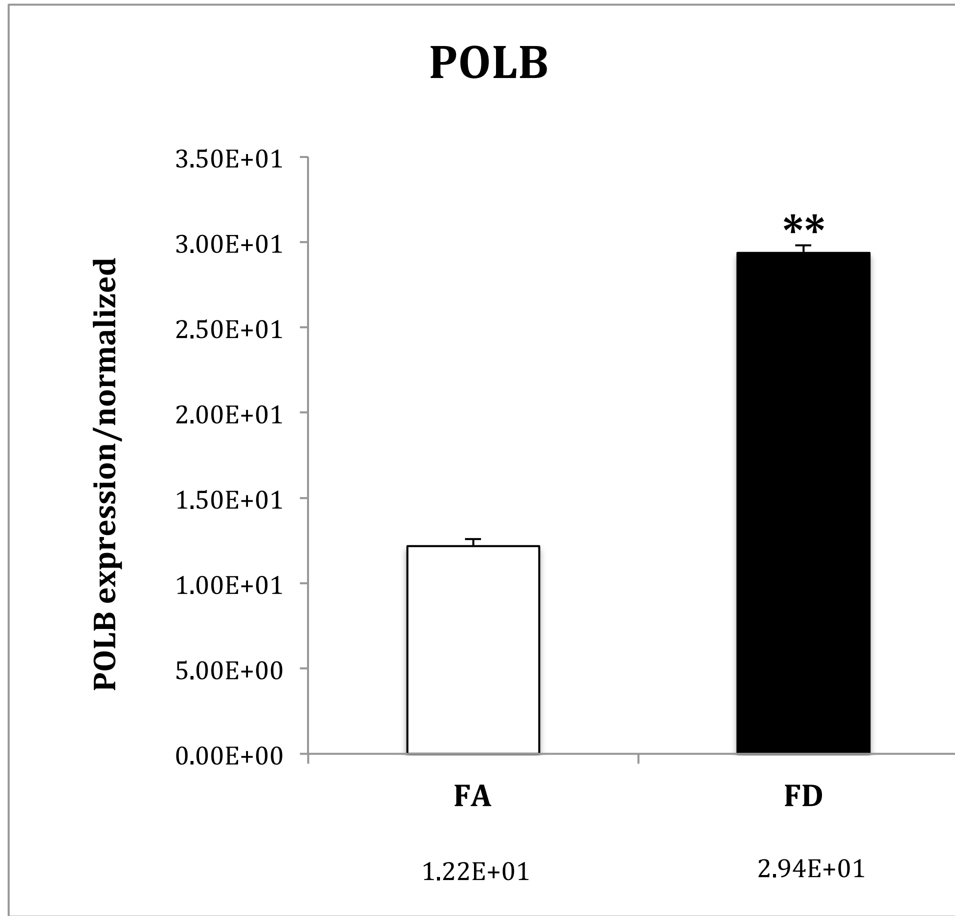


Figure 4.13: Relative POLβ expression in response to Folate depletion in normal LCLs. Folate depletion induces POLβ expression. Cells were grown in absence and presence of Folate. RNAs were isolated from cells grown for 3 passages. POLβ expression was determined by RT-qPCR analysis. Data were normalized to the geometric mean of HPRT1 and GAPDH. Data were replicated and presented, as mean ± SEM. Statistical significant was determined via T-test. **$p < 0.0001$. POLβ relative expression was elevated 2.4 fold in FD cells comparing to control FA.

Figure 4. 14 Relative RNaseH2 expression in response to Folate depletion in normal LCLs.

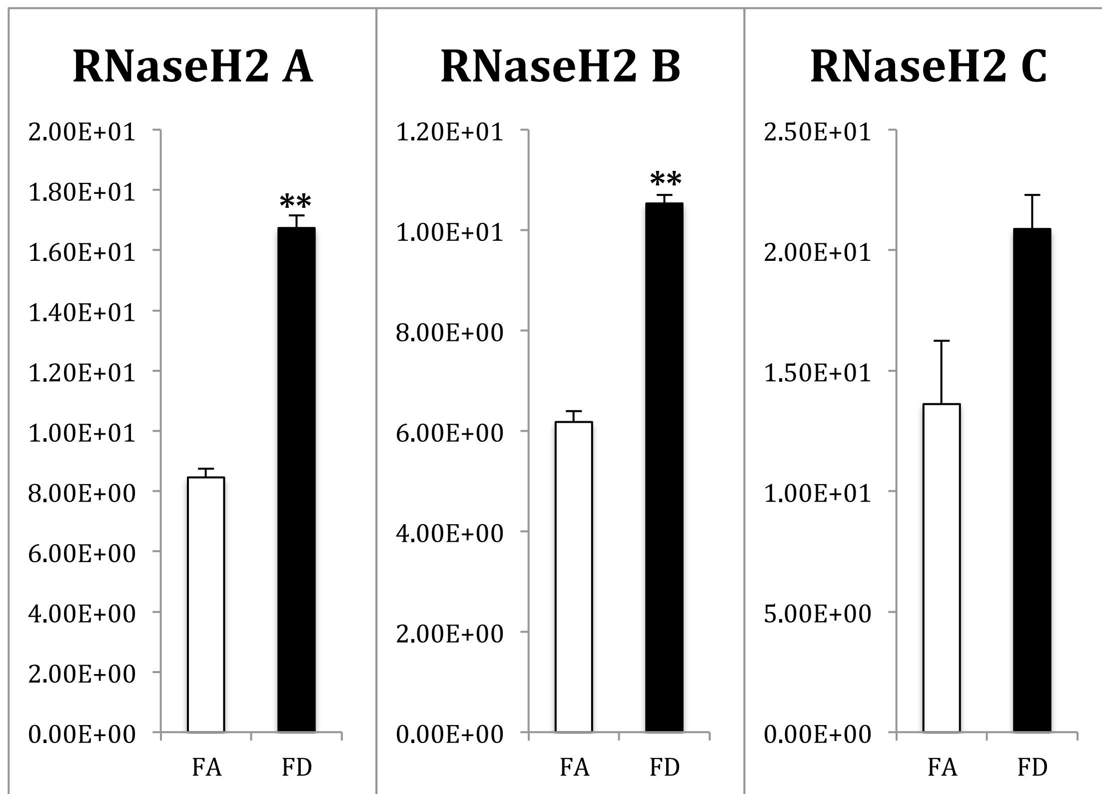


Figure 4.14: **Relative RNaseH2 expression in response to Folate depletion in normal LCLs.** Folate depletion induces RNaseH2 expressions. Cells were grown in absence and presence of Folate. RNAs were isolated from cells grown for 3 passages. RNaseH2 expressions for 3 subunits were determined by RT-qPCR analysis. Data were normalized to the geometric mean of HPRT1 and GAPDH. Data were replicated and presented, as mean ± SEM. Statistical significant was determined via T-test. RNaseH2 A relative expression was elevated 1.98 fold in FD cells comparing to control FA,** $p < 0.0001$. RNaseH2 B relative expression was elevated 1.7 fold in FD cells comparing to control FA, **$p < 0.0001$. RNaseH2 C relative expression was elevated 1.5 fold in FD cells comparing to control FA, yet not significant. T-test= 0.0756.

Figure 4. 15 Relative RNaseH2 gene expression in DS cells.

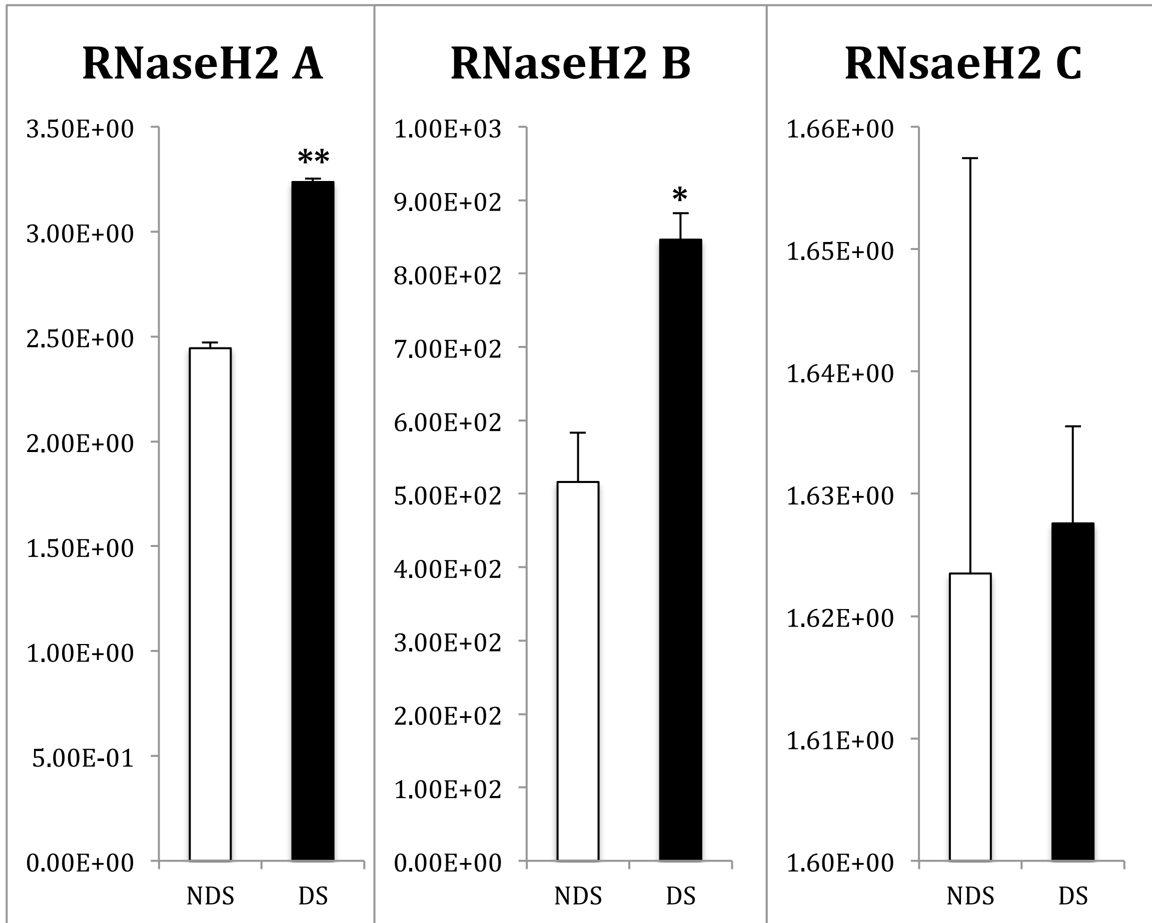


Figure 4.15: Relative RNaseH2 gene expression in DS cells. RNAs were isolated from cells grown for 3 passages. RNaseH2 expressions for 3 sub units were determined by RT-qPCR analysis. Data were normalized to the geometric mean of HPRT1 and GAPDH. Data were triplicated and presented, as mean ± SEM. Statistical significant was determined via T-test. RNaseH2 A relative expression was elevated 1.32 fold in DS, **$p<0.0001$. RNaseH2 B relative expression was elevated 1.64 fold in DS, *$p<0.01$. RNaseH2 C relative expression was elevated 1.00 fold in DS, yet was not significant, T-test= 0.9362.

Figure 4. 16 Relative Folate level in DS and NDs B cells.

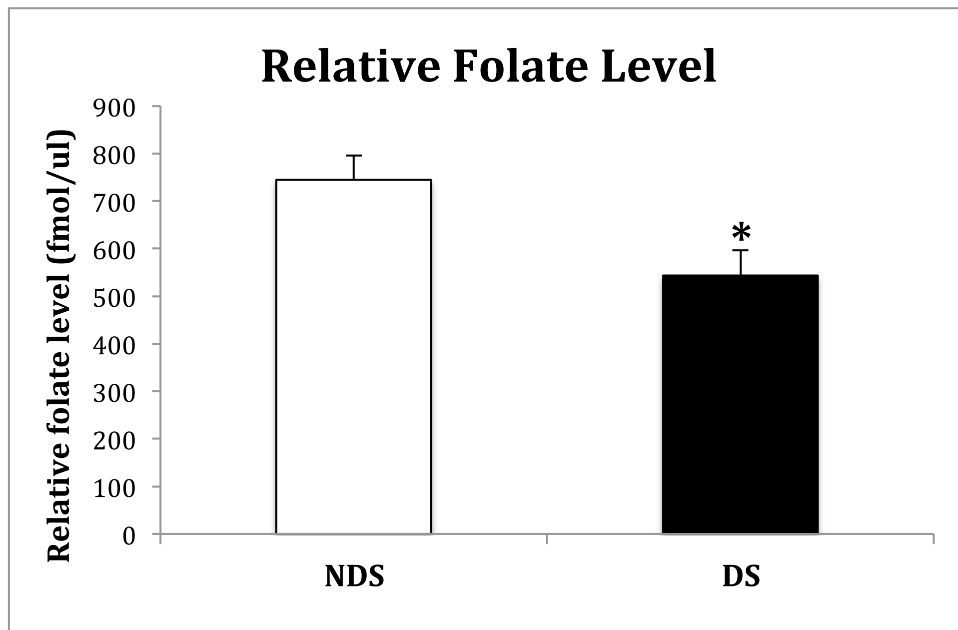


Figure 4.16: Relative Folate level in DS and NDS B cells. Cells were grown in presence of Folic acid in media. Folate level in B cells was measured using the microbiological assay as described in methods. Data were quadruplicated and presented, as mean ± SEM. Statistical significant was determined via T-test (*p value < 0.05). DS cells were lower in total folate level by 0.76 fold in compared to NDS cells

CHAPTER 5: DISSCUTION

We screened SOD1 gene relative expression in five different cell lines; three non Down syndrome and two Down syndrome, in the purpose of selection good representative models for our experiments. We chose cell lines that were age-matched, and that overexpressed MIR155 in an appropriate dosage relative to gene triplication. Also, we normalized our data for two different valid housekeeping genes that are B-lymphocyte-specific, GAPDH and HPRT1, in order to find best reference gene as in **Figure 4.1**. Sod1 gene that encodes Superoxide dismutase 1 protein is localized on chromosome 21, and due to the fact that DS result in an extra copy of this chromosome, SOD1 is overexpressed in DS. While not all genes on Chromosome 21 exert gene-dosage effects in response to Trisomy 21, SOD1 is known to exert this gene dosage effect. As shown in **Figures 4.1** and **4.2**, we can truly see that these cell lines are appropriate lines to evaluate for DS phenotypes due to the elevation level of SOD1 over 1.4 fold compared to the NDS lines. We chose to move forward with analyses in two lines that were age matched as age-dependent effects of DS have been well-described. Further we normalized our data to the geometric mean of the previously selected house keeping genes, GAPDH and HPRT1 according to Vandesopmele *et al*. [47] that using a single gene for normalization leads to relatively large error in significant proportion samples tested, and that using the geometric mean, not the arithmetic mean, of multiple carefully selected house keeping genes was validated as an accurate normalization factor.

Figure 4.3 represents the significant differences level of Sod1 gene in DS cells comparing to NDS, and this observation was repeated for further verification. We can see that Sod1 gene was elevated 1.4 fold in DS cells, and this is consistent with previous experiments that reported that there is a significant increase in Sod1 activity between 1.4 and 1.8 times higher in trisomic cells than normal lymphocyte cells [3]. This is consistent with the anticipated gene-dosage effect, based on the presence of 3 copies of SOD1 in lieu of the normal 2 copies.

From Figure 4.4, the relative expression of miR-155 gene, which was measured using Δ Ct method as descried in methods, was elevated significantly in DS cells more than 1.7 fold, demonstrating a gene dosage effect for miR-155 due to location on chromosome 21 as well. Also, there is evidence that miR-155 is triplicated and over expressed in in Ts65Dn mouse model of Down syndrome as well [15].

AID gene is a direct target of miR-155, so the expression level of AID gene was examined in order to evaluate whether the high dosage of miR-155 in DS cell has an impact on AID expression. From **Figure 4.5**, AID gene was almost 50% depressed as compared to control cells, and the high level of miR-155 could explain this observation. This finding was consistent with previously reported data that AID gene is subject to post-transcriptional regulation by miR-155, and the main role of miR-155 in controlling the germinal center of B cell is due in part by repression of AID expression [10,13].

As miR-155 is considered a negative regulator of AID gene, this would lead us to anticipate that the lesions formed by AID activity are reduced, at least in part, so that the enzymes participating in excision and repairing these lesions, such as Uracil DNA Glycosylase (UDG) encoded by UNG2 gene, would be down regulated as well. Our data from **Figure 4.6** confirm this hypothesis. UNG2 gene relative expression was significantly depressed more than 50% in DS cells comparing to the control cell, and this finding confirm also the reports that indicate UDG is specifically processes U: G lesions raised upon the activity of AID gene [49].

Once UDG initiates BER by excision of the altered base and creation an abasic site, its activity catalyzes subsequent enzymes to repair the abasic site formed, via the base excision Repair (BER) pathway. Of these enzymes, DNA Polymerase β (POLβ) is of the utmost importance since it is the rate limiting enzyme for BER and its activity is error –free repair, short-patch repair, so it was important to investigate the level of expression of this enzyme. **Figure 4.7** shows us that there was a trend of more than 50% down regulation of POLβ gene that encodes DNA polymerase β, yet this reduction was not statically significant as determined by T-test.

CBS gene located on chromosome 21, which encodes cystathionine β-synthase, is over expressed in trisomy 21. A 157% increase in CBS enzymes activity has been previously documented in individuals with DS [4,42]. **Figure 4.8,** we can see that the extra copy of chromosome 21 on DS accounts for the

elevation level of CBS gene expression more than 2.2 fold comparing to the non DS cells.

The elevation expression of CBS gene induces the so-called “Folate trap“, which induces a functional folate deficiency. The mechanism underlying the folate trap stems from the low level of homocytocine in DS as a result of elevated activity of cystathionine β-synthase, which converts homocysteine to cystathionine promoting accumulation of 5-methylterahydrofolate (5-MTHF) [1], which an essential precursor to drive the one carbon metabolism pathway. Upon the functional folate deficiency derived from CBS overexpression, we hypothesized that will be an imbalance in ribonucleotide/ deoxyribonucleotide (rNTP/dNTPs) pool. This imbalance facilitates the misincorporation of ribonucleotide in DNA inducing high level of RNaseH2 activity, which is responsible for removing the misincorporated ribonucleotide and initiating Ribonucleotide Excision Repair RER [38]. From **Figure 4.15**, we can report an overexpression of all three subunits of this enzyme (A,B,and C) in DS cells . However, it was only significant increase for subunit A and B with 1.3 and 1.64 fold increase, respectively while it was not statically significant for subunit C. **Figure 4.16**, we can see that Folate level was significantly lower in DS cell in compared to NDS cell even though both cell lines were grown in media supplemented with Folic acid. Interestingly, another study showed an increase of CBS gene in folate-depleted cells [50].

Since we proposed the impact of folate trap induced by the hyper activity of CBS, it is crucial to examine the impact of folate depletion on normal

lymphocyte cells. **Figure 4.9** and **Figure 4.10** show that folate depletion depletes the intercellular level of folate and reduces dNTP levels in normal B-lymphocytes. This finding from Thomas Kunkel's lab that rNTP misincorporation is more likely to happen when cellular concentrations of dNTPs are low [51]. This reduction of dNTPs affects the balanced ratio of dNTPs:rNTPs causing more opportunity for ribonucleotides to be misincorporated in the DNA, which would hypothesized to trigger RNaseH2 activity for removal and repair. RNaseH2 is multimeric compromised of three subunits, A, B, and C, and the loss of any of three subunits results in reduced activity of RNaseH2 [37,38]. It has been found that ablation of RNaseH2B in mice causes replication stress, embryonic deadliness, and micronuclei creation [38]. **Figure 4.14**, we see a significant increase in RNaseH2 gene expression in both A and B subunits with 1.9 and 1.7 fold, respectively, in folate depleted cells FD comparing to the Folate added cells FA; however, there was no statically significant difference for subunit C even though the expression was 1.5 fold higher FD cells than FA cells. This elevation of RnaseH2 expression was an indicator of ribonucleotide misincorporation in DNA that triggers RNaseH2 activity for removal and repair by RER pathway [38,41].

As we mentioned previously, DNA damage that occurs upon AID gene activity of deamination of cytosine to uracil trigger UDG activity to remove the altered base recruiting BER protein for further repair of the damage. We examined the effect of folate depletion on BER capacity subsequent the DNA damage by measuring the expression of AID, UNG2, and POLβ genes. From **Figure 4.11 and Figure 4.12,** Folate depletion significantly induces AID activity

by 4 fold higher in FD cells than FA cells, and induces UNG2 significantly in FD cells more than 1.7 fold as well. Also, from **figure 4.13**, Polβ expression was elevated 2.4 fold in FD cells comparing to FA cells. These observations could be explained by the role of folate depletion in inducing uracil misincorporation in lymphocyte DNA [61], which in turn triggers UDG for further removal of uracil and then repairing the abasic site by subsequent BER mechanism. This explanation could be applied to the increase level of POLβ expression in response to Folate depletion. It appears that centroblast B cells have evolved a mechanism to suppress Polβ activity where the mutation is required for antibodies development during Somatic hypermutation SHM since POLβ is error free repair polymerase. However, POLβ is highly required in the rest of the genome for faithful repair. AID is inducing mutation in many genes; mutations induced in non-Ig genes are faithfully repaired in an error-free way [18].

CHAPTER 6: CONCLUSION

Many studies have explored the impact of an extra copy of chromosome 21 on DNA repair capacity in DS, and some of these studies point out to the low level of DNA Polymerase β (POLβ), which is a rate limiting enzyme for BER activity, associated with low incorporation of thymidine, an indicator of decrease DNA synthesis and cell proliferation [35,43,52]. In our study, we investigate more to propose a mechanism of folate deficiency and POLβ inhibition derived from high dosage of MIR155 and CBS genes by which explains the reduced activity of BER and thymidine incorporation in DS lymphocytes. In DS cells, our data show that there was a reduction in AID gene expression associated with high expression level of MIR155 gene; AID is a direct target of MIR155 in B-lymphocytes. AID low expression was also associated with low expression level of UNG2 and POLβ; albeit, the down regulation of POLβ is not statically significant; we might see significant effect with time as other studies indicate the progressive reduction of Polβ over time so that we need more investigation to see the effect of MIR155 via AID on POLβ directly. However, in normal lymphocytes undergoing folate depletion (FD), AID, UNG2, and POLβ were all significantly overexpressed. Even though reasonable due to the high level of UNG2, POLβ high expression was dislike many reports that indicate low POLβ expression in response to FD, which might be specific for Lymphoblastoid cells. A notable finding that overexpression of AID in response to FD could be related to AID activity as demethylation factor for 5-methyl –cytosine where its activity is associated with hypomethylation condition [53], which known to be associated

with folate deficiency [54]. Also, FD depleted dNTPs pool which in turn impaired dNTP:rNTP pool, and is hypothesized to induce RNaseH2 activity as repair mechanism. Interestingly, we find an increase in RNaseH2 activity in DS lymphoblastoid cells, which may indicate ribonucleotide misincorporation in DNA in DS, and which may be related to the overexpression of CBS gene, further connecting FD to DS phenotypes. MIR155 over expression and Folate depletion in DS may cooperate to alter dNTP levels and facilitate rNTP misincorporation in DNA could explain the low BER capacity and low thymidine incorporation in DS lymphocytes. However, more in depth investigations are needed to prove this mechanism; studying the impact of DS on enzymes and substrates involved in folate one-carbon metabolism, and studying the direct effect of MIR-155 on POLβ by directing AID could revels a lot about this proposed mechanism and resolve the question about low BER in DS.

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ABSTRACT

THE IMPACT OF DOWN SYNDROME AND FOLATE DEPLETION ON GENOMIC STABILYZING PATHWAYS OF LYMPHOBLASTOID CELLS

by

KHADIJAH ALNABBAT

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Understanding the role of nutrition plays in Down syndrome (DS) could help in the development and implementation of strategies that help overcome the negative consequences phenotypes of Down syndrome. Conserving genome integrity is crucial for cells to survive, and thus understanding how genetic defects induce damage to genomic DNA and impair subsequent repair of this damage is important. Evidence accumulated points to increased DNA damage and mutation accumulation associated with a decline in DNA repair capacity, Base Excision Repair (BER) in particular. Thus, the successful clinical management of DS resides in understanding the metabolic imbalance provoked by overexpression of genes on chromosome 21. In our study, we investigate more to propose a mechanism of folate deficiency and POLβ inhibition derived from high dosage of MIR155 and CBS genes, which we hypothesized to provoke some kind of metabolic imbalance in DS by which may explain the reduced activity of BER and reduced thymidine incorporation in DS lymphocytes.

AUTOBIOGRAPHICAL STATEMENT

Khadijah Ibrahim Alnabbat

2008 : Bachelor in Nutrition and Food science, King Faisal University, Kingdom of Saudi Arabia.

2009: A faculty member in King Faisal University, Kingdom of Saudi Arabia.

2014: Master of Science in Nutrion and Food Science, Wayne State University, Detroit, USA