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Investigating The Role Of Dna Polymerase Beta In The Aging Phenotype Of Down Syndrome

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**INVESTIGATING THE ROLE OF DNA POLYMERASE BETA IN THE AGING
PHENOTYPE OF DOWN SYNDROME**

by

AQILA A. AHMED

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2016

MAJOR: NUTRITION AND FOOD SCIENCE

Approved By:

Advisor	Date
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_____	_____

DEDICATION

I dedicate this thesis to my amazing little boy, Jacob.

Our passion for learning ... is our tool for survival. -Carl Sagan

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TABLE OF CONTENTS

Dedication ii

Acknowledgments iii

List of Tables vi

List of Figures vii

List of Abbreviations ix

Chapter 1: Introduction

 A. Down syndrome (DS) 1

 B. Senescence/premature aging 3

 C. Oxidative stress 5

 D. DNA repair capacity 8

 F. Micro-RNA 155 13

Chapter 2: Loss of DNA Polymerase β Induces Senescence: Implications in Down Syndrome

 A. Summary 20

 B. Intro 21

 C. Methods And Materials 23

 D. Results 25

 E. Discussion 28

Chapter 3: Regulation of DNA Polymerase β by miR-155

 A. Summary 39

 B. Intro 40

 C. Methods and Materials 41

D. Results.....	47
E. Discussion.....	50
CHAPTER 4: Ts65Dn Mouse Model	
A. Summary.....	61
B. Intro.....	61
C. Methods and Materials	69
D. Results.....	71
E. Discussion.....	74
Chapter 5: Discussion And Future Direction	101
References	106
Abstract.....	135
Autobiographical Statement	136

LIST OF TABLES

Table 1.2: Pathophysiological and cellular criteria of ageing.....	16
Table 1.3: Genes on chromosome 21 implicated in oxygen metabolism.....	17
Table 2.1: Quantitative RT-PCR Primer sequences.....	31
Table 3.1: Primer sequences	53
Table 3.3.5 Validated miR-155 targets shown in our dataset	59
Table 3.3.6: Impact of miR-155 overexpression on proteins involved in DNA repair	60
Table 4.2: Summary of segmental trisomy mouse models and features of Down Syndrome that have been documented in each model.....	76
Table 4.3: Summary of transgenic Mouse models.....	77
Table 4.4: Primer sequences	78
Table 4.7.1: Summary of Segmental trisomy Mouse models.....	100

LIST OF FIGURES

Figure 1.1: Nondisjunction	16
Figure 1.4: Base Excision Repair pathway.....	19
Figure 2.1: Primary Down syndrome fibroblasts exhibit gene dosage effects for oxidative stress response genes on chromosome 21	32
Figure 2.2: Premature cellular senescence in Down syndrome fibroblasts. A. Down syndrome fibroblasts exhibit a premature senescence phenotype. B. Down syndrome fibroblasts exhibit an amplified senescence response to hydrogen peroxide and HU	33
Figure 2.3: Primary Down syndrome fibroblasts exhibit reduced DNA Polymerase β transcript abundance.....	34
Figure 2.4: Homozygous loss of DNA polymerase β induces premature cellular senescence. A. Loss of DNA polymerase β is sufficient to induce senescence. B. DNA polymerase β null mouse embryonic fibroblasts exhibit an amplified senescence response to hydroxyurea.....	35
Figure 2.5: DNA polymerase β protects cells from hydroxyurea-induced DNA double strand breaks. A. DNA polymerase β is upregulated in response to hydroxyurea. B. Loss of DNA polymerase β induces DNA double strand breaks	36
Figure 3.1: Proposed model of Pol β by miR-155	39
Figure 3.2 SILAC-based quantitative proteomics. Flowchart of double SILAC coupled with LC-MS/MS	54
Figure 3.3.1: Ectopic overexpression of miR-155 in MEFs	55
Figure 3.3.2: Validation of C/EBP β downregulation in miR-155	56
Figure 3.3.3: Pol β transcript and protein expression is downregulated in miR-155 Cells	57
Figure 3.3.4: Pol β Promoter activity is inhibited by miR-155 overexpression.....	58
Figure 4.1: Major Mouse Models of Trisomy 21	76
Figure 4.6.0: Ts65Dn are smaller than WT controls.....	79
Figure 4.6.1: Liver wet weights in Ts65dn (DS) and WT (NDS)	80

Figure 4.6.2: Weight of Lung in three cohorts	81
Figure 4.6.3: Ts65Dn mice have underdeveloped Testes	82
Figure 4.6.4: Kidney weight did not differ	83
Figure 4.6.5: Ts65Dn heart is smaller than WT	84
Figure 4.6.6: No difference was seen in Brain weight	85
Figure 4.6.7: PolB expression in Ts65Dn mice tissues at 16 week	86
Figure 4.6.8: PolB expression in Ts65Dn mice tissues	87
Figure 4.6.9: PolB expression in Ts65Dn mice tissues	88
Figure 4.6.10 : PolB expression in Ts65Dn Skin	89
Figure 4.6.11: p16 expression in Ts65Dn Tissues	90
Figure 4.6.12 : p16 expression in Ts65Dn Tissues	91
Figure 4.6.13: p16 expression in Ts65Dn Tissues	92
Figure 4.6.14: p16 expression in Ts65Dn Tissues	93
Figure 4.6.15: p16 expression in Ts65Dn Tissues	94
Figure 4.6.16: p16 expression in Ts65Dn Tissues	95
Figure 4.6.17: Incidence of lymphoma in Ts65Dn Spleen	96
Figure 4.6.18: Incidence of malignant tumors of lung in Ts65Dn	97
Figure 4.6.19: Incidence of lymphoma in stomach of Ts65Dn	98
Figure 4.6.20: Incidence of lymphoma in Intestine of Ts65Dn	99
Figure 5.1.1: The proposed mechanism by which miR-155 regulates PolB	104

LIST OF ABBREVIATIONS

DS-Down syndrome

HSA21- Human chromosome 21

miR-microRNA

SOD1- superoxide dismutase 1 (Cu-Zn

BACH1- BTB Domain And CNC Homolog 1)

PolB/POLB- DNA polymerase beta

Creb1/CREB1- Cyclic AMP-responsive element-binding protein 1

MMS- methane methylsulfonate

BER- Base excision repair

RT-PCR- real time polymerase chain reaction

MEF- mouse embryonic fibroblast

WT- wild type

C/EBP β - CCAAT/enhancer-binding protein beta

AD- Alzheimer disease

ROS- reactive oxygen species

NAD- Nicotinamide adenine dinucleotide

SA- β -gal -Senescence-Associated β -Galactosidase

APP - β -amyloid precursor protein

TTFs -terminal-tip fibroblasts

CAT -catalase

GPx -glutathione peroxidase

HO-1-downregulates heme oxygenase-1

UDG - uracil DNA glycosylase

APE1- Apurinic/apyrimidinic endonuclease

XRCC1- X-Ray Repair Complementing Defective Repair In Chinese Hamster Cells 1

PCNA - Proliferating cell nuclear antigen

PARP1- Poly(ADP-Ribose) Polymerase 1

SP-BER -short patch BER

MNNG- Methylnitrosoguanidine

MN- micronucleus formation

DSB- double strand breaks

SSB- single strand break

DSCR1-Down Syndrome Critical Region 1

BrdU- 5-bromo-2'-deoxyuridine

DDR -DNA damage response

MMR- Mismatch repair

HR – homologous repair

NHEJ- non-homologous end joining

FOXO3a- Forkhead box O3 a

CHAPTER 1: INTRODUCTION

A. Down syndrome (DS)

Down syndrome (DS) is one of the most common genetic disorders in the US, with 400,000 Americans living with DS and 6,000 babies born with DS each year (S. E. Parker et al., 2010). DS is a condition in which the affected individual has a full or partial extra copy of chromosome 21 (Trisomy 21). John Langdon Down first described the condition in 1866, while the underlying cause for it was discovered by Jerome Lejeune in 1959. Ninety five percent of DS cases are caused by an error in cell division during either meiosis I or meiosis II, when an embryo receives three copies of chromosome 21 instead of two. This meiotic division error is commonly known as trisomy 21, a form of chromosomal nondisjunction. The extra chromosome is then replicated in every cell of the body unlike the mosaic trisomy (5%), where triplicated Hsa21 is only found in certain tissues. Chromosome 21 is the smallest human chromosome, spanning about 48 million base pairs and representing 1.5 -2% of the total DNA in a cell. Chromosome 21 contains 200 - 300 known protein-coding genes involved in a wide range of processes.

Individuals with DS suffer from both physical and cognitive disabilities (Table 1.1). Common conditions seen in people affected with Down syndrome are: altered immune system function, muscular hypotonia, dysmorphic otolaryngologic features, and premature aging (Esbensen, 2010; Patterson & Cabelof, 2012; Roth, Sun, Greensite, Lott, & Dietrich, 1996; Scoggin & Patterson, 1982; Shin et al., 2009; Shott, 2006; Zigman, Schupf, Lubin, & Silverman, 1987). These individuals are highly susceptible to infections, particularly of the respiratory and the gastrointestinal tract. Congenital heart defects are seen in almost 50% of DS newborns (Shin et al., 2009) and many, even without congenital heart defects, develop mitral valve disease.

Nearly 50% of DS individuals have hypothyroidism and about 20% celiac disease. Obstructive sleep apnea syndrome occurs in approximately half of the cases (Shott 2006). DS individuals have high prevalence of obesity during childhood and adolescence (van Gameren-Oosterom et al., 2012). Sensori-neural hearing loss and cataract may develop before the age of 30 (Shott 2006). Behavioral changes with loss of skills, withdrawal, psychomotoric retardation, and mutism occur frequently from the age of 30 and may represent either symptoms of mental illness or onset of Alzheimer's dementia (Hill et al., 2003; Levine, Saltzman, Levy, & Ginsberg, 2009; Lott, 1982; Odetti et al., 1998; Qureshi & Parvez, 2007; Rabinowe, Rubin, George, Adri, & Eisenbarth, 1989; Scoggin & Patterson, 1982; Shin et al., 2009; Zigman et al., 1987) By the age of 40, virtually all individuals with DS will have neuropathology of AD (Head, Powell, Gold, & Schmitt, 2012).

To dissect the pathogenesis of DS, two hypotheses have been proposed: the amplified developmental instability hypothesis and the gene-dosage effect hypothesis (Pritchard & Kola, 1999). The amplified developmental instability hypothesis advocates that a non-specific disturbance of the chromosomal balance leads to development homeostasis disruption (Pritchard & Kola, 1999; N. Rueda, Florez, & Martinez-Cue, 2012). This hypothesis states that the features of DS are likely due to the overall abundance of genetic material and not to specific genes.

The gene-dosage effect hypothesis suggests that the HSA21 trisomy causes a general alteration in developmental homeostasis that leads to the DS phenotypes (Pritchard & Kola, 1999). It states that these alterations result from overexpression of a subset of genes located in the critical region on chromosome 21 (Greber-Platzer, Schatzmann-Turhani, Wollenek, & Lubec, 1999). Genes implicated in oxidative stress regulation have been shown to play key roles in the pathogenesis of DS. This hypothesis aligns with the free radical theory of aging, which states

that energy consumption causes in time buildup of waste, such as ROS (Harman, 1981), which leads to constant attack on cells, causing them to cease proliferation and thus, age. Over the years, evidence of the role of caloric restriction in longevity has helped to support this theory (Heilbronn & Ravussin, 2003).

Premature Aging

DS exhibits many of the typical aging signs such as atrophy (skin thinning and loss of elasticity), loss of cutaneous fat, wrinkling, greying hair, and loss of hair ((Bittles & Glasson, 2004; Brown, 1987; Esbensen, 2010; Franceschi et al., 1992; Gilchrest, 1981; Hill et al., 2003; Horvath et al., 2015; Lott, 1982; Murdoch & Evans, 1978; Patterson & Cabelof, 2012; Roth et al., 1996)). DS was first characterized as a segmental progeria in 1982 (Martin, 1982) and was found to be the only genetic disorder that had 14 of the 17 features of a true premature aging syndrome, according to Martin (Table 1.2) (Martin 1977). In the early 1900s, the life expectancy of DS individuals was about 12 years of age (Carmeli, Kessel, Bar-Chad, & Merrick, 2004). By 1983 it was 35 years, an increase accredited to improvements in medical care for both children and adults (Head et al., 2012). Current life expectancy for a one year old with DS is 43-55 years (Uppal, Chandran, & Potluri, 2015). The shortened lifespan is said to be due to DS mortality rate doubling every 6.4 years, as compared to every 9.6 years for the rest of the population (Head et al., 2012).

Evaluating *in vitro* aging/senescence of cultured fibroblasts is widely utilized as a surrogate model for *in vivo* aging. Segal and McCoy were first to show that DS fibroblasts had a slower growth rate and decreased life span (Segal & McCoy, 1974). Chapman *et al* showed that NAD levels and proliferation ability were altered in DS lymphocytes and fibroblasts (Chapman, Zaun, & Gracy, 1983). The senescence phenotype of DS has been confirmed in

human tissue and mouse DS models (Chapman et al., 1983; Contestabile, Fila, Cappellini, Bartesaghi, & Ciani, 2009; Fuchs, Ciani, Guidi, Trazzi, & Bartesaghi, 2012). The immune dysfunction seen in DS has been linked to the early senescence of immune system, as characterized by small thymus and abnormal lymphocytes (S. S. Agarwal et al., 1970; da Rosa Utiyama et al., 2008; Rabinowe et al., 1989). Utilizing proliferation marker Ki-67, Contestabile et al showed a reduced number of proliferating cells in the hippocampal dentate gyrus and in the neocortical germinal matrix of fetuses with Down syndrome. A well-known method to assess senescence is the Senescence-Associated β -Galactosidase (SA- β -gal), which relies on the detection of beta galactosidase at pH =6 (Dimri et al., 1995; Itahana et al., 2003). This enzyme is specifically produced by senescent cells only and is not observed in quiescent or transformed cells (Dimri et al., 1995). Fibroblasts collected from DS donors are positive for SA- β -Gal in higher numbers than controls (Rodriguez-Sureda, Vilches, Sanchez, Audi, & Dominguez, 2015). Two other established markers of senescence, p16Ink4a and p19Arf, are involved in senescence-induced loss of proliferation and have been shown to be overexpressed in DS (Adorno et al., 2013; Baker et al., 2011; Itahana et al., 2003; Y. Liu et al., 2009; Yeo et al., 2000).

Similar findings were seen in a widely used mouse model of DS, Ts65dn. Ts65Dn model phenocopies much of DS, even though it is trisomic for only about 60% of the HSA21 genes. Mouse embryonic fibroblasts (MEFs) and terminal-tip fibroblasts (TTFs) from this model showed a high number of SA- β -Gal-positive cells and increased p16Ink4a expression (Adorno et al., 2013; Contestabile et al., 2009). Interestingly, the reduced proliferation in Ts65Dn is widespread as multiple tissues were affected (heart, liver, skin and intestine) and appears early in life (postnatal) (Contestabile et al., 2009; Contestabile et al., 2007). More work is needed to fully evaluate the accelerated aging phenotype of this model.

Alzheimer's disease (AD) is considered a disease of old age, with increasing risk for individuals over 65 years (Head et al., 2012). It is the most common cause for dementia and overall cognitive decline (Larson, Kukull, & Katzman, 1992). AD neuropathology appears in virtually all DS adults older than 40 years of age (Head et al., 2012). DS individuals have early AD onset, some showing signs in their 30s (Head et al., 2012; Hill et al., 2003; Jackson, Holland, Williams, & Dickerson, 1988). AD is a major cause of mortality in DS, third to congenital defects and leukemia (Esbensen, 2010; Hermon, Alberman, Beral, & Swerdlow, 2001; Hill et al., 2003; Uppal et al., 2015; Q. Yang, Rasmussen, & Friedman, 2002). AD risk has been attributed to β -amyloid precursor protein (APP), which is encoded on chromosome 21. The extra chromosome leads to overexpression of APP and thus, to generation of shortened β -amyloid ($A\beta$) peptides that cause senile plaques (Head et al., 2012). Another DS trait responsible for increased AD risk is the high oxidative stress (Head et al., 2012).

B. Oxidative Stress

Oxidative stress is considered one of the main causes of aging and DS provides the best evidence for this hypothesis. DS is a disorder characterized by constitutive oxidative stress, which causes damage to DNA, lipid and protein oxidation (Amparo Gimeno et al., 2014; Odetti et al., 1998; Praticò et al., 2000; Marianna Zana, Janka, & Kálmán, 2007; M. Zana et al., 2006). This is likely due to the presence of many genes on HSA21 that are involved in regulation of oxygen metabolism (See Table 1.3) (Patterson & Cabelof, 2012).

One of the best studied enzymes is superoxide dismutase 1 (Cu-Zn), SOD1. SOD1 is encoded on the distal portion of HSA21 and is responsible for destroying free superoxide radicals by converting them to hydrogen peroxide (H_2O_2) (F. L. Muller, M. S. Lustgarten, Y. Jang, A. Richardson, & H. Van Remmen, 2007). The ~ 50% overexpression of SOD1 in DS,

without a coordinated upregulation of the two downstream enzymes, catalase (CAT) and glutathione peroxidase (GPx), causes an abundance of H₂O₂, which leads to hydroxyl radical damage (Campos & Casado, 2015; de Haan, Cristiano, Iannello, & Kola, 1995; Druzhyna, Nair, LeDoux, & Wilson, 1998; A. Gimeno et al., 2014; Kedziora & Bartosz, 1988; Patterson & Cabelof, 2012). In addition, when SOD1 is overexpressed in human neuroblastoma cell line, the proliferation rate was reduced by 30% compared to untransfected cells (Lee, Hyun, Jenner, & Halliwell, 2001). The increased activity of SOD1 also underlies the neural abnormalities observed in individuals with Down syndrome (de Haan et al., 1995; Lee et al., 2001; Zitnanova et al., 2004). Other DS traits attributed to SOD1 overexpression were demonstrated in transgenic mouse models overexpressing SOD1. In three independently derived transgenic SOD1 mice, overexpression caused thymus and bone marrow abnormalities similar to those seen in DS (Peled-Kamar, Lotem, Okon, Sachs, & Groner, 1995). Another study showed that transgenic animals exhibited significant pathological changes in tongue neuromuscular junctions (NMJ) similar to those observed in the tongue muscle of patients with Down's syndrome (Groner et al., 1990).

For years, overexpression of SOD1 was thought to be the major cause of increased oxidative stress in DS. Bach1, a transcription factor, acts as a repressor of oxidative stress response and was recently implicated in DS pathogenesis (Domenico et al., 2015). Bach1 binds to antioxidant response elements (AREs) of DNA and inhibits transcription of specific genes involved in the cell stress response (Dohi et al., 2008). Thus, its overexpression can potentially play a role in the constitutive state of oxidative stress of DS. Bach1 was shown to be upregulated in the brain of DS individuals where it downregulates heme oxygenase-1 (HO-1)(Domenico et al., 2015). HO-1 is up-regulated in response to oxidative stress in order to protect cells against

ROS-induced damage and thus, its downregulation in DS brain could be a contributing factor to the early onset of Alzheimer's disease (Dohi et al., 2008; Domenico et al., 2015; Hefti, Quinones-Lombrana, Redzematovic, Hui, & Blanco, 2016).

DS exhibits many makers of oxidation. In lipids, isoprostane 8,12-iso-iPF(2 α)-VI is a specific marker of lipid peroxidation. Elevated levels of this isoprostane were found in the urine of DS subjects compared with controls ((Praticò et al., 2000). DS neurons exhibit a three-fold increase in intracellular reactive oxygen species and show elevated levels of lipid peroxidation (Busciglio & Yankner, 1995). Protein oxidation is assessed by carbonyl assays (Marianna Zana et al., 2007), which measure the amino acid oxidation by ROS. Cerebral cortices from DS fetuses, showed a 2.9 fold increase in carbonyl groups (M. Zana et al., 2006). Using a proteomics approach, Perluigi et al showed that lipid peroxidation damages brain proteins by modify them and rendering them nonfunctional (M. Perluigi et al., 2009). The accumulation of oxidation damage was shown to start very early, as evidenced by the presence of isoprostane in fetal liver (Cabelof et al., 2009).

ROS can cause multiple DNA modifications ranging from single base damage, single strand breaks, and double strand breaks. Single base modifications due to oxidative stress include: 8-oxoguanine (8-oxoG), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG), and 2,2- diamino-4-(2-deoxy-b-D-erythropentofuranosyl)amino]-5 (2H)-oxazolone (oxazolone, Oz) (Jena, 2012). In fibroblasts from both adult and fetal DS samples, levels of 8-oxodG were 3-fold higher than in their matched controls (Necchi et al., 2015). Similar results were observed in brains of DS individuals (Domenico et al., 2015). Numerous studies have shown an overall increase in DNA damage in DS samples. In one study, DS lymphocytes showed twice as much constitutive DNA damage in G2 over control (Pincheira, Romero, Marcelain, Salazar, & de la

Torre, 2007) A recent study showed that DS patients had significantly higher DNA damage, as assayed by comet assay (El-Bassyouni et al., 2015).

DNA repair capacity

Maintaining the integrity of DNA is vital for cell survival. There are numerous pathways that facilitate repair of damaged DNA. ROS are considered a major source of spontaneous DNA damage. A few sources of oxidative stress in the cell include: lipid peroxidation (Campos & Casado, 2015; Praticò et al., 2000; Marianna Zana et al., 2007), oxygen radicals produced during cellular respiration (Perrone et al., 2007) , and increased release of amyloid beta-peptide (A β) (Marzia Perluigi & Butterfield, 2012). The primary DNA repair pathway responsible for repair of oxidative stress damage is base excision repair (BER) (Figure 2). BER is responsible for the repair of single base or gap lesions, including abasic sites, single-strand breaks, deamination and alkylation damage (Cabelof, 2007; Dianov et al., 2001; Horton, Baker, Berg, Sobol, & Wilson, 2002; Krokan & Bjoras, 2013; Ochs, Sobol, Wilson, & Kaina, 1999; Sobol et al., 1996; Wilson et al., 2000; Xu, Herzig, Rotrekl, & Walter, 2008). BER can be classified as either short patch or long patch, depending on the number of nucleotides replaced (Figure 1.4). The excision repair is initiated by DNA glycosylases, enzymes which recognize and excise the damaged base (Krokan & Bjoras, 2013). They are grouped in two classes: mono-functional or bifunctional, based on the distinct biochemical mechanism by which they repair damage. There are 11 known human glycosylases, with overlapping substrates, able to recognize more than one type of DNA damage (Jacobs & Schär, 2012). Mono-functional glycosylases, such as uracil DNA glycosylase (UDG), excise the damaged base by nucleophilic attack of N-glycosylic bond, via an activated water or hydroxyl ion (Savva, McAuley-Hecht, Brown, & Pearl, 1995), without nicking the DNA backbone. The resulting abasic site (Lindahl, Ljungquist, Siegert, Nyberg, & Sperens, 1977) is

then processed by apurinic or apyrimidine (AP) endonucleases. AP endonucleases (APE1) create nicks in DNA by β -elimination, to yield 3' OH adjacent to a 5'-dRP (Bailly & Verly, 1988; Y. J. Kim & Wilson, 2012). Bifunctional glycosylases possess AP lyase activity that allows them to nick the DNA in addition to excising the damaged base. These enzymes use the amino group of a lysine side chain as a nucleophile for base cleavage, forming a covalent Schiff-base intermediate with the damaged DNA strand (Jacobs & Schär, 2012). The product of this reaction is a strand break with 3'-phosphate and 5'-OH ends that are later processed by Ape1 to create 3'-OH and 5'-phosphate. DNA polymerase beta (POLB) is the next enzyme. The 5'-dRP flap produced by APE1 will be processed by the dRP-lyase activity of POLB, creating a 5'phosphate. The polymerization activity of POLB will then add the correct base creating a 3'-OH adjacent to the 5'-phosphate. The final step is DNA ligase sealing the DNA strands and completing the repair. XRCC1, PCNA and PARP1, are also involved in the repair process, by working to recruit and coordinate proteins in the BER process. LIGIII α , the DNA ligase used in short patch BER (SP-BER) requires XRCC1 for full activity (Y. J. Kim & Wilson, 2012).

The rate limiting enzyme in this pathway is DNA polymerase beta (POLB). POLB is a 39 kDa protein containing a 31 kDa nucleotidyl-transferase domain and an 8 kDa dRP-lyase domain. Both polymerization and dRp-lyase activity are crucial to efficiently complete the repair process (Srivastava et al., 1998). The generation of the dRP-flap is a function of repair initiation by DNA glycosylases. POLB is regulated by a TATA-less promoter and also lacks a CAAT box. The minimal promoter contains 3 SP1 sites and a cAMP-response element (Widen, Kedar, & Wilson, 1988). Promoter deletion studies have clearly established that the TGACGTCA palindrome is essential for β -pol induction by alkylating (MNNG) and oxidizing (H₂O₂) agents (Kedar, Widen, Englander, Fornace, & Wilson, 1991; Widen & Wilson, 1991). A precise

sequence including the CRE palindrome and flanking residues (7 on each side of the palindrome) is required for ATF/CREB binding. As such, proteins that bind to ATF/CREB sites in other genes do not recognize the POLB promoter (Widen & Wilson, 1991). In 1997, it was determined that CREB1 and ATF1 were the transcription factors required for CRE-mediated TPA activation of the POLB promoter (X. P. Yang, He, Rawson, & Wilson, 1997). Importantly, following MNNG treatment, CREB1 levels increased but ATF1 levels did not. Additionally, recombinant expression of CREB1 increased POLB expression, even in the absence of MNNG (Narayan, Widen, Beard, & Wilson, 1994). In addition to the CRE site, POLB promoter has functional binding elements for transcription factors Sp1 and NF- κ B (Faumont et al., 2009; Narayan et al., 1994; Pei et al., 2011). Three NF- κ B binding sites were identified on POLB promoter (Faumont et al., 2009). Only the proximal kappaB binding site (-211 to -199nt) was found cause activation upon viral factors release in EBV-infected cells. Sp1 binds to DNA at consensus GC-boxes (Narayan et al., 2000) and on POLB promoter it acts as a stabilizer of the transcription complex (Narayan et al., 2000). HIV-1 transactivator protein Tat transactivation of POLB is dependent on Sp1 binding site on POLB promoter (Srivastava et al., 2001).

Most experiments on PolB functionality were done in mouse models. Homozygous PolB null (PolB^{-/-}) mice are embryonically lethal, while the heterozygous mice (PolB^{+/-}) are viable and have been vital in elucidating the phenotype associated with PolB loss. Mouse Embryonic Fibroblast (MEFs) from PolB^{-/-} collected at day 14 of gestation have been cultured and show sensitivity to alkylating agents, MMS and MNNG, and oxidative stress (Horton et al., 2002; Ochs et al., 1999; Pascucci, Russo, Crescenzi, Bignami, & Dogliotti, 2005; Podlutzky, Dianova, Wilson, Bohr, & Dianov, 2001; Sobol et al., 1996). PolB^{-/-} MEFs not only have deficient repair phenotype but also have a higher mutation frequency post MMS treatment (Sobol et al., 2002).

Studies on the PolB^{+/-} mouse and cultured cells show that heterozygosity leads to ~50% reduction of PolB transcript and protein (Cabelof et al., 2003). These cells exhibit a significant reduction in the ability to repair damage due to MMS as evidenced by increased mutation frequency (Cabelof et al., 2003). Tissues collected from PolB^{+/-} mouse displayed higher levels of DNA single-Strand breaks as well as increased chromosomal aberrations as compared to control littermates (Cabelof et al., 2003). PolB^{+/-} mice also showed an accelerated onset of age-dependent lymphoma and accelerated rate of mortality (Cabelof, Ikeno, et al., 2006). The loss of BER and POLB as a driving factor in accelerated aging has been the basis for my thesis.

The DNA repair capacity of various organisms and their rate of aging were first shown to be correlative by Hart and Setlow in 1974 (Hart & Setlow, 1974). Since then, various studies have shown that the DNA repair capacity declines with age and it might be underlying old age diseases (Brosh & Bohr, 2007; Brown, 1987; Bucholtz & Demuth, 2013; Cabelof, 2007; Cabelof, Ikeno, et al., 2006; Cabelof, Raffoul, et al., 2006; Carre & Pieau, 1979; Franceschi et al., 1992; Gilchrest, 1981; Hanaoka et al., 1983; Harman, 1981; Intano, Cho, McMahan, & Walter, 2003; Li, Mitchell, & Hasty, 2008; Martin, 1982; Florian L. Muller, Michael S. Lustgarten, Youngmok Jang, Arlan Richardson, & Holly Van Remmen, 2007; Murray, 1981; Qureshi & Parvez, 2007; Rao, Annapurna, & Raji, 2001; Sykora et al., 2015; Vyjayanti, Swain, & Rao, 2012; Weirich-Schwaiger, Weirich, Gruber, Schweiger, & Hirsch-Kauffmann, 1994; Weissman et al., 2007; Xu et al., 2008). The brain is highly affected by a DNA repair decline with age (Sykora, Wilson, & Bohr, 2013), shown by increase of neurodegenerative diseases with age. Defective or lost BER is suspected to have a role in dementia related disease, such as Alzheimer's (Sykora et al., 2015; Weissman et al., 2007). This reduction in repair capacity furthermore correlates with decrease in the enzymatic activity of PolB enzymatic and low protein

and mRNA levels (Cabelof et al., 2002; Kisby et al., 2010; Krishna et al., 2005; Rao et al., 2001; Rao, Vinay Kumar, Bhaskar, & Sripad, 1994; Subba Rao & Subba Rao, 1984).

DS is characterized by accelerated aging and abundant evidence appoints toward a DNA repair defect as the driving force of this phenotype. Many of the premature aging syndromes have been linked to a defect in either DNA damage response or repair (Brosh & Bohr, 2007). Unlike the other premature aging diseases, which are monogenic, DS genotype makes the identification of critical DNA repair genes difficult, but not impossible. Reduced DNA repair had been demonstrated in people with DS by accumulation of DNA repair intermediates (strand breaks), increased chromosomal damage, and reduced repair capacity, as demonstrated by *in vitro* methods and/or by expression levels of key DNA repair genes. Leukocytes from individuals with DS contain more DNA strand breaks than leukocytes from control patients (Maluf & Erdtmann, 2001). The ability to repair single strand breaks induced by gamma irradiation is reduced by DS (Athanasίου, Sideris, & Bartsocas, 1980). These unrepaired strand breaks might be expected to induce chromosomal aberrations. Indeed, patients with DS exhibit higher baseline levels of micronucleus formation (MN) and greater induction of MN in response to mitomycin C, cyclophosphamide, and quercetin than their age-matched controls (Caria, Chaveca, & Rueff, 2001). Further, the MN response to mitomycin C is significantly impacted by age in DS (Shafik, Au, & Legator, 1988). Chromosomal aberrations are greater in lymphocytes exposed to ionizing radiation from DS individuals than age-matched controls (Scarfi et al., 1990). Actual measures of DNA repair capacity in DS have been addressed by what are now considered crude methods in which thymidine incorporation provides an estimate of unscheduled DNA synthesis (i.e., DNA repair). By these methods, investigators have shown reduced ability to repair DNA in response to PHA stimulation (S. S. Agarwal et al., 1970) and MNNG exposure (Raji & Rao, 1998).

Interestingly, this loss of repair capacity is accelerated with advancing age in DS (Raji & Rao, 1998).

In support to the hypothesis of this thesis, Raji and Rao have shown that young DS individuals experience a 50% reduction in POLB activity (Raji & Rao, 1998). The aged DS individuals experience an even greater deficit in POLB activity. Additionally, the inducibility of POLB in response to DNA damaging agents is blunted in DS. Importantly, they showed no effect of DS on another DNA polymerase that is not involved in DNA repair (POL ϵ) Since then, we have shown that down regulation of POLB starts *in utero* (Cabelof et al., 2009). DS fetal liver (a surrogate for the hematopoietic system) expresses reduced transcript, protein, and enzymatic activity of POLB (Cabelof et al., 2009). These data support a connection between the DNA repair defect of DS and the BER pathway. As mentioned above, Trisomy 21 causes an abundance of ROS/oxidative stress, damaging lipids, proteins and DNA. Typically damage caused by ROS is predominately repaired by BER, but this pathway is defective in DS. Is the lack of an adaptive response to oxidative stress in DS a curse or blessing? Across age, sex, and racial groups, individuals with DS develop tumors less than one-tenth as often as expected (Hill et al., 2003), with the exceptions of childhood leukemia and testicular cancer. In DS, leukemia is a childhood cancer initiated *in utero*, and thus likely unrelated to senescence. Testes resist the increased expression of senescence-related biomarkers that occurs with age (Krishnamurthy et al., 2004). It is hypothesized that the reduced DNA repair causes a shift towards senescence instead of tumorigenesis.

microRNA-155

What is the cause of POLB downregulation in DS? The two proposed hypotheses for DS pathogenesis, the amplified developmental instability and the gene-dosage effect hypotheses

(Pritchard & Kola, 1999), point to faulty transcriptional regulation of POLB. The extra chromosome does lead to overexpression of many HSA21 genes (Aït Yahya-Graison et al., 2007). HSA21 also contains 14 microRNAs: hsa-miR-99a, let-7c, miR-125b, miR-155, miR-802, miR-3197, miR-3648, miR-3687, miR-4327, miR-4759, miR-4760, miR-3118-5, miR-3156-3 and miR-548x (Kozomara & Griffiths-Jones, 2011). MicroRNAs (miRNA) are single strand RNA molecules composed of about 19-24 nucleotides (Bartel, 2004) that repress translation of its target genes via binding sites on 3'untranslated regions of mRNA targets. Studies have shown that 5 of 14 HSA21 miRNAs (hsa-miR-99a, let-7c, miR-125b-2, miR-155 and miR-802) play a significant role in the variable phenotypes of DS (Elton, Sansom, & Martin, 2010). Studies of miRNA expression, using microarrays and quantitative PCR (qPCR), revealed that these 5 miRNAs are overexpressed in DS. Sethupathy et al evaluated expression in fibroblasts collected from monozygotic twins, where trisomy penetrance varied and one twin had no aneuploidy in skin. DS twin exhibited a 2-fold increase in miR-155 expression compared to nonDS twin (Sethupathy et al., 2007). miR-155 and miR-802 were also found to be overexpressed in DS fetal brain and heart tissues, as well as in the hippocampus and whole blood of the DS mouse model, Ts65Dn(Keck-Wherley et al., 2011; Kuhn et al., 2008).

miR-155 is one of the five miRNAs with the potential to regulate POLB and BER. miR-155 is a multifaceted microRNA with numerous target genes ((Faraoni, Antonetti, Cardone, & Bonmassar, 2009)). It has been implicated in inflammation, cancer, hematopoiesis, immunity and DNA repair (Faraoni et al., 2009; Hefti et al., 2016; Keck-Wherley et al., 2011; Khamaneh, Alipour, Sheikhzadeh Hesari, & Ghadiri Soufi, 2015; Lashine, Salah, Aboelenein, & Abdelaziz, 2015; S. Liu, Yang, & Wu, 2011; Teng et al., 2008; N. Valeri et al., 2010; Velazquez et al., 2016; Yan et al., 2016). Validation studies reveal overexpression of miR-155 leads to

downregulation of genes like AID, CEBPB, Ets-1, SHIP-1, FOXO3a, MLH1 and CREB1 (Faraoni et al., 2009; Hefti et al., 2016; Keck-Wherley et al., 2011; Khamaneh et al., 2015; S. Kim et al., 2016; Kuhn et al., 2008; Lashine et al., 2015; S. Liu et al., 2011). Transcription factor CREB1, activator of POLB, was shown to be regulated by miR-155 (Elton et al., 2010; Lashine et al., 2015; S. Liu et al., 2011). Overexpression of miR-155 causes a decrease in protein levels of CREB1, as well as its targets (Lashine et al., 2015; S. Liu et al., 2011). POLB promoter studies demonstrated that when levels of CREB1 are compromised, POLB levels are also affected (K. H. Chen et al., 1998; Narayan et al., 1994; Pei et al., 2011; Podlutzky, Dianova, Podust, Bohr, & Dianov, 2001; Sobol et al., 1996; Srivastava et al., 1998). The question becomes, does miR-155 overexpression in DS cause a down regulation of POLB and does this play a role in the accelerated aging phenotype of DS?

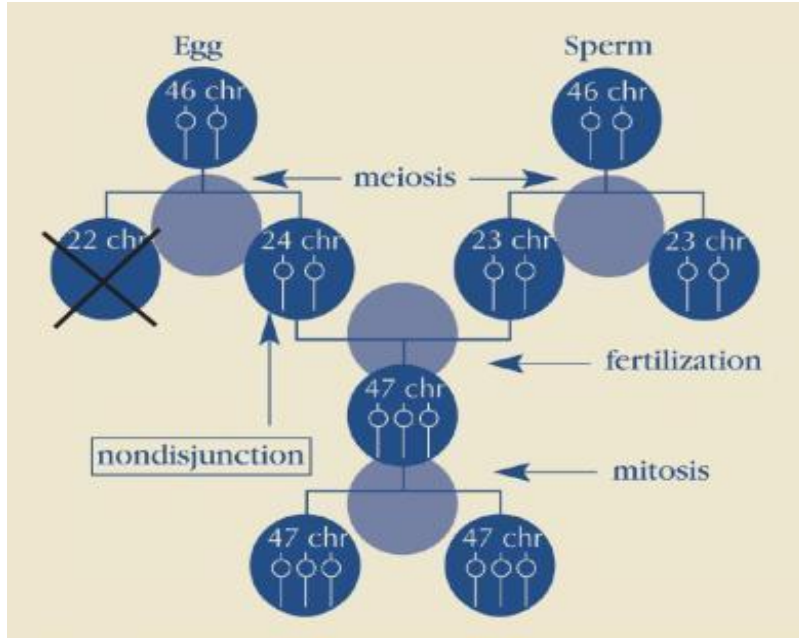


Figure 1.1: Nondisjunction. Failure of the maternal chromosome to separate during meiosis causing a zygote with an extra chromosome to be fertilized and Trisomy 21 to arise (" National Down Syndrome Society," 2012).

1 Increased frequency of non-constitutional aberrations
2 Increased susceptibility to certain types of neoplasm
3 Premature greying or loss of hair
4 Dementia or degenerative neuropathology related to senile changes
5 Increased amyloid deposition
6 Increased lipofuscin pigments
7 Diabetes mellitus
8 Disordered lipid metabolism
9 Hypogonadism
10 Autoimmunity
11 Hypertension
12 Degenerative vascular disease
13 Osteoporosis
14 Cataracts
15 Mitochondrial abnormalities
16 Regional fibrosis
17 Abnormal amounts/distribution of adipose tissue

Table 1.2: Pathophysiological and cellular criteria of ageing, adapted from G. M. Martin (1977)

Gene Name

ATP synthase F0 coupling factor

NF-E2 related factor (NRF2) (GABPA)

Amyloid precursor protein (APP)

Bach1-repressor of oxidative stress response

TIAM1

Cytosolic superoxide dismutase (SOD1)

NADPH: quinone reductase-like (CRYZL1 or NORL1)

ATP synthase OSCP subunit

RCAN1 (DSCR1)

Carbonyl reductase 1 (CBR1)

Carbonyl reductase 3 (CBR3)

DYRK1A

Thioredoxin-like protein (SH3BGR)

Mitochondrial NADH: Oxidoreductase 10kDa subunit (NDUFV3)

MIR155HG

Table 1.3: Genes on chromosome 21 implicated in oxygen metabolism(Patterson & Cabelof, 2012)

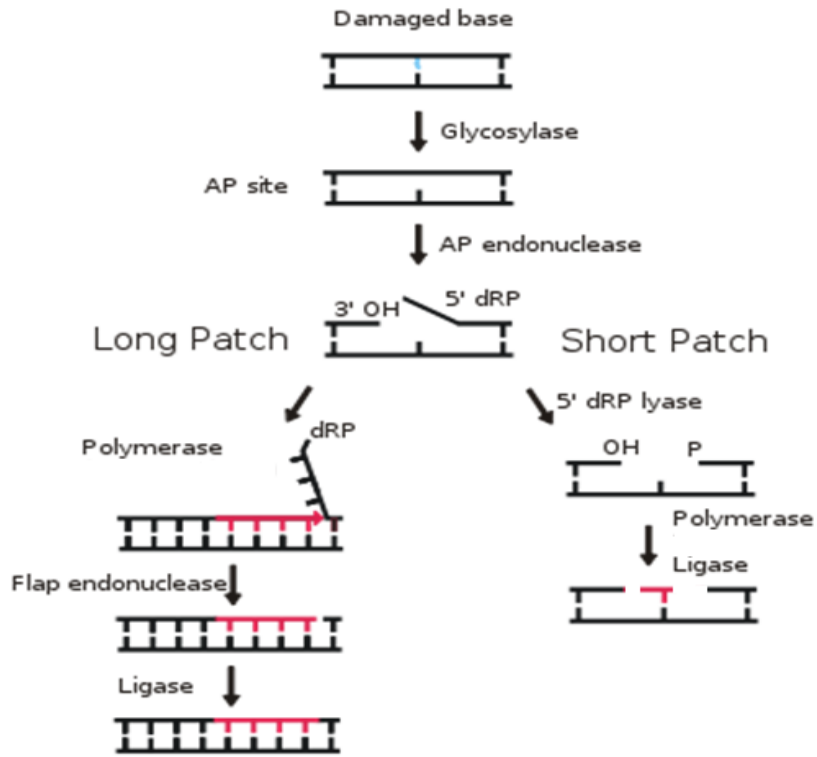


Figure 1.4: Base Excision Repair pathway

CHAPTER 2: LOSS OF DNA POLYMERASE β INDUCES SENESCENCE: IMPLICATIONS IN DOWN SYNDROME

SUMMARY

Individuals with Down syndrome exhibit accelerated aging and premature cellular senescence. Correlative evidence for reduced DNA repair capacity, specifically base excision repair, is reported in Down syndrome, but no direct connection has emerged. We report here that primary fibroblasts from Down syndrome individuals exhibit greater SA- β -Gal staining (20% increase, $p < 0.001$) and p16 transcript abundance (3-fold, $p < 0.01$). We also find that Down syndrome fibroblasts senesce more than their diploid counterparts in response to both hydrogen peroxide and hydroxyurea. To correlate this increased predisposition to senesce to a specific DNA repair deficiency, we measured POL β transcript levels and find that expression is significantly reduced in Down syndrome (~50% decline, $p < 0.01$). To mechanistically connect this loss of POL β to senescence, we assessed pol β -null primary mouse embryonic fibroblasts (MEFs) for a senescence phenotype. Here we report that untreated pol β -null MEFs display an approximate 2-fold increase in number of senescent cells ($p < 0.001$) and a 4-fold increase in senescent cells in response to hydroxyurea ($p < 0.05$) demonstrating that loss of pol β induces senescence. Pol β has not been reported to process DNA damage induced by hydroxyurea. We report here an induction in pol β transcript abundance in response to hydroxyurea exposure (2.5-fold, $p < 0.01$). Additionally, we report a 5-fold increase in DNA double strand breaks in untreated pol β MEFs ($p < 0.0001$) which is amplified another 2-fold by hydroxyurea ($p < 0.001$). Our findings firmly establish pol β as causative in senescence induction, reasonably establishing POL β and DNA base excision repair as important factors driving aging in Down syndrome.

Introduction

A correlation between DNA repair capacity and longevity was first proposed by Hart and Setlow (Hart & Setlow, 1974), who correlated mammalian DNA repair capacity to maximum lifespan. This association launched an entire field of study into the relative roles of DNA repair capacity on lifespan. In turn, many human conditions of premature and/or accelerated aging have been directly linked to defective DNA repair (Brosh & Bohr, 2007). Down syndrome is a genetic disorder caused by an extra copy of chromosome 21 (trisomy 21), and is characterized by a shortened lifespan and biomarkers of precocious aging. The mean life expectancy for individuals with Down syndrome has improved greatly from the mid-teens in 1940s (Penrose, 1949) to nearly 60 today (Bittles & Glasson, 2004). In addition to shorter lifespan, individuals with Down syndrome exhibit signs of accelerated aging. Early onset of Alzheimer's disease is highly penetrant in Down syndrome (Powers et al., 2015). Earlier skin changes, hair changes, menopause, vision and hearing impairment, thyroid dysfunction, and immune dysfunction (Esbensen, 2010; Hermon et al., 2001; Hill et al., 2003) as well. While other disorders of accelerated aging have been connected to specific DNA repair defects, Down syndrome has not. As a polygenic condition, the ability to identify a specific gene or pathway responsible for aging in Down syndrome is difficult, but there is evidence for reduced DNA repair in Down syndrome including: accumulation of DNA repair intermediates in the form of strand breaks (Athanasidou et al., 1980; Maluf & Erdtmann, 2001), increased chromosomal damage (Caria et al., 2001; Shafik et al., 1988), reduced *in vitro* measures of repair capacity (S. S. Agarwal et al., 1970; Raji, Surekha, & Rao, 1998), and reduced expression of DNA base excision repair genes in human Down syndrome samples (Cabelof et al., 2009; Raji et al., 1998).

Segal and McCoy first observed that fibroblasts from Down syndrome donors exhibited slower doubling time and had characteristics of fibroblasts from old, healthy donors (Segal &

McCoy, 1974). Over the years, evidence accumulated that the proliferative potential of fibroblasts and lymphocytes from individuals with Down syndrome is reduced (de Arruda Cardoso Smith et al., 2004). When cultured, they exhibit many of the features of premature aging, such as enlarged and flattened morphology, increased number of multinucleated cells and early appearance of SA- β -gal activity (Cristofalo, Lorenzini, Allen, Torres, & Tresini, 2004; Kalanj-Bognar, Rundek, Furac, Demarin, & Cosovic, 2002). In fibroblasts isolated from a mouse model of Down syndrome, TS65Dn, there is increased SA- β -gal activity and reduced proliferation, also seen in skin from these TS65Dn mice (Contestabile et al., 2009). Like Cockayne and Werner syndromes, the Down syndrome aging phenotype is characterized by subtle reductions in both lifespan and doubling time (Weirich-Schwaiger et al., 1994). In a longitudinal evaluation of biological aging in Down syndrome, the estimated rate of aging was found to be two-fold greater in Down syndrome (Nakamura & Tanaka, 1998). The impact of Down syndrome on aging has also been confirmed at the epigenetic level (Horvath et al., 2015). This premature aging has been attributed to the high levels of constitutive, endogenous oxidative stress in Down syndrome.

The DNA repair pathway primarily responsible for processing oxidative DNA damage is the base excision repair (BER) pathway. We have demonstrated inducibility of BER and DNA polymerase β (POL β) in response to oxidative stress (Cabelof, 2007; Cabelof et al., 2003; Cabelof et al., 2002). Accordingly, we should anticipate an adaptive upregulation in POL β in response to the constitutive oxidative stress of Down syndrome, but this does not occur. Lack of an adaptive DNA repair response over the lifespan of the individual with Down syndrome could conceivably contribute to a stress induced senescence. Our objective in this work is to establish

direct evidence that loss of POL β drives premature cellular senescence and can mechanistically explain the accelerated aging phenotype of Down syndrome.

Methods and Materials

Tissue Culture. Down syndrome (DS) primary fibroblasts (AG06872, AG05397) and their age- and sex-matched controls (GM00969, GM05659) were acquired from Coriell Cell Repositories. Results are pooled from both cell lines where stated. Cells were maintained in MEM medium (Life Technologies, Cat#10370-021) supplemented with 15% fetal bovine serum (Hyclone, Cat# SH3007003) and 1% penicillin/streptomycin (Gibco, Cat# 15240-062). DNA polymerase β (Pol β) null cells, a generous gift from Samuel H. Wilson, were maintained in DMEM (Gibco, Cat# 11995081) supplemented with 10% FBS. Pol β -null cells were derived from embryonic tissue of homozygous *Pol β* knockout mice (Sobol et al., 1996). Isogenic MEF cell lines, provided by Andrew Jackson, were cultured in DMEM (Gibco, Cat# 11995081) supplemented with 10% FBS and 0.1 mM β -mercaptoethanol. All cells were maintained in a 5% CO₂ and 3% O₂ humidified incubator at 37°C.

SA- β -gal: Senescence-associate β -galactosidase activity was assessed by using a senescence detection kit from BioVision (Milpitas, CA), according to the manufacturer's instructions. Briefly, cells seeded at 5×10^4 density were treated for 10-14 days with either hydroxyurea (HU) or hydrogen peroxide (H₂O₂). Cells were fixed for 5 minutes at room temperature and incubated with staining solution containing X-gal and staining supplement, overnight at 37°C at ambient CO₂. Blue-stained cells were identified as senescent. A minimum of 300 cells in at least 5 fields of view were photographed and counted at 20X magnification, by two blinded counters. Data shown here represent the average of three independent experiments. Results are expressed as percentage of stained cells in the total number of cells. Cells cultured and treated concomitantly

with those used to assess senescence were passaged for additional 2 weeks to evaluate their ability to recover/emerge from senescence.

Immunofluorescence staining: 4×10^4 cells were seeded on coverslips in 12-well plates and fixed in ethyl: acetate (80:20) for 5 min. After three washes, cells were blocked in PBST containing 1% BSA for 30 min at room temperature and incubated with primary anti- γ H2AX antibody (Millipore, Cat# 05-636) for 1.5 hr at room temperature or overnight at 4°C. Cells were then incubated with Alexa Fluor 488-conjugated anti-mouse IgG (1:400) (Invitrogen, Cat# A-11001) for 1 hr at room temperature and mounted with Pro-Long Gold anti-fade reagent (Invitrogen, CA). Slides were photographed under the Nikon Eclipse 80i microscope (Nikon, CA) and processed using the Nikon Elements built-in software. Approximately 300 cells were counted in more than 10 fields of view and those cells displaying more than 7 distinct foci/nucleus were considered positive for γ -H2AX.

Expression analysis: cDNA was synthesized, as described previously (Cabelof et al. 2006a), from 2 μ g RNA using random hexamer primers and purified with the QIAquick PCR Purification columns (Qiagen, Valencia, CA). Transcripts were amplified and quantitated with a LightCycler Real Time PCR machine (Roche). PCR reactions contained 2 μ l purified cDNA, 0.5 μ M of each sense and antisense primer, and 2 μ l FastStart DNA Master SYBR Green I enzyme-SYBR reaction mix (Roche). For all amplifications, PCR conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 40–45 cycles at 95°C for 10s, primer specific annealing temperatures for 10s, and elongation at 72°C for 10s. Melting curves from 65°C to 95°C confirmed specificity. External standards were prepared for all genes from cDNA amplicons cloned into pCRII TOPO cloning vector (Invitrogen, Carlsbad, CA). All transcripts were quantitated and normalized to GAPDH or Rpl4 expression where stated. Primer sequences are

detailed in Table 1, with the exception of primer sets used for amplification of human POL β and human p16, which were purchased from SA Biosciences (Frederick, MD) (Cat#PPH13735F-200 and Cat#PPH00207C, respectively).

Data Analysis

Results are expressed as mean \pm SEM and were analyzed using Student's t-test. Statistical comparisons between groups were conducted using one-way ANOVA, using GraphPad Prism 5.0 (Graph Pad Software, La Jolla, CA). Values of $p < 0.05$ were considered statistically significant and individual p-values are shown in the figures.

Results

Primary fibroblasts from Down syndrome donors recapitulate the gene dosage phenotype of Down syndrome.

To determine whether primary Down syndrome (DS) fibroblasts recapitulate the gene dosage effect of trisomy 21, we evaluated expression of three genes located on chromosome 21 in cells from age, sex, and race matched individuals without Down syndrome (NDS) and with Down syndrome (DS). All three genes are involved in oxidative stress responses: Superoxide dismutase 1 (SOD1), Cystathionine- β -synthase (CBS), and BACH1. While not all triplicated genes in Down syndrome demonstrate gene dosage effects, SOD1, CBS and BACH1 do. SOD1 overexpression has been verified in human tissues and DS cell lines (Ait Yahya-Graison et al., 2007). CBS is involved in the conversion of homocysteine to cystathionine and its upregulation in Down syndrome is responsible for the low plasma levels of homocysteine (Perrone et al., 2007; Pogribna et al., 2001). BACH1 is a transcription factor upregulated in Down syndrome (Domenico et al., 2015) that modulates oxidative stress-induced cellular senescence (Dohi et al., 2008). In Figure 2.1 we show upregulation of SOD1 (5-fold, $p < 0.01$), CBS (>5 -fold, $p < 0.001$),

and BACH1 (2-fold, $p < 0.001$) in our Down syndrome fibroblasts, confirming the gene dosage effect of trisomy 21.

Premature cellular senescence in Down syndrome fibroblasts

Senescence is an irreversible cell cycle state that can be induced by multiple endogenous and exogenous signals. Widely used biomarkers of senescence are senescence-associated beta-galactosidase (SA- β -gal) and p16 expression (Alcorta et al., 1996). SA- β -gal is expressed only in senescent cells and is not observed in pre-senescent, quiescent, or transformed cells (Dimri et al., 1995). Elevated expression of p16 maintains the senescence phenotype and is an established marker of premature aging (Alcorta et al., 1996). Further, removal of p16 from senescing tissues can reverse senescent phenotypes (Baker et al., 2011).

Cells were stained for SA- β -Gal and the percentage of X-Gal-positive cells was calculated for each cell line. In the absence of exogenous stressors, 5% of NDS fibroblasts stain positive while 20% of DS fibroblasts stain positive for senescence (Figure 2.2A, 4-fold increase, $p < 0.001$). These data are consistent with previous reports that Down syndrome fibroblasts are prone to early cellular senescence (Kalanj-Bognar et al., 2002). In parallel to SA- β -Gal detection, we evaluated p16 expression as a function of genotype and find a significant increase in p16 in primary DS fibroblasts (Figure 2.2A, 3-fold, $p < 0.01$). When exposed to hydrogen peroxide (10 μ M H₂O₂), we observe 10% senescent cells in NDS fibroblasts and 35% senescent cells in the DS fibroblasts (Figure 2B, 3.5-fold increase $p < 0.05$). In response to hydroxyurea (HU), we observe 21.4% of NDS fibroblasts are senescent while 58.2% of the DS fibroblasts are senescent (Figure 2.2B, >2-fold, $p < 0.01$). All SA- β -Gal data were collected 48 h after recovery from the final exposure. Cells grown and treated in parallel were maintained in culture for an additional two weeks, but never reentered the cell cycle.

Reduced DNA polymerase β (POLB) expression in Down syndrome fibroblasts

As described above, loss of DNA repair capacity, in particular loss of POL β , has been observed in several model systems of Down syndrome. To evaluate a possible DNA repair defect in our model system, we examined POL β expression in two primary fibroblast cell lines from each genotype, and find a significant reduction in POL β expression in Down syndrome (Figure 2.3, 2.9-fold, \pm SEM, $p < 0.01$). This reduction is similar to levels previously reported in the Pol $\beta^{+/-}$ mouse which has been shown to be sufficient to induce a moderate increase in the rate of aging (Cabelof, Ikeno, et al., 2006). However, no study of POL β has yet evaluated a direct role for this gene in premature senescence.

Homozygous loss of DNA polymerase β induces premature cellular senescence

To elucidate a possible role for POL β in cellular senescence, we evaluated Pol $\beta^{-/-}$ primary mouse embryonic fibroblasts gifted by Samuel H. Wilson (Sobol et al., 1996) for biomarkers of senescence, at baseline and in response to exogenous DNA damage. We anticipated a reduced DNA damage threshold in response to senescence-inducing agents in the absence of Pol β , but were surprised to see a large number of SA- β -gal positive cells in untreated Pol $\beta^{-/-}$ cells (Figure 4A, > 11-fold, $p < 0.001$). p16 was also significantly upregulated in the absence of Pol β , further confirming the premature senescence induced by Pol β deficiency (Figure 2.4A, 1.69-fold, $p < 0.001$). This is the first direct evidence that Pol β loss is sufficient to induce premature cellular senescence. In addition, treatment of Pol β null cells with hydroxyurea resulted in a greater increase in both SA- β -gal positive cells (Figure 2.44B, >2-fold, $p < 0.05$) and p16 expression (Figure 4B, > 3.5 fold, $p < 0.001$). These data demonstrate both a senescence phenotype induced by the absence of Pol β alone and an increased susceptibility to toxic exposures that could further contribute to accelerated aging when pol β is inhibited.

DNA polymerase β protects cells from hydroxyurea-induced DNA double strand breaks.

This sensitivity of Pol β null cells to HU suggests a role for this gene product in the processing of damage induced by HU. To investigate, we evaluated the impact of HU on Pol β expression in two isogenic MEF cell lines and found a significant increase in Pol β (Figure 2.5A, 2.5-fold, $p < 0.01$). Hydroxyurea inhibits ribonucleotide reductase, which alters dNTP/NTP ratios. This imbalance generates strand breaks and stalled replication forks, both known drivers of senescence (Yeo et al., 2000). As such, we investigated the impact of HU on DNA double strand break (DSB) formation, both in the presence and absence of Pol β . In wildtype cells, 20% of cells contain DSB when exposed to 300 μ M HU, while in the Pol β null cells this same exposure results in over 50% of cells with DSB (Figure 2.5B, >2.5-fold, $p < 0.001$). This provides an explanation for the increased sensitivity and accelerated senescence of Pol β null cells in response to HU. Perhaps more notable is the significant induction of DSB in untreated Pol β null cells. While fewer than 5% of wildtype cells show distinct foci, over 20% of pol β null cells contain DSB (Figure 2.5B, 4-fold, $p < 0.0001$). Although loss of Pol β is known to induce DSB and drive recombination in response to DNA damaging agents (Horton et al., 2002), this is the first report that these breaks arise spontaneously. These data point to a mechanism by which loss of Pol β both induces senescence and reduces the threshold of damage able to induce senescence.

Discussion

We show here that loss of Pol β is sufficient to induce senescence, both in response to accumulating endogenous DNA damage and in response to hydroxyurea exposure, likely by reducing the DNA damage threshold for senescence. The offending DNA damage is most likely to be DNA double strand breaks (DSB), as hydroxyurea is known to induce senescence through a strand break-mediated mechanism (Yeo et al., 2000). The 10-fold increase in level of DSB in the

Pol β null cells supports this (Figure 2.5B). Pol β performs both the DNA synthesis and dRPlyase steps of base excision repair (Sobol et al., 1996). In the absence of either enzymatic activity, DNA repair intermediates in the form of strand breaks accumulate, as evidenced by accumulation of DSB in Pol β ^{-/-} cells exposed to methylmethane sulfonate (MMS) (Pascucci et al., 2005). Our finding that DSB accumulate in unexposed Pol β ^{-/-} null cells (Figure 2.5B) is the first report that loss of base excision repair results in spontaneous DSB.

Down syndrome presents an accelerated aging model in which there is both increased endogenous damage, and reduced DNA base excision repair capacity, a perfect recipe for early senescence. The level of oxidative stress in Down syndrome is constitutively high, and begins *in utero* (Cabelof et al., 2009; Domenico et al., 2015; Nizetic & Groet, 2012; Pogribna et al., 2001). The high level of oxidation likely results from the presence of over twenty genes involved in oxidative metabolism located on Chromosome 21 (Patterson & Cabelof, 2012). Likewise, the base excision repair defect also begins *in utero* (Cabelof et al., 2009). Under typical conditions, both base excision repair and POL β are induced in response to oxidative stress. The lack of this adaptive response provides a plausible explanation for the early senescence in Down syndrome.

Base excision repair capacity and Pol β abundance decline by approximately 50% with age (Cabelof et al., 2003). Further, the pol β heterozygous mouse expresses 50% less Pol β and ages at a slightly faster rate than its wildtype littermates (Cabelof, Ikeno, et al., 2006)). This is coincident with an approximate 50% decline in POL β levels observed in Down syndrome (Raji et al., 1998). Thus, POL β haploinsufficiency in Down syndrome is sufficient to induce aging and premature senescence. The question becomes why POL β is reduced in Down syndrome, and why tissues and cells from individuals with Down syndrome fail to respond properly to DNA damage. The combination of increased, chronic levels of oxidative stress and reduced DNA repair likely

make individuals uniquely susceptible to environmental exposures. In support, children with Down syndrome who develop AMkL are highly responsive to chemotherapy (Taub et al., 2000). While increased cytotoxicity is desirable in the treatment of pediatric leukemias, it is hazardous with respect to unintended environmental exposures. With a reduced threshold to senescence, the aging observed in Down syndrome may be the result of unknown gene/environment interactions in this population. With the incidence of Down syndrome at 14.47 per 10,000 live births (S. E. Parker et al., 2010) that is increasing an average of 0.9% per year (Shin et al., 2009), this is a highly relevant question and a potentially interesting model for addressing questions pertaining to mechanisms of aging as it relates to environmental exposures.

TABLES

Gene	Sense primer 5'–3'	Anti-sense primer 5'–3'
<i>Human</i>		
CBS	Ggggctgagattgtgaggac	cggtactggcttaggatgtga
SOD1	Ggtgggccaaggatgaagag	ccacaagccaacgacttcc
BACH1	Ctcagccttaatgaccagcgg	gcctacgattcttgagtggaag
<i>Mouse</i>		
p16 UPL probe #91	Aatctccgcgaggaaagc	gtctgcagcggactccat
Pol β (exon 12–13)	Agcgagaaggatggaaaggaa	cgtgcgctctcatgttcttat
<i>Housekeeping genes</i>		
Gapdh	Aggtcgggtgaacggatttg	tgtagaccatgtagttgaggtca
GAPDH	aaggtgaaggtcggagtcaac	ggggtcattgatggcaacaata
Rpl4	ccgtcccctcatatcgggtga	gcatagggctgtctgttgtttt
Rpl4 UPL probe # 75	tggtggtgaagataaggtga	ccaagcttgagtttcttgagc

Table 2.1: Quantitative RT-PCR Primer sequences

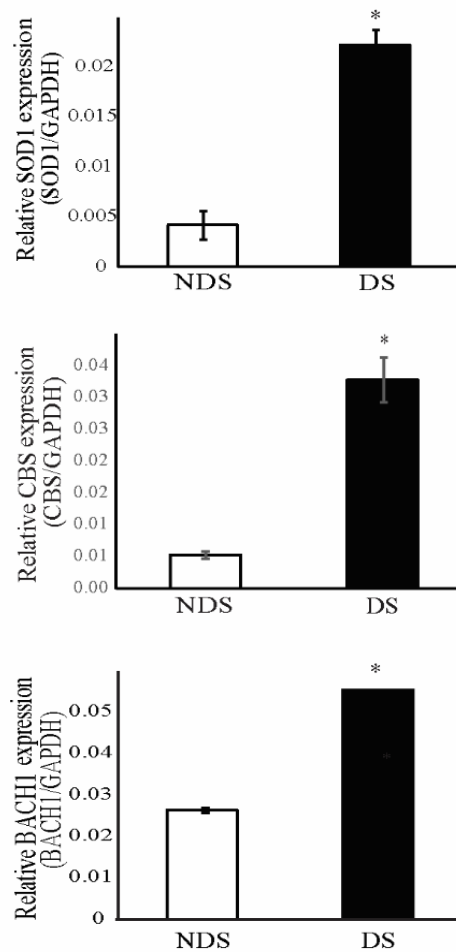
FIGURES

Figure 2.1: Primary Down syndrome fibroblasts exhibit gene dosage effects for oxidative stress response genes on chromosome 21. Gene expression was evaluated in primary fibroblasts from age, sex and raced matched donors either without Down syndrome (NDS, GM00696 and GM05659) or with Down syndrome (DS, AG06872 and AG05397). Transcript levels were determined by quantitative RT-PCR and normalized to GAPDH. Data are presented as the average of pooled samples from each genotype \pm SEM. *Values significantly different from control (NDS) at $p < 0.01$.

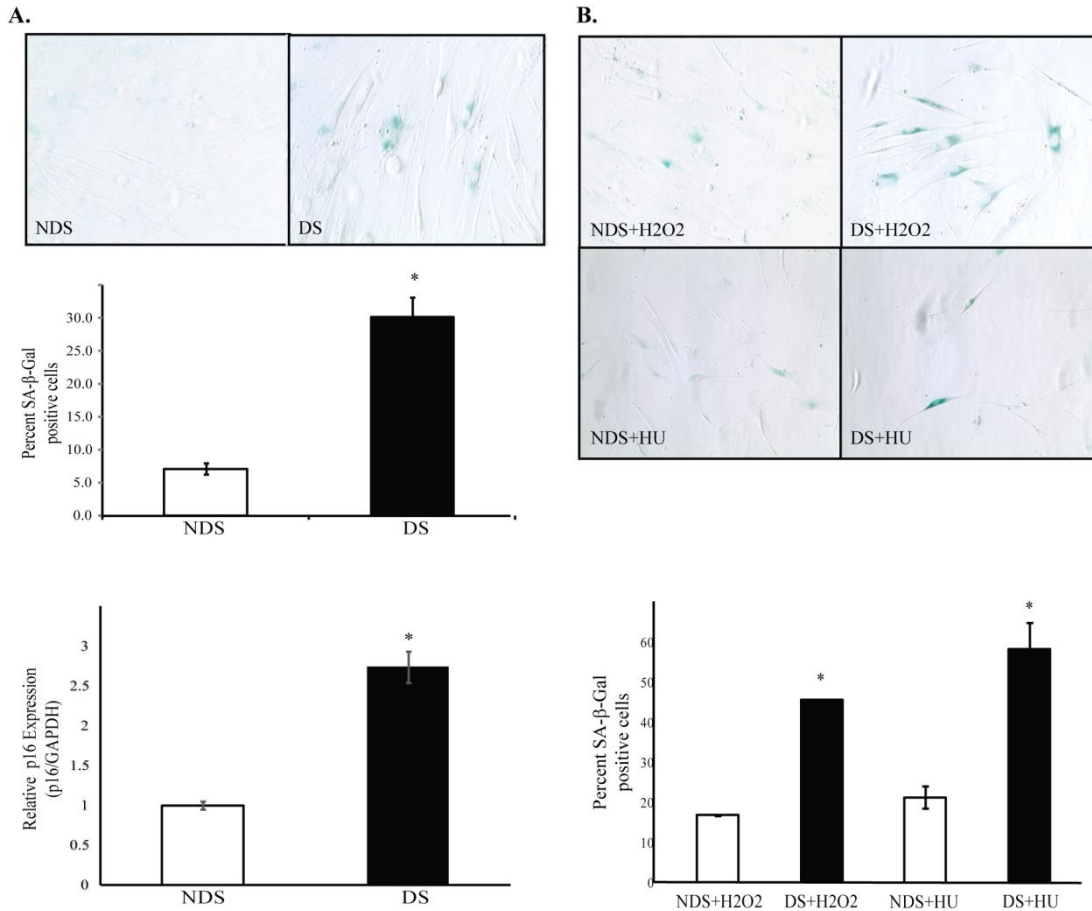


Figure 2.2: Premature cellular senescence in Down syndrome fibroblasts. A. Down syndrome fibroblasts exhibit a premature senescence phenotype. Senescence-associated β -galactosidase (SA- β -Gal) activity and p16 transcript abundance were measured in primary fibroblasts from donors either without Down syndrome (NDS, GM05659) or with Down syndrome (DS, AG05397) as described in Methods. A minimum of 300 cells was counted in random fields by a technician blinded to genotype to quantify the proportion of SA- β -Gal-positive cells. Quantification is presented as the average of three independent experiments \pm SEM and is expressed as a percent of positive cells $[(SA-\beta-Gal\text{-positive cells}/total\ cells)*100]$. p16 transcript abundance was determined by quantitative RT-PCR and normalized to GAPDH. B. Down syndrome fibroblasts exhibit an amplified senescence response to hydrogen peroxide and hydroxyurea. Cells were seeded 24 hours prior to treatment with 10 μ M H₂O₂ or 300 μ M Hydroxyurea (HU) as described in Methods. Briefly, cells were exposed to H₂O₂ for two hours and allowed to recover for 5 days, and to HU every 2 days over a 14 day period. 300 cells were counted in random fields by a technician blinded to both genotype and treatment. Quantification is presented as the average of three independent experiments \pm SEM and is expressed as a percent of positive cells $[(SA-\beta-Gal\text{-positive cells}/total\ cells)*100]$. *Values significantly different from control at $p < 0.01$.

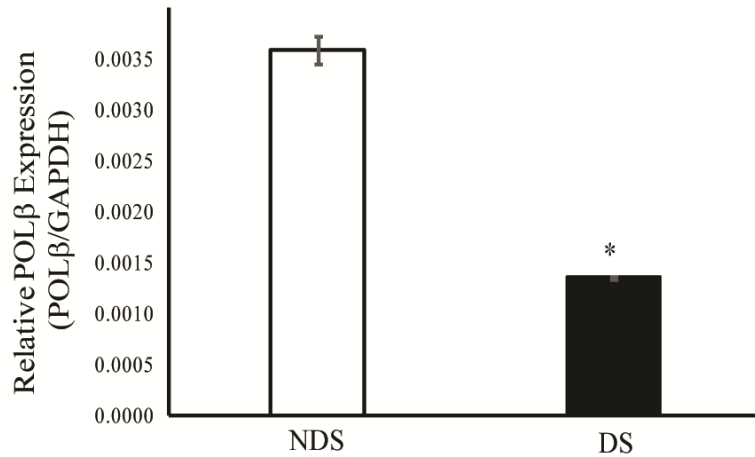


Figure 2. 3. Primary Down syndrome fibroblasts exhibit reduced DNA polymeraseβ transcript abundance. Gene expression was evaluated in primary fibroblasts from age, sex and raced matched donors either without Down syndrome (NDS, GM00696 and GM05659) or with Down syndrome (DS, AG06872 and AG05397). POLβ transcript levels were determined by quantitative RT-PCR and normalized to GAPDH. Data are presented as the average of pooled samples from each genotype \pm SEM. *Values significantly different from control (NDS) at $p < 0.01$.

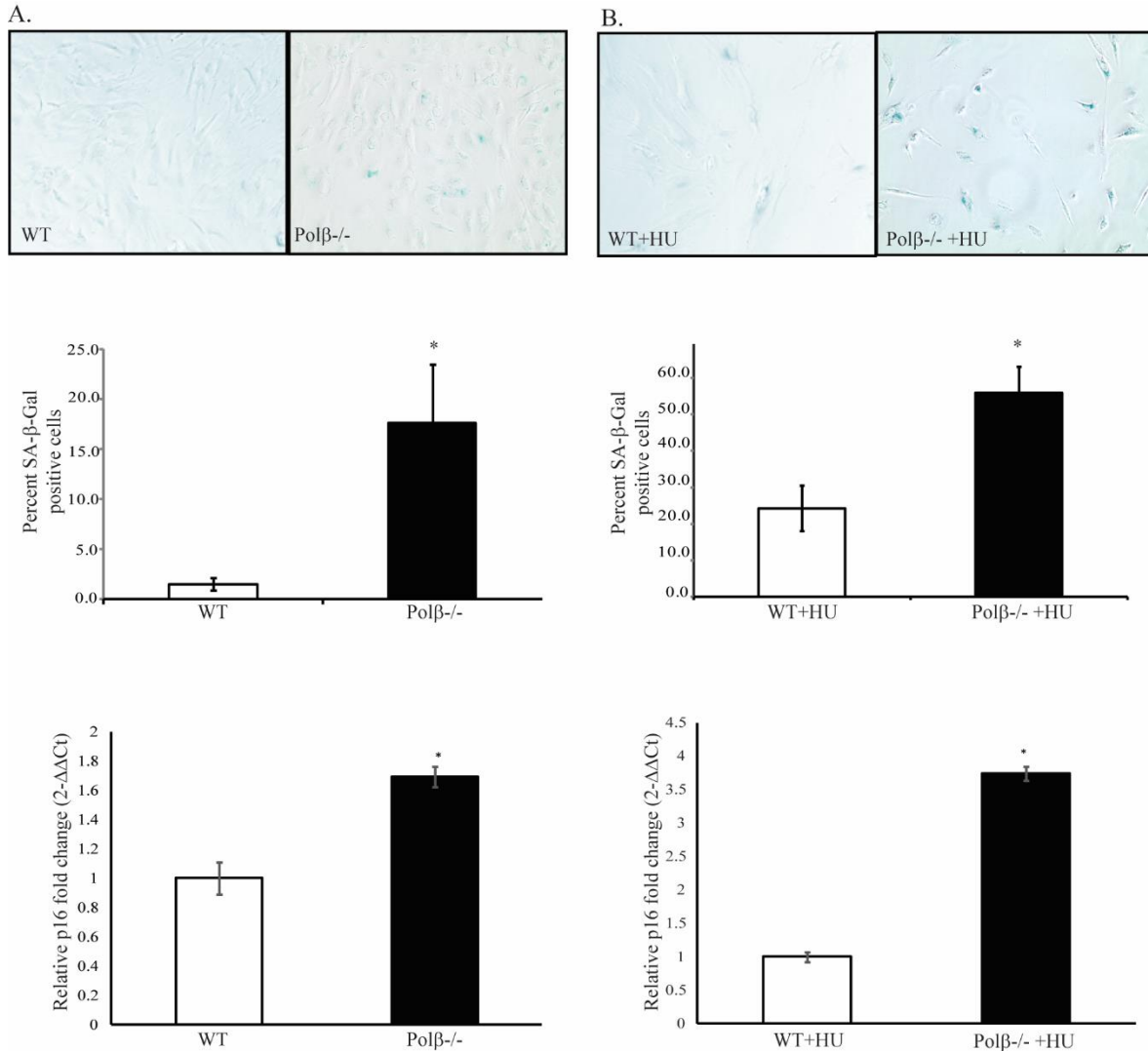


Figure 2.4: Homozygous loss of DNA polymerase β induces premature cellular senescence.

A. Loss of DNA polymerase β is sufficient to induce senescence. Senescence-associated β -galactosidase (SA- β -Gal) activity and p16 transcript abundance were measured in primary mouse embryonic fibroblasts from wildtype or from DNA polymerase β null embryos as described in Methods. 300 cells were counted in random fields by a technician blinded to genotype to quantify the proportion of SA- β -Gal-positive cells. Quantification is presented as the average of three independent experiments \pm SEM and is expressed as a percent of positive cells [(SA- β -Gal-positive cells/total cells)*100]. p16 transcript abundance was determined by quantitative RT-PCR using a probe-based system as described in Methods. B. DNA polymerase β null mouse embryonic fibroblasts exhibit an amplified senescence response to hydroxyurea. Cells were seeded 24 hours prior to treatment with 300 μ M Hydroxyurea (HU) as described in Methods. Briefly, cells were exposed to HU every 2 days over a 14 day period. 300 cells were counted in random fields by a technician blinded to both genotype and treatment. Quantification is presented as the average of three independent experiments \pm SEM and is expressed as a percent of positive cells [(SA- β -Gal-positive cells/total cells)*100]. p16 transcript abundance was

determined by quantitative RT-PCR using a probe-based system as described in Methods.
*Values significantly different from control at $p < 0.01$.

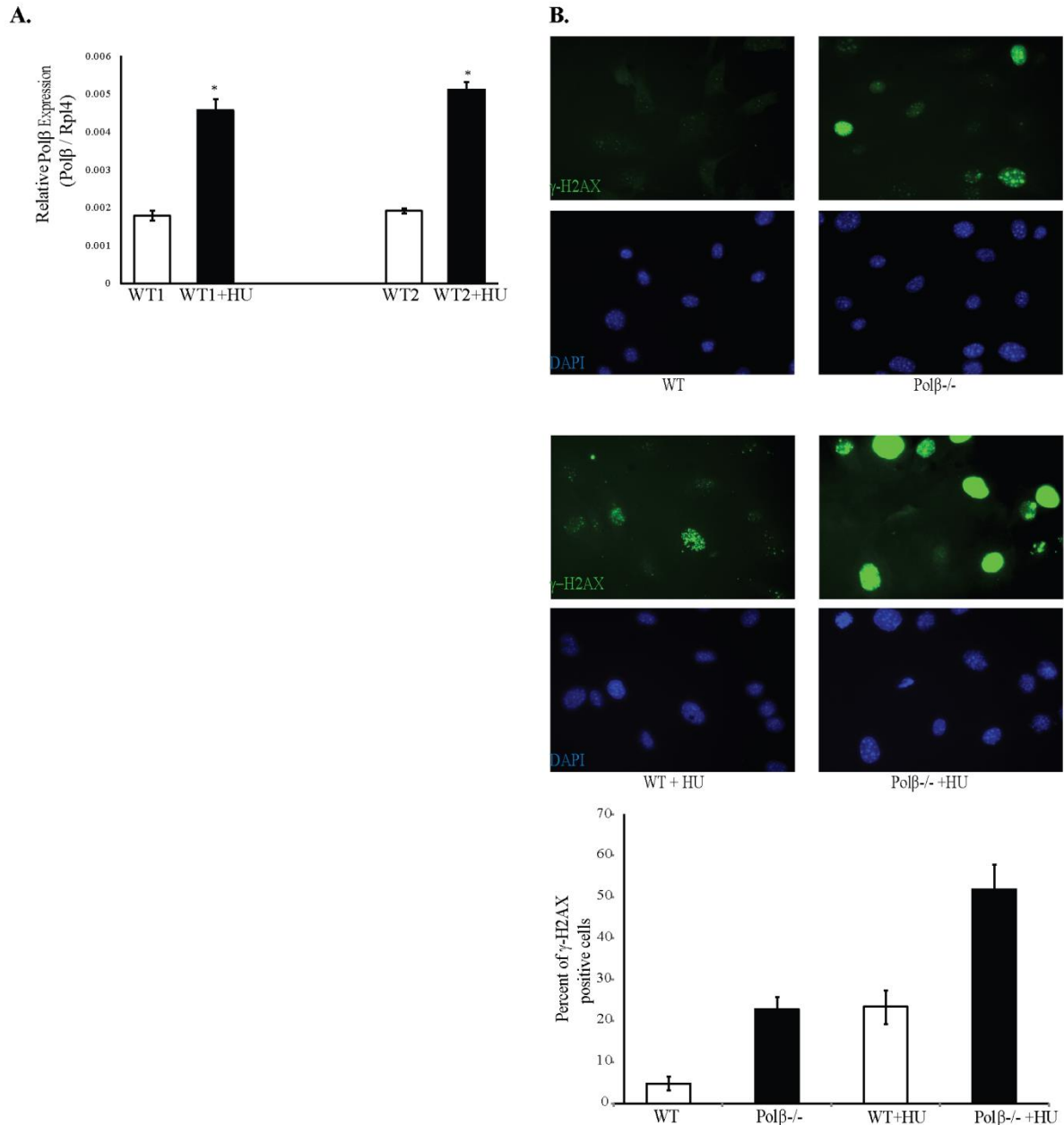


Figure 2.5: DNA polymerase β protects cells from hydroxyurea-induced DNA double strand breaks. A. DNA polymerase β is upregulated in response to hydroxyurea. Pol β transcript abundance was determined by quantitative RT-PCR in two isogenic MEF lines following HU exposure. Expression was normalized to Rpl4, and data is presented as mean \pm SEM. *Value significantly different from control at $p < 0.01$. B. Loss of DNA polymerase β induces DNA double strand breaks. DNA double strand breaks were detected by γ -H2AX immunostaining in primary mouse embryonic fibroblasts from wildtype or from DNA polymerase β null embryos as described in Methods. Cells were either untreated or were treated with 300uM HU for 24 hrs, fixed and stained with DAPI (blue). Cells were probed with antibody against γ -H2AX (green; anti- γ H2AX antibody (Millipore, Cat# 05-636)). A total of ≥ 300 cells in > 10 fields were

counted. Cells were considered positive for double strand breaks if >7 foci per nucleus were positive. Data are presented as mean \pm SEM. *Values significantly different from control at $p < 0.01$.

CHAPTER 3: REGULATION OF DNA POLYMERASEB BY MIR-155

Summary

In this chapter we elucidate the link between Pol β downregulation and Down syndrome genotype. DS, unlike other premature aging syndromes, provides a non-monogenic model to study the effects of trisomy 21 on aging and DNA repair. Here, we set out to determine whether HSA21-localized miR-155 played any role in regulating Pol β . Although Pol β does not have a miR-155 seed on its 3'UTR, there is ample evidence that Creb1, a transcriptional regulator of Pol β , is a direct target of miR-155. miR-155 overexpression causes a reduction in Pol β promoter activity, as well as a decrease of both Creb1 and Pol β protein levels. Data from proteomics study reveals that other BER genes are also differentially affected by miR-155 overexpression. Further investigation into the precise mechanisms of Pol β inhibition by miR-155 is necessary in order understand the effects of Trisomy 21 on DNA repair.

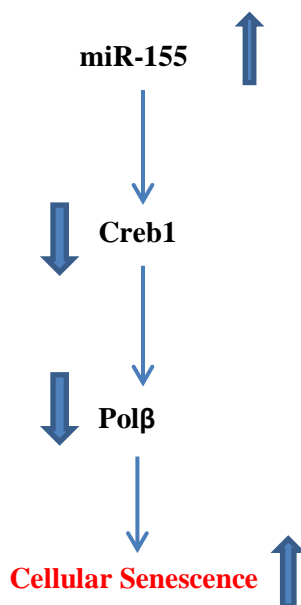


Figure 3.1: Proposed mechanism of miR-155 regulation.

3.1 Introduction

Down syndrome is a genetic disorder characterized by an accelerated aging, as result of increased genomic instability. This is due to an imbalance between increased DNA damage and decreased repair. Individuals with DS have high constitutive oxidative stress linked to the overexpression of HSA21 genes SOD1, APP1, and BACH1 (Domenico et al., 2015; El-Bassyouni et al., 2015; Nizetic & Groet, 2012; Patterson & Cabelof, 2012; Rodriguez-Sureda et al., 2015; N. Rueda et al., 2012). Typically, damage induced by oxidative stress is repaired by base excision repair (BER), a process in which POLB is the rate limiting enzyme. We have established that DS and Pol β null fibroblast exhibit high levels of senescence markers: SA beta gal staining and p16 expression. DS fibroblasts show a reduced POL β expression that correlates with increase in senescence. Data from Pol β nulls cells confirm that Pol β loss alone is sufficient to induce senescence.

In this study, we explore the underlying mechanism responsible for the negative regulation of Pol β . Non-protein coding RNAs have recently been shown to play an important role in regulating multiple DNA repair pathways. The 20-nucleotide long miRNAs bind to specific 3'-UTR sites on target RNAs and limit the activity of targeted proteins, by either inhibiting translation or degrading the transcript. It is estimated that miRNAs regulate approximately 30% of protein-coding genes (Bartel, 2004).

Five miRNAs are found on chromosome 21: miR-99a, let-7c, miR-125b2, miR-155 and miR-802. miR-155 is overexpressed in the fetal brain, fetal heart, and fibroblasts of DS individuals (Hefti et al., 2016; Sethupathy et al., 2007), as well as in the hippocampus and whole blood of the Ts65DN mouse (Keck-Wherley et al., 2011). miR-155 has been shown to negatively regulate DNA mismatch repair (MMR)(Nicola Valeri et al., 2010). It does so by binding to

mismatch repair genes, hMSH2, hMSH6, and MLH1, causing their downregulation. This results in impaired MMR (N. Valeri et al., 2010). MiR-155 also affects DSB repair, by inhibiting Rad51 (Gasparini et al., 2014), a gene responsible for strand invasion during homologous recombination (HR). A recent study showed that miR-155 is also involved in non-homologous end-joining (NHEJ), via transcription factor FOXO3a (Czochor, Sulkowski, & Glazer, 2016), which modulates DNA repair through Gadd45a and PCNA. This paper also demonstrated that miR-155 overexpression results in significant reduction in DNA polymerase delta (POLD) (Czochor et al., 2016).

MiR-155 could potentially regulate BER, via translational inhibition of transcription factor Creb1. Creb1 has 3 putative binding sites for miR-155 on its 3'-UTR and has been shown to be negatively regulated by miR-155 (Y. Chen et al., 2013; Gaudet et al., 2016; Lashine et al., 2015; S. Liu et al., 2011). miR-155 overexpression causes decreased levels of CREB1 and its targets (Lashine et al., 2015; S. Liu et al., 2011), while miR-155 knockout results in restored Creb1 (Gaudet et al., 2016). BER activity is dependent on Creb1 binding to the c-AMP-response element (CRE) site at positions -49 to -40 in the mouse Pol β promoter (Kedar et al., 1991; Narayan et al., 1994; Sobol et al., 1996; Widen & Wilson, 1991). Promoter studies from Wilson group have clearly demonstrated that Creb1 binding to the Pol β promoter is crucial for Pol β transcription and efficient BER activity (Srivastava et al., 1998) A zebrafish study also showed that reduced Creb1 in *Ape1* knockdown results in low Pol β expression (Pei et al., 2011), a finding we have also seen in brain and liver of *Ape1* heterozygous mice (Raffoul et al., 2004). Thus, we sought to establish whether miR-155 overexpression, characteristic of the overexpression seen in DS, could explain the low Pol β levels observed in these individuals.

3.2 Materials and Methods

Tissue Culture SV-40 transformed mouse embryonic fibroblasts (MEFs; Tag 92), a generous gift from Robert W. Sobol, were maintained in DMEM (Gibco, Cat# 11995081) supplemented with 10% FBS, in a 5% CO₂ and 20% O₂ humidified incubator, at 37°C.

Transfections SV40 transformed mouse embryonic fibroblasts (MEFs) were electroporated with either pEGP-miR-155 or pEGP-miR-null. Cells incorporating the construct were selected and pooled by treatment with puromycin. Briefly, cells were grown to 75% confluence, then collected and counted. 1×10^6 cells in 200 μ l volume were transferred into a sterile Bio-Rad GenePulser electroporation cuvette (0.2 cm; Bio-Rad) along with 10 μ g of pEGP-miR-155 or control pEGP-miR-null (Cell Biolabs, San Diego, CA) plasmid DNA. The mixture was gently mixed, then pulsed at 220 V and 500 μ FD, using a Bio-Rad GenePulser electroporation apparatus (Bio-Rad). Freshly electroporated cells were then transferred into 6-well plates containing 2ml of complete growth media and incubated at 37°C and 5% CO₂ for 24 hours. The media was changed after 24 h and total RNA was extracted using Trizol (Invitrogen) at 72h posttransfection. Stable clones were selected beginning at 48h posttransfection with 3 μ g/ml puromycin (Sigma-Aldrich, St. Louis, MO), for 2 weeks.

Plasmid constructs A plasmid containing the Polb core promoter (positions -114 to +62), which exhibits the same CAT activity as a 4.6kb fragment, was kindly gifted by Dr. Samuel Wilson. The promoter sequence was PCR-amplified using the following primers: forward 5'CCGCGCCGGCACGCCTCACAAACAGTA-3' and reverse 5'GAGCCGGAGGGCGGCCCGGGACTCAC-3'. Subsequently, it was cloned into the pGL3 basic vector, upstream of the luciferase gene. The CRE site in the minimal promoter was mutated

(TGACGTCA →TGAatTC) by using the Q5 Site-Directed Mutagenesis kit (NEB, Ipswich, MA) with the following primers: forward TGGCGCGTGAATTCACCGCGCTGCGCC and reverse GGCTACGGGGCGGGGCTA. Both constructs were sequenced to verify accuracy.

MicroRNA 155 Expression Total RNAs was isolated using Trizol. 50 ng of total RNA were used for reverse transcription (RT). RT reactions comprised 0.15 µL dNTP, 1.5 µL buffer (10x), 9 µL RNase-free water, 0.2 µL RNase inhibitor, 1 µL Multiscribe Reverse Transcriptase, and 3 µL of specific miR-155 RT primers (Applied Biosystems). RT reactions for primer extension and synthesis of the first cDNA strand were set at 16°C for 30 minutes, 42°C for 30 minutes, and 85°C for 5 minute, using a BioRad thermal cycler. The TaqMan miR-155 assay (Applied Biosystems) was used to quantify miR-155. 5 µL of RT reaction were mixed with 10 µL of TaqMan Universal PCR master mix (Applied Biosystems), 4 µL of RNase-free water, and 1 µL of TaqMan miRNA Assay (Applied Biosystems). Quantitative real-time PCR analysis was performed using LightCycler 480 (Roche) at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 60 seconds. Sno202 (Applied Biosystems), a noncoding RNA, was used as endogenous control.

Expression analysis cDNA was synthesized, as described previously (Cabelof et al. 2006a), from 2µg RNA, using random hexamer primers and purified with the QIAquick PCR Purification columns (Qiagen, Valencia, CA). Transcripts were amplified and quantitated with a LightCyclerReal Time PCR machine (Roche). PCR reactions contained 2µl purified cDNA, 0.5 µM of each sense and antisense primer, and 2µl FastStart DNA Master SYBR Green I enzyme-SYBR reaction mix (Roche). For all amplifications, PCR conditions consisted of an initial denaturation step at 99°C for 10 min, followed by 35–45 cycles at 96°C for 10s, primer specific annealing temperatures for 10s, and elongation at 72°C for 5s. Melting curves from 40°C to 99°C

confirmed specificity. External standards were prepared for all genes from cDNA amplicons cloned into pCRII TOPO cloning vector (Invitrogen). All transcripts were quantitated and normalized to GAPDH or RPL4 expression. Primer sequences are detailed in Table 3.1.

Western blotting Cells overexpressing miR-155 and null were pelleted and stored at -80°C until further use. Nuclear proteins were extracted using the NucBuster™ Protein Extraction Kit (Novagen, Darmstadt, Germany) and quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific), as per the manufacturer protocols. 40 ug of nuclear extracts were ran on 10% TGX Stain-free precast gels (BioRad) and transferred to 0.2um PVDF by using the Trans-Blot Turbo Transfer System (BioRad), according to the manufacturer's recommendations. Loading and transfer consistency was determined by UV-activation of the gel and membrane. Following activation, blots were incubated for 30 minutes in blocking buffer (5% BSA in PBST) and overnight at 4C in primary antibodies, at 1:500 dilutions: CEBP α (Abcam, ab18336) and Pol α (Abcam, ab3181). Blots were subsequently washed in PBST and incubated for 90min at room temperature in anti-mouse IgG-HRP secondary antibody (Cell Signaling, #7076), diluted at 1:5000 in PBS. Bands were detected using a ChemiImager, following activation with the SuperSignal Chemiluminescent Substrate luminol/enhancer (BioRad). Optical density was determined using ImageJ (Schneider, Rasband, & Eliceiri, 2012) and data were expressed as band intensity normalized to total protein intensity.

Luciferase Assay Stable miR-155 and control clones were co-transfected with 5ug of pGL3-Pol β promoter plasmid, which includes the CRE binding site (TGACGTCA), and 25ng of Renilla vector, which acts as control for transfection efficiency. The mixture was gently mixed, then pulsed at 200V and 500 μ FD, using a Bio-Rad GenePulser electroporation apparatus (Bio-Rad, Hercules, CA). Media was changed 24 hours posttransfection and cells were grown for

additional 24 hours before analysis. Luminescence was detected using a Dual Luciferase assay (Promega, Madison, WI, USA) and quantified with a Turner Biosystems 20/20 Luminometer (Promega). Relative light units (RLUs) were calculated as luciferase activity normalized to Renilla activity. Data represent the average \pm standard deviation of triplicate samples.

Proteomics

Stable isotope labeling with amino acids in cell culture (SILAC): Select miR-155 overexpressing and null cell lines were maintained in DMEM media supplemented with 10 % dialyzed fetal bovine serum, 0.46 mM l-Lys-HCl or 0.46 mM 13C6, 15N2-Lys-HCl, 0.47 mM l-Arg-HCl or 0.47 mM 13C6-Arg-HCl, 200 mg/L l-proline, 2 mM glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin, in a humidified 5 % CO₂ atmosphere (G. C. Parker, Carruthers, Gratsch, Caruso, & Stemmer, 2016). Cells were passaged three times a week and harvested for experiments after six passages in the SILAC media. Cells were harvested by washing and scraping, then spun and stored in pellets at -80°C until analysis.

Sample preparation Cells were resuspended in 100 μ L of water, followed by addition of 100 μ L of 2 % LiDS, and incubation in 95 $^{\circ}\text{C}$ water for 5 min. Proteins in the lysates were determined using a BCA protein assay (Pierce, Rockford, IL). Equal amounts of protein from SILAC heavy and light cell lysates were combined and treated with 10 mM DTT and alkylating agents with 30 mM iodoacetamide, before adding 10 mM additional DTT. Samples were fractionated on 10 % polyacrylamide SDS-PAGE gels and stained with Coomassie blue. Each of the three sample lanes was divided into 30 fractions with the edges of each lane removed prior to slicing for analysis. Proteins in the gel were digested overnight with 0.04 μ g trypsin per slice in buffer containing 20 mM Tris (pH 8.0) and 10 % acetonitrile. Eluted peptides solubilized in 0.1 % formic acid were analyzed by LC-MS/MS, without further purification.

LC-MS/MS analysis Two GelC-30 SILAC experiments were performed: C1 with miR-155 heavy and null light and C2 with null heavy and miR-155 light. Mass spectra were searched against the Uniprot mouse database downloaded 2014.06.24, using MaxQuant version 1.5.28. Cysteine carbamido methylation was specified as a fixed modification and protein N-terminal acetylation and methionine oxidation were specified as variable modifications (Carruthers, Parker, Gratsch, Caruso, & Stemmer, 2015). Unique and razor peptides were both used for protein quantification. We identified 3342 proteins that were present in both SILAC experiments. An abundance ratio (miR-155/null) was determined for each protein group using ratios from all peptides that could be assigned to it. Ratios were log-transformed and normalized so that the median log-fold change for each experiment was 0.

Immunofluorescence staining 4×10^4 cells were seeded on coverslips in 12-well plates and fixed in ethyl: acetate (80:20) for 5 min. After three washes, cells were blocked in PBST containing 3% BSA for 30 min at room temperature and incubated with primary anti- γ H2Ax antibody (Millipore, Cat# 05-636) for 1.5hr at room temperature or overnight at 4°C. Cells were then incubated with Alexa Fluor 488-conjugated anti-mouse IgG (1:400) (Invitrogen, Cat# A-11001) for 1 hr at room temperature, and mounted with Pro-Long Gold anti-fade reagent (Invitrogen, CA). Slides were photographed under the Nikon Eclipse 80i microscope (Nikon, CA) and processed using the Nikon Elements built-in software. Approximately 300 cells were counted in more than 10 fields of view and those cells displaying more than 7 distinct foci/nucleus were considered positive for γ -H2AX.

Data Analysis

Results are expressed as mean \pm SEM analyzed and were analyzed using Student's t-test. Statistical comparisons between groups were conducted using one-way ANOVA, using

GraphPad Prism 5.0 (Graph Pad Software, La Jolla, CA). Values of $p < 0.05$ were considered statistically significant and individual p-values are shown in the figures.

3.3 Results

We generated several miR-155-overexpressing clones, exhibiting a range of 2-12 fold miR-155 overexpression (Figure 3.3.1a, $p < 0.05$). Most of the subsequent experiments were done in clone 3, which overexpresses miR-155 at levels 2-3 fold above control, consistent with the level of overexpression observed in DS. This level of miR-155 overexpression is therefore relevant to our objective of studying gene dosage of Trisomy 21. Additionally, we confirmed our miR-155 overexpression by quantifying protein levels of previously validated targets of miR-155. Cebp β , previously shown to be negatively regulated by miR-155 (Jiang et al., 2012; S. Kim et al., 2016), had reduced protein levels in our engineered miR-155 MEFs (Figure 3.3.1b).

Next, we analyzed changes in Pol β mRNA and protein in two stable miR-155 clones (2 – and 12-fold). The low overexpressing cells showed an approximate 50% reduction in Polb transcript ($p < 0.01$ Figure 3.2a), reflected also by reduced PolB protein. However, cells overexpressing miR-155 at ~12 fold showed no difference in Polb transcript and protein, suggesting a dose dependent regulation (Figure 3.2b).

To evaluate the impact of miR-155 overexpression on promoter activation, we have cloned the PolB core promoter, including the CRE site, into a luciferase vector as described in Methods. We find that overexpression of miR-155 effectively inhibits PolB promoter activity by almost 5 fold (Fig 3 $p < 0.01$). In addition, when the Creb1 binding site in the core promoter is mutated, the luciferase activity becomes undetectable. Taken together, these *in vitro* results suggest that miR-155 inhibition of Creb1 translation could be responsible for PolB inhibition.

We show in Chapter 2 that Pol β null cells exhibit a high number of DSBs, both at baseline and in response to DNA damage. A recent study also showed that cells overexpressing miR-155 leads to initiation of the error prone NHEJ repair (Czochor et al., 2016). The mutational specificity of miR-155 overexpression is consistent with upregulated NHEJ (Czochor...), but not consistent with a MMR deficiency, suggesting that the DNA repair defects induced by miR-155 overexpression are related more to strand break resolution and less to post replication repair. To further investigate this point, we measured DSB by γ -H2AX staining in miR-155 overexpressing cells. In the miR-155 clone that mimicked DS conditions, we saw an almost 17% increase in γ -H2AX foci compared to ~ 4% increase in the null control (Figure 3.4a). In the miR-155 clone with 12-fold overexpression, we did not see either an increase in DSB or a loss of Pol β (Figure 3.4b), strengthening the connections between Pol β loss and DSB formation. Further, this creates an opportunity to connect the mutagenicity and DNA damage of miR-155 overexpression to polB and altered BER.

In order to conduct an unbiased evaluation of the impact of miR-155 overexpression on DNA repair genes, we employed an unbiased proteomic approach to discover which DNA repair proteins and pathways are affected by miR-155 overexpression. Stable isotope labeling with amino acids in cell culture (SILAC) is a tool that allows for differential labeling of amino acids. Amino acids with substituted stable isotopic nuclei labeled either “light” or “heavy “are introduced into the growth medium. Cells in culture will incorporate the labeled amino acid instead of the natural amino acid into all newly synthesized proteins (Mann, 2006). LC-MS/MS allows for analysis of the ratio between light and heavy in each identified peptide. This approach generated 4000 differentially expressed proteins in response to miR-155 overexpression. Preliminary analysis of these 4000 proteins revealed the pleiotropic effects

known to be exerted by this promiscuous miRNA. Multiple pathways and numerous gene products are differentially impacted by miR-155 overexpression, even at low levels of overexpression. Using the beta version of DAVID 2008, we established that these proteins belong to these processes such as: oxygen metabolism, dNTP metabolism, DNA damage response, and DNA repair. We have validated our proteomics findings both by comparing the proteins identified in our screen to those that have been previously validated as direct targets of miR-155 (Table 3.5.1), and by western analysis. Using the DIANA tool in TarBase (Vlachos et al., 2015), we set protein identification filters for: *mus musculus*, directly validated targets of miR155, and a prediction score of >0.95. This generated a list of 31 total mouse proteins identified as direct miR-155 targets that have been published in the literature. Table 3.5.1 lists the proteins that overlap with our proteomics data, and 12 of our genes match the list generated by DIANA. 39% of all prior validated targets for miR-155 in *mus musculus* are represented in our data set. This is particularly interesting in light of the fact that our system employed a completely different cell type (fibroblasts), pointing to the potential universality of the effect of miR-155 on these specific gene products. In addition, by western analysis we measured protein levels of CEBP β , a known human target of miR-155 (S. Kim et al., 2016), and find that in our system we likewise see downregulation of this miR-155 target.

Next, we evaluated our data set for differential expression of DNA repair proteins. i We find that theMMR proteins MSH6 and MSH2 are reduced in miR-155 overexpressing cells, consistent with published reports(N. Valeri et al., 2010). Interestingly, we found that several BER genes downstream of Pol β are also affected (Table 3.5.2). However, a search using the TargetScan database revealed that none of theBER genes contain seed sequence for miR-155 in their 3'-UTR, suggesting an indirect regulation by miR-155(V. Agarwal, Bell, Nam, & Bartel,

2015). Notably, all the upregulated BER proteins shown in Table 3.5.2 are downstream of the PolB reaction in BER.

3.4 Discussion

miR-155 is one of the most widely studied microRNAs. Based on its broad importance and on its known impact on another DNA repair pathway, mismatch repair, we set out to determine whether HSA21-localized miR-155 played any role in regulating Pol β . While there is no indication that Pol β would be a direct target of miR-155, there was abundant evidence that the transcription factor, Creb1, was directly, negatively regulated by miR-155 (S. Liu et al., 2011). Because Creb1 and the CRE in the Pol β promoter are essential for transactivation of polB transcription, we hypothesized that inhibition of Creb1 by miR-155 would negatively impact Pol β expression. Our data clearly establish that miR-155 overexpression results in downregulation of Pol β expression. In miR-155 overexpressing MEFs, we show downregulation in both Pol β transcript and protein. This is the first reported effect of miR-155 on a DNA repair protein outside the MMR pathway, and points to a role for Pol β in the phenotypes induced by miR-155 overexpression. In chapter 2 we demonstrated clearly that loss of Pol β results in a significant accumulation of DNA DSB. Here we find that miR-155 overexpression likewise causes accumulation of DSBs, conceptually tying the loss of Pol β in miR-155 overexpressors to the DSB. This is interesting and counterintuitive within the context of a recent paper in which strand breaks were suppressed in miR-155 overexpressing cells, but were elevated in cells with miR-155 silenced (Czochor et al., 2016). However, in that work the level of miR-155 overexpression was more than 50-fold overexpression. In that same paper, the authors found no impact of miR-155 on Pol β , but did see a reduction in Pol δ . Our data set, on the other hand, showed no effect on Pol δ . Many studies investigating the roles of miRs on various outcomes

utilize systems that largely overexpress the miR of interest (hundreds of folds overexpression). This may be relevant to the study of cancer, as some types of cancer exhibit large fold increases in miR-155 expression. But for our purposes of studying the impact of a trisomy 21-induced gene dosage effect of miR-155, those studies are irrelevant. One important finding to come from our studies is that the level of overexpression can drastically vary the impact of miR-155 on its targets, and that blanket statements about the role(s) of this miRNA must take into consideration dosage context.

To extend our investigation into a more complete evaluation of the entire BER pathway, we looked in Target Scan for BER(V. Agarwal et al., 2015) genes that might have miR-155 seed sequence in their 3'UTR, but found none. However, this approach does not take into consideration potential indirect targets, like in the case of Pol β . So we proceeded to compare our proteomic data set against all known DNA repair proteins (Wood RD, 2014) and found several BER proteins downstream of the Pol β step of BER that are also affected by miR-155 overexpression (Table 3.5.2). Notably, these proteins are all upregulated. Each of these genes conceivably plays a role in helping to resolve the DNA repair intermediates induced by loss of Pol β (i.e., DNA single strand breaks). We suggest that the loss of Pol β generates damage that induces an adaptive response in these particular BER genes to minimize the impact of accumulating repair intermediates on the formation of DSB. Because single strand breaks at the replication fork can cause replication stress and DSB accumulation, we further suggest that eliminating these proteins should amplify the DSB accumulation induced by miR-155 overexpression.

To conclude, our data definitively show that miR-155 overexpression inhibits Pol β and induces DSB. It remains unclear what the mechanism of Pol β inhibition may be. We propose

two possible mechanisms. The first is through Creb1 downregulation induced by miR-155 overexpression, as described above and supported by the reduced protein levels of Creb1 we observe when we overexpress miR-155 (data not shown). Another, not mutually exclusive mechanism could be through Foxo3. miR-155 could regulate BER via FOXO3a and its target, Gadd45a. Gadd45a has been shown to activate BER and play a role in recruiting other DNA repair genes. As such, inhibition of Gadd45a by Foxo3 could block BER. Further investigation into the precise mechanisms of Pol β inhibition by miR-155 is necessary in order to move toward developing interventional strategies to protect genome integrity in individuals with Down syndrome.

Gene	Sense primer 5'–3'	Anti-sense primer 5'–3'
<i>Mouse</i>		
B-pol (exon 12–13)	agcgagaaggatggaaggaa	Cgtgcgctctcatgttcttat
<i>Housekeeping genes (mouse)</i>		
Gapdh	aggtcggtgtgaacggatttg	Tgtagaccatgtagttgaggta
Rpl4	ccgtcccctcatatcgggtga	Gcatagggctgtctgtgttttt
Rpl4 UPL probe # 75	tggtggtgaagataagggtga	Ccaagcttgagttcttgagc

Table 3.1: Primer sequences

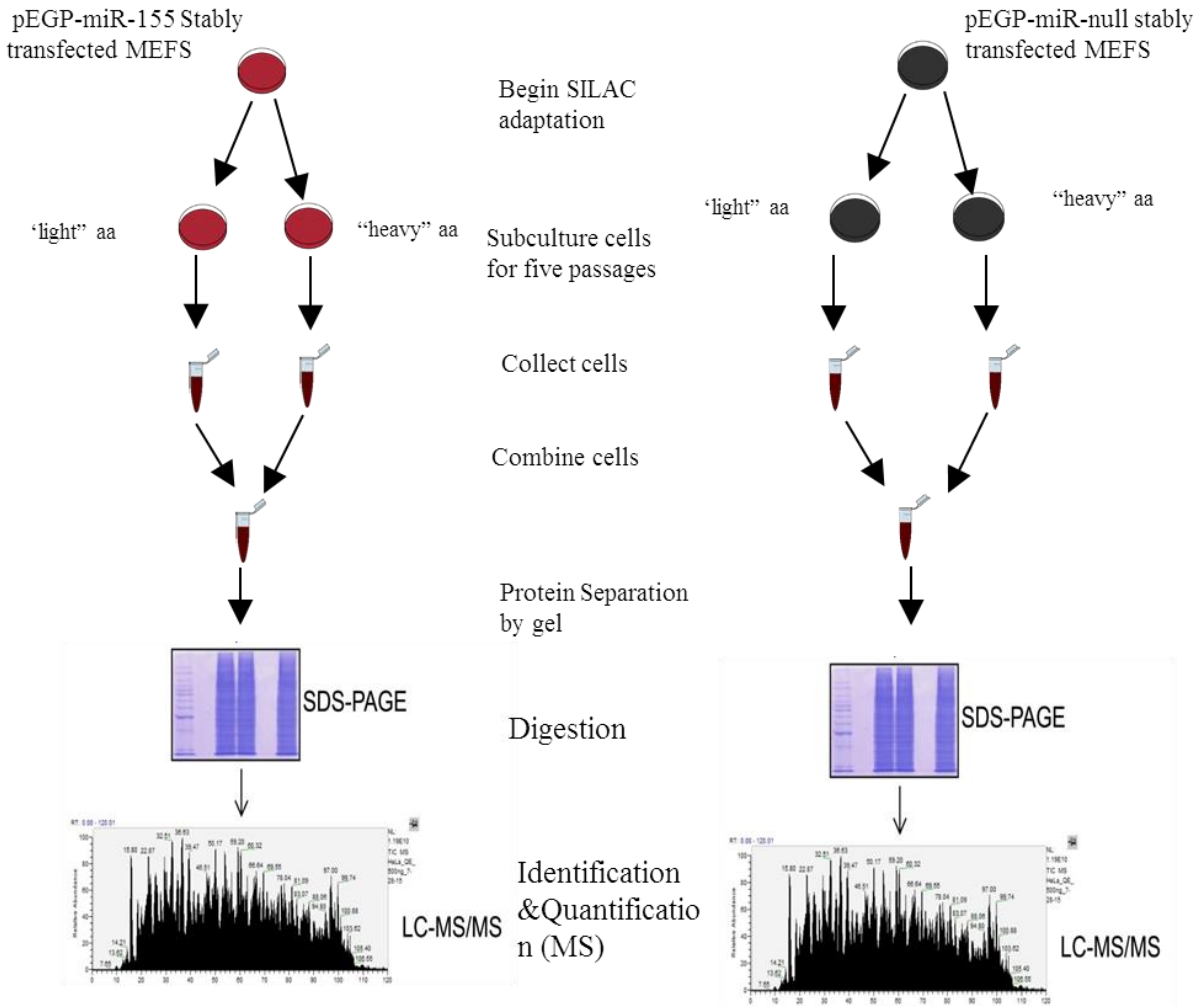


Figure 3.2 SILAC-based quantitative proteomics. Flowchart of double SILAC coupled with LC-MS/MS .

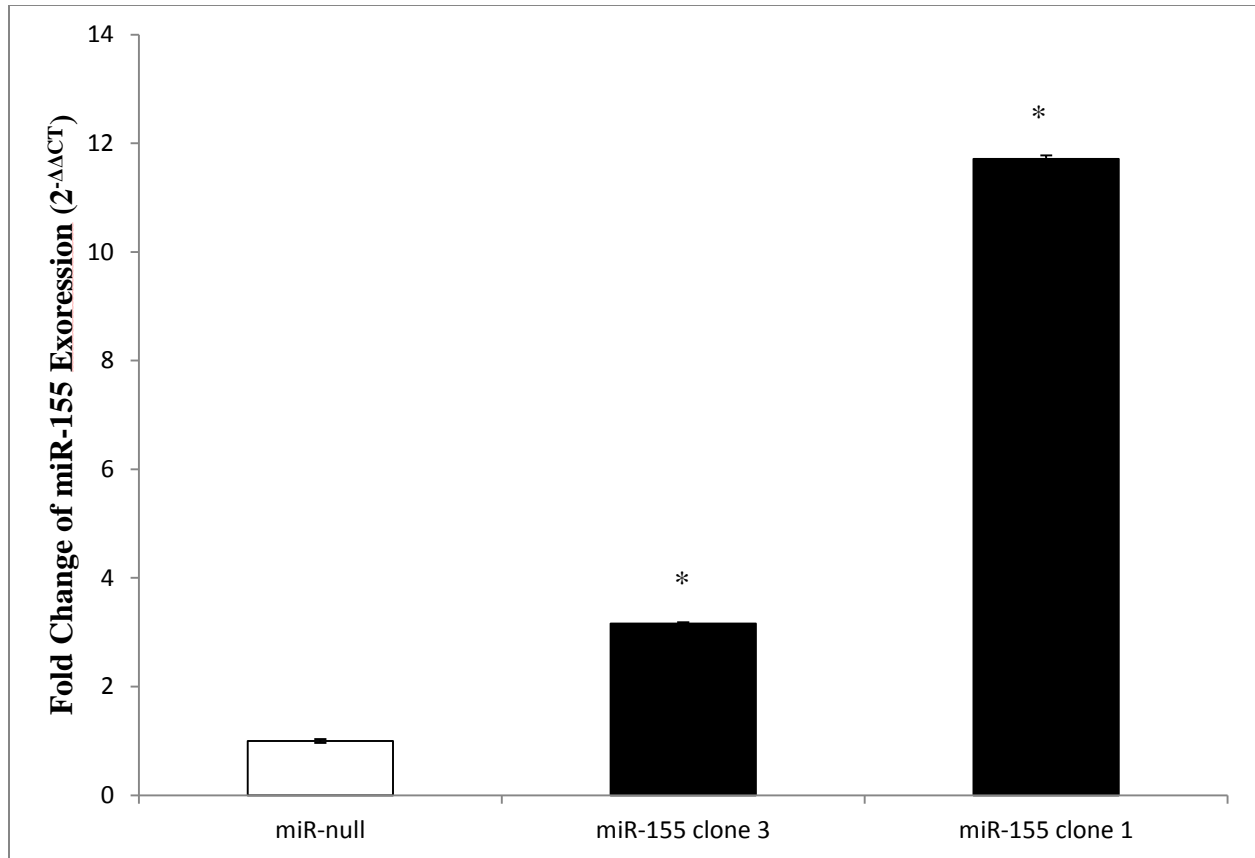


Figure 3.3.1: Ectopic overexpression of miR-155 in MEFs. miR-155 overexpression in MEFs by electroporation is confirmed by qRT-PCR and normalized endogenous control, sno202. Data presented is average \pm SEM. Delta $\Delta\Delta$ CT method was used to calculate fold change.

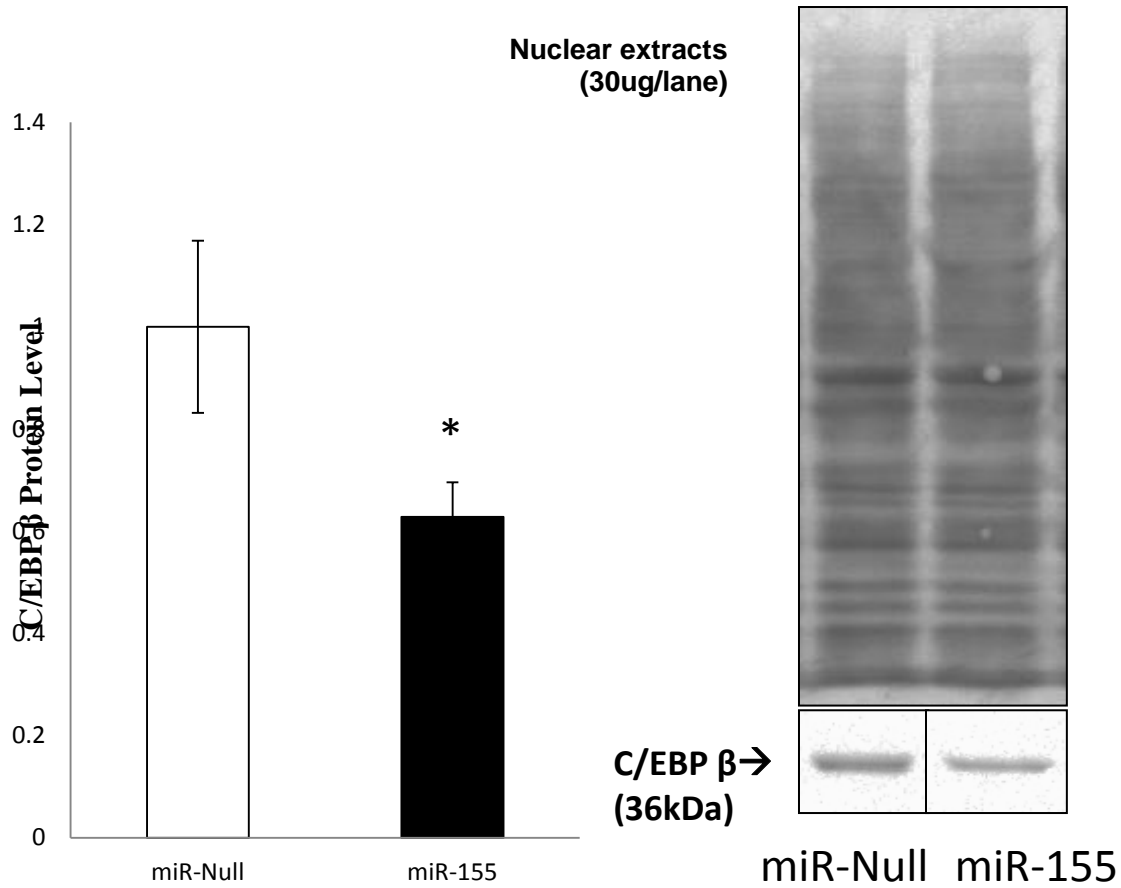


Figure 3.3.2: Validation of C/EBP β downregulation in miR-155. The levels of C/EBP β protein from miR-null and miR-155 overexpression cells was determined by western blot analysis. The level of C/EBP β protein was normalized to whole protein. Data are presented as means \pm SEM of four replicates.

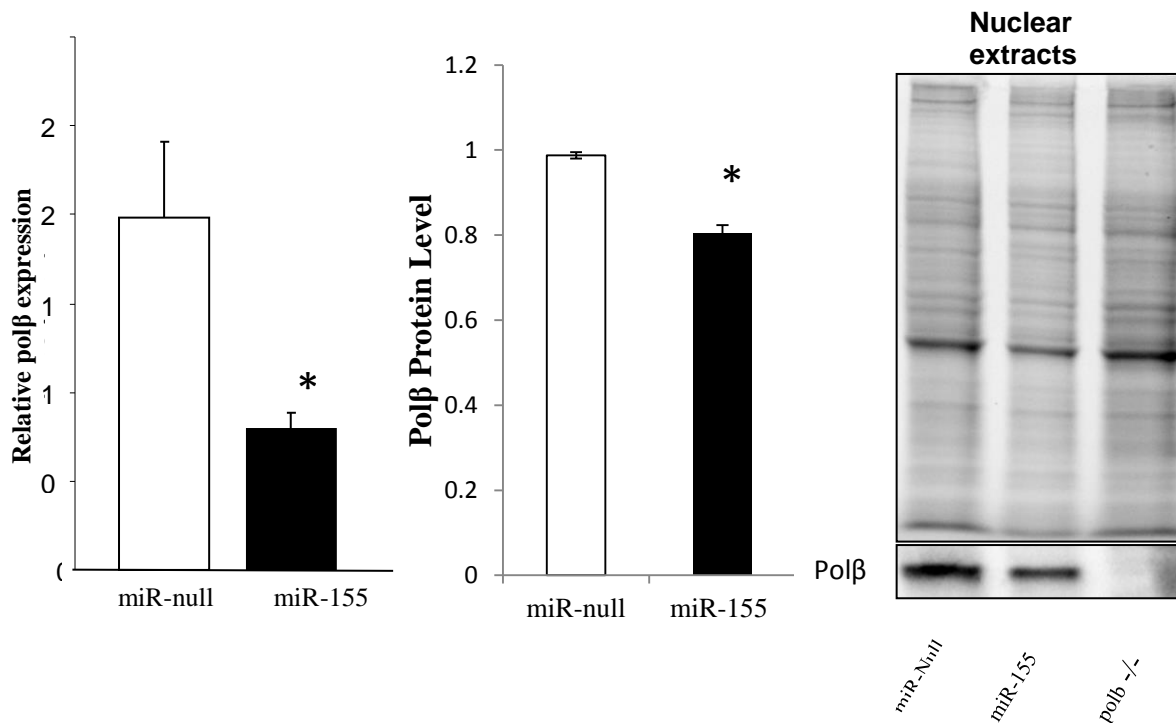


Figure 3.3.3: Polβ transcript and protein expression is downregulated in miR-155 Cells.

Polβ transcript expression was determined by q RT-PCR in miR-null and miR-155 overexpression cells. Expression was normalized to Rpl4, and data is presented as mean \pm SEM. *Value significantly different from control at $p < 0.01$. The levels of Polβ protein was determined by western blot analysis. The level of Polβ protein was normalized to whole protein. Data are presented as means \pm SEM of three biological replicates.

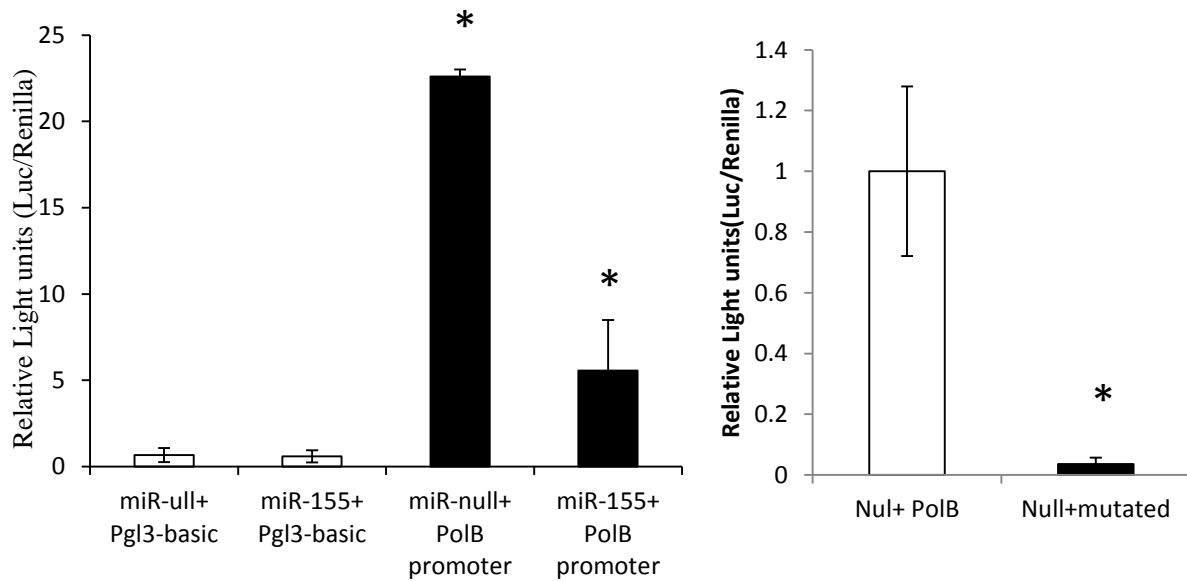


Figure 3.3.4: Pol β Promoter activity is inhibited by miR-155 overexpression. A. Pol β constructs (2 μ g) were co-transfected with a Renilla luciferase plasmid, serving as an internal control for transfection efficiency, into miR-null and miR-155 cells, and luciferase activity was measured 48 h post-transfection. . B. Luciferase expression in Mutated PolB construct transfected into miR-null cells.

<u>Validated miR-155 targets</u>	<u>up/down</u>	<u>cell type(s)</u>	<u>Our data</u>	<u>fold change</u>
Myo1d	Down	Muscle	Down	2.68
Nr2f2	Down	Muscle	Down	1.5
Strn3	Down	Muscle	Up	1.41
Rps6ka3	Down	Muscle	Down	1.28
Trps1	Down	Muscle	Up	1.24
Rcor1	Down	T cells; muscle	Up	1.22
Tab2	Down	T cells; stem cells	Down	1.19
Fosl2	Down	T cells; muscle	Down	1.18
Kdm2a	Down	T cells; muscle	Up	1.18
Irf2bp2	Down	T cells; muscle	Down	1.1
Cdc73	Down	T cells; muscle	Up	1.05
Pkn2	Down	T cells	Up	1.03

Table 3.3.5 Validated miR-155 targets shown in our dataset.

DNA REPAIR	Fold change
<i>Mismatch repair</i>	
MSH3	-1.8
MSH2	-1.3
MSH6	-1.3
<i>BER/strand break</i>	
DNA ligase3	1.31
DNA ligase 1	1.41
Xrcc1	1.46
Pnkp	2.12
Parp2	1.76
Fen1	1.26
Xrcc4	1.37
DNA polk	1.5

TABLE 3.3.6: Impact of miR-155 overexpression on proteins involved in DNA repair.
 Proteins involved in MMR and BER/SSB

CHAPTER 4: TS65DN MOUSE MODEL

4.1 Summary

It is important to consider that no mouse model can be a perfect model of DS, for the reasons enumerated below, and as summarized in Vacano et al. (Vacano, Duval, & Patterson, 2012). That said, the Ts65Dn model phenocopies much of DS, even though it is trisomic for only about 50% of the HSA21 genes. This model is particularly appropriate as it exhibits signs of premature aging (Adorno et al., 2013): fibroblasts from these mice senesce early (Contestabile et al., 2009). Our objective was to do an extensive evaluation of aging and senescence in tissues from these mice and their disomic littermates (WT) at different ages. Experiments were conducted in male mice only, to preserve female animals for breeding purposes. Expression levels of p16 and Pol β will allow us to correlate premature aging and reduced DNA repair capacity.

4.2 Introduction

Mouse models have been one of the most useful tools in studying DS pathogenesis due to the homology existing between human genes on chromosome 21 and mouse genes on chromosome 16, chromosome 10 and chromosome 17 (Rachidi & Lopes, 2007). Mouse models allow scientist to dissect many of the features of DS as well as develop therapeutic drugs for ailments that afflict individuals with DS. In the last 40 years researchers have been able to study the molecular, cellular, physiological, and behavioral phenotypes observed in human DS through the use and manipulation of mouse models.

Two types of mouse models have been developed to study the molecular genetics of DS: segmental trisomic models and transgenic models. Each model has its limitation but each offer insight into the molecular mechanisms driving the DS phenotype. The segmental trisomic

models have the advantage of mimicking many of the clinical phenotypes observed in DS individuals. Despite this, their drawback remains species-specific gene differences between mice and humans (Rachidi & Lopes, 2007). Alternatively, the transgenic mouse models, overexpressing only one or few genes, allow a direct genotype–phenotype relationship, but the disadvantage of these mouse models is the loss of critical interactions between genes present at three copies (Rachidi & Lopes, 2007).

4.3 Trisomic mice

The first mouse model of DS developed was Ts16 using the rationale that MMU 16 and HSA21 were close in sentry. A breeding scheme using a balanced Robertsonian translocation of MMU 16 was devised. A male mouse carrying Robertsonian translocations of MMU 16 was mated with a normal female thus producing approximately one-third of the progeny with Trisomy of MMU16. The model presents several characteristics of DS such as increased oxidative stress, mitochondrial dysfunction and nervous system impairment (Cox, Smith, Epstein, & Epstein, 1984; Pagano & Castello, 2012). Ts16 mice develop cardiovascular anomalies similar to those seen in DS (Villar et al., 2005). Severe thymic hypoplasia and delayed maturation of thymic lymphocytes are also observed in Ts16 mouse (Epstein, Cox, & Epstein, 1985). The key function of the thymus is to provide an area for T-lymphocyte maturation and in DS this process is significantly impaired (Kusters, Verstegen, Gemen, & de Vries, 2009; Peled-Kamar et al., 1995). DS children display many signs of thymic hypoplasia such as T-cell dysfunction and increased risk for infections, lymphoproliferative disorders, and autoimmune diseases (De Leon-Luis et al., 2011).

With respect to cognitive function, Gearhart et al demonstrated impairment in the development of the basal forebrain cholinergic neuron (impaired learning) and increased

susceptibility of these mice to develop AD. They exhibit a two fold increase in amyloid precursor protein (APP) accumulation, a protein implicated in senile plaque formation in AD. Cells cultured from these Ts16 mice have led to substantial insight on neuronal development in DS as well as in Alzheimer disease (AD). Ts16 lines overexpress other key genes involved in the pathogenesis of AD. These include Cu/Zn superoxide dismutase (SOD-1), Ets-2 transcription factors and Down Syndrome Critical Region 1 (DSCR1) stress-inducible factor (Lott, Head, Doran, & Busciglio, 2006). The overabundance of these genes due to Trisomy causes a pathological cascade that cause oxidative stress and a neurogeneration typical of AD (Lott et al., 2006). These findings are analogous to what is observed in DS brain.

Nonetheless Ts16 is not an ideal model due to its many imperfections vis-à-vis human DS. It does not accurately recapitulate human DS aneuploidy because Mmu16 is bigger than HSA21 and contains many genes homologous with HSA3, HSA8, HSA16, and HSA21 (Noemí Rueda, Flórez, & Martínez-Cué, 2012). The extra genes that are not implicated in DS may contribute to the perceived DS phenotype in the mouse models, confounding the story. Further, one key characteristic of DS is degeneration of basal forebrain cholinergic neurons (BFCNs). The Ts16 mouse does not exhibit this trait, and many others as well, so it does not faithfully recapitulate enough of the DS phenotype to be an ideal model. Another disadvantage is that the Ts16 genotype is embryonic lethal limiting the ability to research postnatal DS phenotypes (Seregaza, Roubertoux, Jamon, & Soumireu-Mourat, 2006; Villar et al., 2005).

Another model, the Ts(16C-tel)1Cje (Ts1Cje) mouse, is created by a translocation between MMU12 and MMU16. When a gamete carrying MMU12 with translocation of MMU16, and another complete MMU16 combines with a normal gamete, a partial Trisomy of the distal region of MMU16 results (Sago et al., 1998). Consequently this scheme allows only a

small region of MMU16 to be trisomic, and about one third of genes seen in the Ts65Dn model are trisomic in Ts1Cje mice (Dierssen et al., 2001). Unlike Ts65Dn (described below), Ts1Cje mice exhibit very few DS phenotypes. Craniofacial anomalies are not visible, learning deficits are not severe, and the age-dependent degeneration of BFCN are absent in these mice. However, this does not mean that Ts1Cje is of no use, as comparison between other trisomic models can allow for the pinpointing of genes involved in some DS phenotypes. For example, neuronal atrophy is absent in Ts1Cje and present in Ts65Dn, suggesting that the missing region, from APP to SOD1, is required for the pathology to develop (Sago et al., 1998).

The newest model mouse to emerge is from a group out of England. Tc1 (Tc = transchromosomal). Unlike the previously discussed models, this mouse carries an almost complete copy of human chromosome 21 (approximately 92% of all genes). It recapitulates almost all DS phenotypes including the heart defects seen in DS newborns (Galante et al., 2009). While this sounds like the ideal model, genetically speaking it's identical to DS in humans, it has many weaknesses. For one, the coexistence of human chromosome and human proteins in mouse environment can be problematic for interpretation, since mice and humans have various differences that underline development and aging. Further, a primary issue with the Tc1 model is that the freely segregating HSA21 is not found in all tissues, making this more relevant to mosaic type of DS.

The DS model we have chosen to use for our studies is the most widely used and studied DS mouse model: Ts(17¹⁶)65Dn mouse (Ts65Dn). This mouse carries a segmental trisomy, created by Davisson et al.. To create this mouse, testes of DBA/2J male mice were cesium irradiated and then bred to C57BL/6J female mice (Lorenzi, Duvall, Cherry, Reeves, & Roper, 2010). The resulting offspring that carried reciprocal translocations for Mmu16 were bred to

B6C3F1 mice (Davisson et al., 1993). This resulted in Ts65Dn heterozygote mice producing offspring with the small marker chromosome consisting of the telomeric Mmu16 attached to a Mmu17 centromere (Dierssen et al., 2001). Ts65Dn is trisomic for roughly 104 genes (from App to Mx1) that are homologous to HSA21 and have been shown to be responsible for several DS phenotypes (Noemí Rueda et al., 2012). Phenotypes exhibited in Ts65Dn mice include developmental abnormalities, cognitive and neurological impairments, craniofacial abnormalities, and age-related deterioration of BFCNs (Roper, St John, Philip, Lawler, & Reeves, 2006). Another characteristic of Ts65Dn is the increase in oxidative stress markers seen in young and old mice (Domenico et al., 2015). What makes this model practical is that the mice survive live birth and age to adulthood allowing for postnatal research. This allows researchers to study many aspects of DS such as the premature aging phenotype as well as the development of AD. Proliferation impairment was reported in cultured fibroblasts from newborn Ts65Dn. Ts65dn fibroblasts uptake of BrdUrd, a thymidine analogue that is incorporated by proliferating cells during the S-phase of cell cycle was reduced by 30% (Contestabile et al., 2009). The fibroblast also exhibited morphologic changes characteristic of senescent cells such as enlarged, flattened shape and increase in number of SA-Beta Gal positive cells (Contestabile et al., 2009). In vivo studies also showed Ts65Dn mice having an impaired proliferation in skin collected from 2 day old Ts65dn post BrdU injection (Contestabile et al., 2009). These findings show a premature aging phenotype that mimics what has been reported in DS. As they age, they develop pathologies consistent with other age related disease such development of AD and increase incidence of lymphomas (Levine et al., 2009).

Even so there are drawbacks associated with Ts65Dn model. For one, male progeny are sterile thus expansion is dependent on female mice. Though most features of DS are

recapitulated, some are nonexistent, notable they do not exhibit the heart defects seen in DS. Also Ts65Dn is only a partial Mmu 16 Trisomy, accounting for approximately 60% of genes on HSA21. Despite these weaknesses, the advantages of the Ts65Dn model have been used/exploited for the study of cognitive impairment, AD, and aging in DS.

Ms1Ts65 is a DS mouse model created by crossing Ts65Dn female (see below) and Ts1Cje male mice. Ms1Ts65 mice have been very useful in separating out the effects of genes present in Ts65Dn but not Ts1Cje mice on DS phenotypes. Comparison studies between Ts65Dn and Ms1Ts65 have demonstrated that the Ts65Dn phenotypes, absent in other segmental models, is most likely due to the interaction between the trisomic genes in the distal end of MMU 16 (Dierssen et al., 2001) On its own, the Ms1Ts65 model displays the fewest similarities to the DS phenotype when compared to other segmental trisomics models, though they have been useful in allowing us to have a better understanding of the genes found in DS critical region (DSCR) and their role of these genes in some phenotypes of DS.

4.4 Transgenic Mice

Despite the overwhelming advantage of full and partial Trisomy models, single gene mouse models overexpressing genes on HSA21 have also generated key findings in deciphering DS pathogenesis. We refer to these models as transgenic DS mice. The transgenic genes are believed to be important in driving DS phenotypes. This type of model allows researchers to examine the role of one gene at a time in the complex phenotype of DS. Though they don't recapitulate all DS traits, they do offer insight into role of single genes on development of DS. The Sod1 mouse was the first of these transgenic mice to be produced. Superoxide dismutase 1 (Cu-Zn), Sod1, is gene encoded on DSCR of HSA21 and MMU 16 is responsible for destroying free superoxide radicals by converting them to hydrogen peroxide (Florian L. Muller et al.,

2007). Transgenic mice over expressing Cu-Zn Sod1 show chronic oxidative stress as well as cognitive impairment (de Haan et al., 1995). The chronic oxidative stress has been linked to accelerated aging of many tissues and organism as a whole. Overexpression of SOD1 has been verified in many, but not all, tissues and cell lines of DS (Campos & Casado, 2015).

The amyloid precursor protein (APP) transgenic model is predominately used in Alzheimer's disease research. The humanized APP mouse is overexpressed through use of active promoters, allowing for overexpression of human APP in the mouse (Quon et al., 1991). In addition to APP overexpressing models, mutants mimicking human APP mutations have also been created, and have been useful in identifying key human APP mutation that may lead to Alzheimer's (Lott et al., 2006). Many of these transgenic models showed promising results but lacked full AD neuropathology, demonstrating pretty effectively that APP overexpression or mutation is not sufficient for development of AD. In 1995 Games et al. used platelet-derived growth factor promoter, (PDGF)-B to generate a transgenic mouse model overexpressing mutant human APP (with valine at residue 717 substituted by phenylalanine) (Games et al., 1995). This model, unlike the previous models, exhibited AD neuropathology, including amyloid beta deposition, dystrophic neuritic components, gliosis and loss of synaptic density (Games et al., 1995). Crosses of SOD1 and APP mice revealed to be more useful than their standalone models in elucidating AD neuropathology. As stated earlier, most transgenic models of APP lacked the formation of beta amyloid deposits, while SOD1-APP model recapitulated this phenomenon seen in DS individuals with AD. Multiple models of APP are actively used today in developing therapeutics to treat not only elderly AD patients but also DS individual with AD.

DYRK1A is a nuclear serine/threonine kinase that is localized to HSA21 and is overexpressed in several DS tissues (Altafaj et al., 2001). DYRK1A is thought by many to be a

key candidate driving nervous system alterations in DS. Altafaj et al., created a transgenic mice overexpressing DYRK1A under the control of the inducible sheep metallothionein-Ia (sMT-Ia) promoter (Altafaj et al., 2001). TgDyrk1A mice exhibit DS phenotypes that include neuromotor development, hyperactivity, and significant impairment in spatial learning and memory (Altafaj et al., 2001). Researchers trying to detect the cellular and molecular events that lead to the phenotype seen in TgDyrk1A mice and other DS models were able to demonstrate that Dyrk1A alters the timing of neural cell proliferation and differentiation (Yabut, Domogauer, & D'Arcangelo, 2010). Additional studies using more sophisticated mice models of Dyrk1a will allow a better understanding of the role of this gene on DS phenotype.

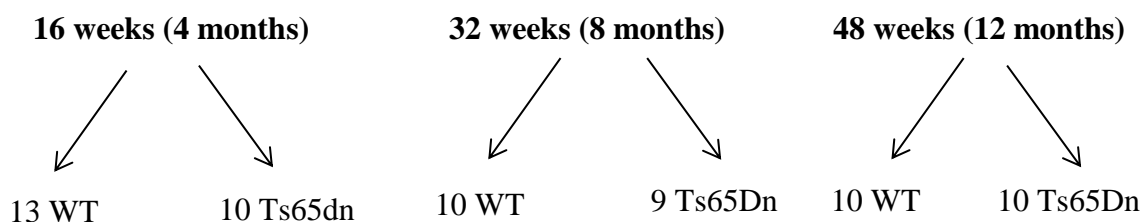
ETS2 is a member of the Ets family of transcription factors and is known to activate apoptosis in DS tissues. It is located within the critical region of DS on HSA2. DS neuronal cultures exhibit a fivefold increase in ETS2 expression (Rueda et al 2012). ETS2 has important roles in cancer, bone development and immune responses (Wolvetang et al., 2003). It should be noted that the overexpression of ETS2 is thought to be key in the low incidence of solid tumors in DS. This is believed to be due to its role in regulation of key apoptotic genes (N. Rueda, Florez, & Martinez-Cue, 2013). Like the other transgenic DS models, tgETS2 displays DS phenotypes such as smaller thymus and lymphocyte abnormalities (Wolvetang et al., 2003). ETS2 mice also display increased neural cell death, which leads to suggestion that it plays a major role in the neural degeneration seen in many mouse models of DS as well as in DS individuals (Rueda et al 2013)

S100 calcium binding protein β (S100 β) is found to affect the nervous system, specifically the glial cells. Transgenic S100 β mice were developed by placing the human S-100 β gene into the mouse genome, under control of its own regulating elements (Friend et al.,

1992). Studies by Whitaker-Azmitia et al. show transgenic mice overexpressing $S-100\beta$ have abnormal neuronal morphology as well as accelerated aging (Whitaker-Azmitia et al., 1997). When comparing the transgenic mice to control, young mice had increased density of dendrites and that as they aged they exhibit a significant loss of dendrite density, suggesting an important role of S100B in AD development in DS individuals (Whitaker-Azmitia et al., 1997).

4.5 Materials and Methods

Animals: Segmental trisomy 16 (Ts65Dn) mice were obtained by mating Ts65Dn females (B6EiC3H – a/ATs65Dn) with (C57BL/6JEi × C3H/HeJ)F1 (JAX # JR1875) males. Ts65Dn mice were thus maintained on the B6/C3H background (Davisson et al., 1993). Experimental male mice were housed at the Eleanor Roosevelt Institute at the University of Denver. Ts65Dn was genotyped using a quantitative PCR protocol. The technique is based on the DDCT calculation method between a control gene present in two copies (ApoB) and a target gene present in three copies (Mx1) (Lorenzi et al., 2010). Mice were organized into three cohorts (16 week, 32 week, and 48 week). A total of 63 mice were placed in three cohorts: 16 week (n=23) with 10 Ts65Dn and 13 WT, 32 week (n=20) with 9 Ts65Dn and 11 WT and 48 week (n=20) with 10 Ts65Dn and 10 WT. In each cohort, there were 9-11 mice in each group (~10 WT and 10 Ts65Dn). Animals were maintained in a 12:12-hour light/dark schedule (lights on at 7 AM) with *ad libitum* access to food and water. The cross-sectional pathological analysis of tissues from trisomic and disomic mice was carried out by Dr. Yuji Ikeno at UTHSCSA.



Analysis of Pathology: The mice were necropsied for gross pathological lesions. Organs and tissues were excised and preserved in 10% buffered formalin. The fixed tissues were processed conventionally, embedded in paraffin, sectioned at 5 μm , and stained with hematoxylin and eosin (Zhang et al., 2009). For each mouse, a list of pathological lesions that included both neoplastic and non-neoplastic diseases was constructed. Based on these histopathological data, the tumor burden, disease burden, and severity of each lesion in each mouse were assessed (Bronson & Lipman, 1991). The severity of neoplastic lesions was assessed using the grading system previously described (Ikeno, Bronson, Hubbard, Lee, & Bartke, 2003).

MicroRNA 155 Expression: Total RNAs was isolated from the brain, testes, heart, kidney, lung and liver of Ts65Dn and WT mice using Trizol method. A total of 50 ng of total RNA was used for reverse transcription (RT) reaction. RT reactions were realized with 0.15 μL dNTP, 1.5 μL buffer (10x), 9 μL RNase-free water, 0.2 μL RNase inhibitor, 1 μL multiscribe Reverse Transcriptase, and 3 μL of specific miR-155 RT primers (Applied Biosystems). RT reactions were set at 16°C for 30 minutes, 42°C for 30 minutes, and 85°C for 5 minutes using a BioRad thermal cycler . The TaqMan miR-155 assay (Applied Biosystems) was used to quantify miR-155. 5 μL of RT reactions was mixed with 10 μL of TaqMan Universal PCR master mix (2x, (Applied Biosystems)) 4 μL of RNase-free water, and 1 μL of TaqMan miRNA Assay (20X , (Applied Biosystems)). Quantitative real-time PCR analysis was performed using the LightCycler 480 (Roche) at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Sno202 (Applied Biosystems), a noncoding RNA, was used as endogenous control.

Expression analysis: Total RNAs were isolated from the brain, testes, heart, kidney, lung and liver of Ts65Dn and WT mice using Trizol method. cDNA was synthesized, as described

previously (Cabelof et al. 2006a), from 2 μ g RNA using random hexamer primers and purified with the QIAquick PCR Purification columns (Qiagen, Valencia, CA). Transcripts were amplified and quantitated with a LightCycler Real Time PCR machine (Roche). PCR reactions contained 2 μ l purified cDNA, 0.5 μ M of each sense and antisense primer, and 2 μ l FastStart DNA Master SYBR Green I enzyme-SYBR reaction mix (Roche). For all amplifications, PCR conditions consisted of an initial denaturation step at 99°C for 10 min, followed by 35–45 cycles at 96°C for 10s, primer specific annealing temperatures for 10s, and elongation at 72°C for 5s. Melting curves from 40°C to 99°C confirmed specificity. External standards were prepared for all genes from cDNA amplicons cloned into pCRII TOPO cloning vector (Invitrogen). All transcripts were quantitated and normalized to GAPDH or RPL4 expression. Primer sequences are detailed in Table 4.4.

Data Analysis:

Results are expressed as mean \pm SEM analyzed and were analyzed using Student's t-test. Statistical comparisons between groups were conducted using one-way ANOVA, using GraphPad Prism 5.0 (Graph Pad Software, La Jolla, CA). Values of $p < 0.05$ were considered statistically significant and individual p-values are shown in the figures.

4.6 Results

Body and Organ Weight of Ts65Dn and WT Mice at 16, 32 and 48 weeks.

We evaluated the body weight of Ts65Dn and WT controls within the three age groups. The body weight of both Ts65Dn and WT mice increased steadily and almost linearly with age (Figure 4.6.1). A comparison of Ts65Dn and WT mice showed that Ts65Dn mice had a smaller body weight than the WT counterparts (Figure 4.6.1). This difference persisted in three age groups. Evaluation of tissues showed some differences. Lung of Ts65Dn at 16 week were

significantly smaller but with age this difference is lost (Figure 4.6.2). The most dramatic difference was in testes size. Ts65Dn testes were significantly smaller than their WT controls and this difference persisted in all three aged cohorts (Figure 4.6.3). These results are consistent with what has been reported earlier and is linked to male sterility that is reported in Ts65Dn (Davission et al., 2007). Kidney and brain did not show any difference between the two genotypes (Figure 4.6.4 and 4.6.7). Heart and liver of Ts65Dn mice at 16 weeks were significantly smaller than WT (Figure 4.6.5 and 4.6.6). These results are consistent with Fuchs et al. study. They showed that not only was heart and liver smaller, there was also an impaired proliferation as assayed by BrdU (Fuchs et al., 2012). At 32 and 48 weeks a trend of small size was seen but was not significantly different from WT. Interestingly, we did not see any differences in spleen or thymus which have been shown to be underdeveloped in Ts65Dn ((Lorenzo, Shatynski, Clark, Yarowsky, & Williams, 2013). Furthermore, no differences were seen in fat tissues between Ts65Dn and WT (data not shown).

Neoplastic incidences

It has been suggested that gene dosage imbalances caused by the presence of an extra copy of chromosome 21 may be protective against the development of certain malignancies in DS individuals. The low incidence of solid tumors in DS individuals has been well documented in population studies worldwide; with the exception to this is testicular cancer (Uppal et al., 2015). Though the incidence of solid tumors is low, DS children have a 10- to 20-fold increased risk of developing acute lymphoblastic leukemia (ALL) or acute myeloid leukemia (AML) compared with non-DS children (Hermon et al., 2001). The mouse models of DS have shown similar results with respect to low incidence of solid tumors and increase in malignant lymphoma (Patterson & Cabelof, 2012). In our study we found that Ts65Dn spleen had an increase minimal

and mild lymphoma at 16 week and 32 week (Figure 4.6.17). By 48 weeks, the difference between ts65dn and WT was lost. 20% of 16 week old Ts6d5n mice also exhibited minimal invasive lymphoma in lung and stomach (Figure 4.6.18 and 4.6.19). Lungs of Ts65Dn mice also showed increase in alveolar/bronchiolar adenoma incidence which was absent in WT mice (Figure 4.6.18). In 48 week old Ts65Dn, the incidence of invasive lymphoma increased for both stomach and intestine (Figure 4.6.20). Our results are in line with that Levine et al., showed in three different mouse models of DS including Ts65dn. The absence of solid tumors in our cohorts of Ts65Dn supports the continued use of this model in studying DS malignances.

p16 expression on peripheral tissues

One of the most commonly used in vivo markers of senescence is the expression of p16INK4A, a selective inhibitor of cyclin D-dependent CDK4 and CDK6 (Y. Liu et al., 2009). The expression of p16 increases with age normally and in the response to stress (Baker et al., 2011). In our study we sought to know if Ts65Dn tissues had increase in p16 expression. In all of tissues evaluated, we did not see a significant increase of p16 in either WT or Ts65Dn mice. Liver and Lung of Ts65dn showed a slight increase at 16 weeks that was not statistical significant (Figure 4.6.11 and 4.6.13). Testes did show an increase in p16 with age independent of genotype differences (Figure 4.6.16). Our results further support previous work that p16 is only detectable in advanced age and is undetectable or very low in most adult tissues (Krishnamurthy et al., 2004). It is possible that 48 week (12 months) old mice are too young to display any aging phenotype regardless of genotype.

Expression of DNA polymerase beta (Pol β)

Evaluation of DNA repair capacity or expression levels of the main players has not been extensively studied in Ts65Dn. DS individuals have been shown to have a defective DNA repair

(Druzhyna et al., 1998; Necchi et al., 2015; Pincheira, Rodriguez, Bravo, Navarrete, & Lopez-Saez, 1994; Weirich-Schwaiger et al., 1994) and in particular have reduced expression and activity of POLB (Cabelof et al., 2009; Raji & Rao, 1998). Our objective was to look at the expression of Pol β in several tissues of Ts65Dn. In the tissues assessed (liver, lung, kidney, testes, brain, heart, and skin), only testes showed a reduction of Pol β . In the 16 week old group, the Ts65Dn mice showed a significant decrease in Pol β compared to control littermates (Figure 4.6.7, $p < 0.01$). These findings are notable as testes are the only tissues to develop cancer in DS. Brain of Ts6dn at 16 week revealed a trend of downregulation of Pol β that was not statistically significant. Skin also showed reduced Pol β expression at 48 weeks though it was not significant (Figure 4.6.10). Unlike DS, the Ts65Dn mouse did not show a progressive downregulation of Pol β over time in the tissues tested. The next step is to look at accumulation of repair intermediates, such as SSB and DSB, as markers of repair capacity. A recent study revealed hematopoietic stem cells (HSCs) of Ts65Dn have an accumulation of DSBs at baseline and that these HSCs are defective in repairing radiation-induced DSBs (Wang et al., 2016). These results allow us to anticipate that Ts65Dn will show an increase in DSBs as they age.

4.7 Discussion

In the 1970s we saw the production of the first model of DS and it had promising results. Many years later, we still do not have a perfect animal model but a compilation of data from distinct models has given us great insight into DS pathogenesis. The limitations with many of the in elucidating the genetic sources of the DS phenotype have been described above. In addition to genetic influences, we have not discussed potential epigenetic factors that may also account for pathogenesis in DS, as well as the many difference between mice and humans (Gunter & Dhand, 2002).

In our studies, we utilize the model considered by most to be the most inclusive model of DS. Though the model lacks certain cardiovascular anomalies it is still a very useful model for studying molecular mechanism of DS. Studies focusing on aging traits have revealed Ts65Dn mice exhibit muscle weakness and motor alterations (sarcopenia) at 12 months and 19 months (Cisterna, Costanzo, Scherini, Zancanaro, & Malatesta, 2013). Systemic pathology in aged Ts65Dn (8 to 24 months) indicated a significant increase in prevalence of adenocarcinoma /lymphoma (Levine et al., 2009). Our study showed that Ts65Dn does mimic some of DS traits such as increase in lymphoma incidence and low incidences of solid tumors. We failed to find any solid tumors in the three cohorts evaluated. Counter to our hypothesis, we did not see any changes in expression of p16 or Pol β over time in most tissues, with the one exception being testes which showed an early significant downregulation of POLB (figure 2.1) and a steady increase of p16 over time (figure 3.6). Screening for other markers of aging such as SA-beta gal and SASP in this model will allow us to consider whether continued use of this model in aging research is beneficial.

Mouse Model	Type of Model	DS Features
Ts16	Trisomic	- impaired learning - increased oxidative stress in utero -mitochondrial dysfunction
Ts65Dn	Segmental Trisomy	-developmental delay -hyperactivity, -craniofacial dysmorphology -impaired learning -behavior deficits -Reduced density of cerebellar granule cells - oxidative stress markers in young and old Ts65Dn mice
Ts1Cje	Segmental Trisomy	-impaired learning and memory -Reduced density of cerebellar granule cells -hypoplasia of the cerebellum and enlarged ventricles
Ms1Ts65	Segmental Trisomy	-Moderate reduction in density of cerebellar granule cells -moderate impaired learning and memory.
Ts1Rhr	Segmental Trisomy	-impaired learning and memory -moderate craniofacial dysmorphology
Tc1	“full Trisomy”	-cardiac malformations -developmental delay -hyperactivity, -craniofacial dysmorphology -impaired learning

Table 4.2: Summary of segmental trisomy mouse models and features of Down syndrome that have been documented in each model.

Mouse Model	Type of Model	DS Features
Dyrk1A BAC	Transgenic	-Abnormal brain structure and locomotors behavior
SOD1	Transgenic	-chronic oxidative stress -Abnormal neuromuscular junction -decreased plasma serotonin level
APP	Transgenic	-Dystrophic neuritis associated with congophilic plaques
ETS2	Transgenic	-Skeletal abnormalities particularly craniofacial abnormalities -brachycephaly
S100 β	Transgenic	-Abnormal dendritic development

Table 4.3: Summary of transgenic Mouse models

Gene	Sense primer 5'–3'	Anti-sense primer 5'–3'
<i>Mouse</i>		
p16 UPL probe #91	aatctccgcgaggaaagc	Gtctgcagcggactccat
B-pol (exon 12–13)	agcgagaaggatggaaaggaa	Cgtgcgctctcatgttcttat
<i>Housekeeping genes (mouse)</i>		
Gapdh	aggtcgggtggaacggattg	Tgtagaccatgtagttgaggca
Rpl4	ccgtcccctcatatcgggtga	Gcatagggctgtctgtgtttt
Rpl4 UPL probe # 75	tggtggtggaagataaggtga	Ccaagctttgagttcttgagc

Table 4.4: Primer sequences

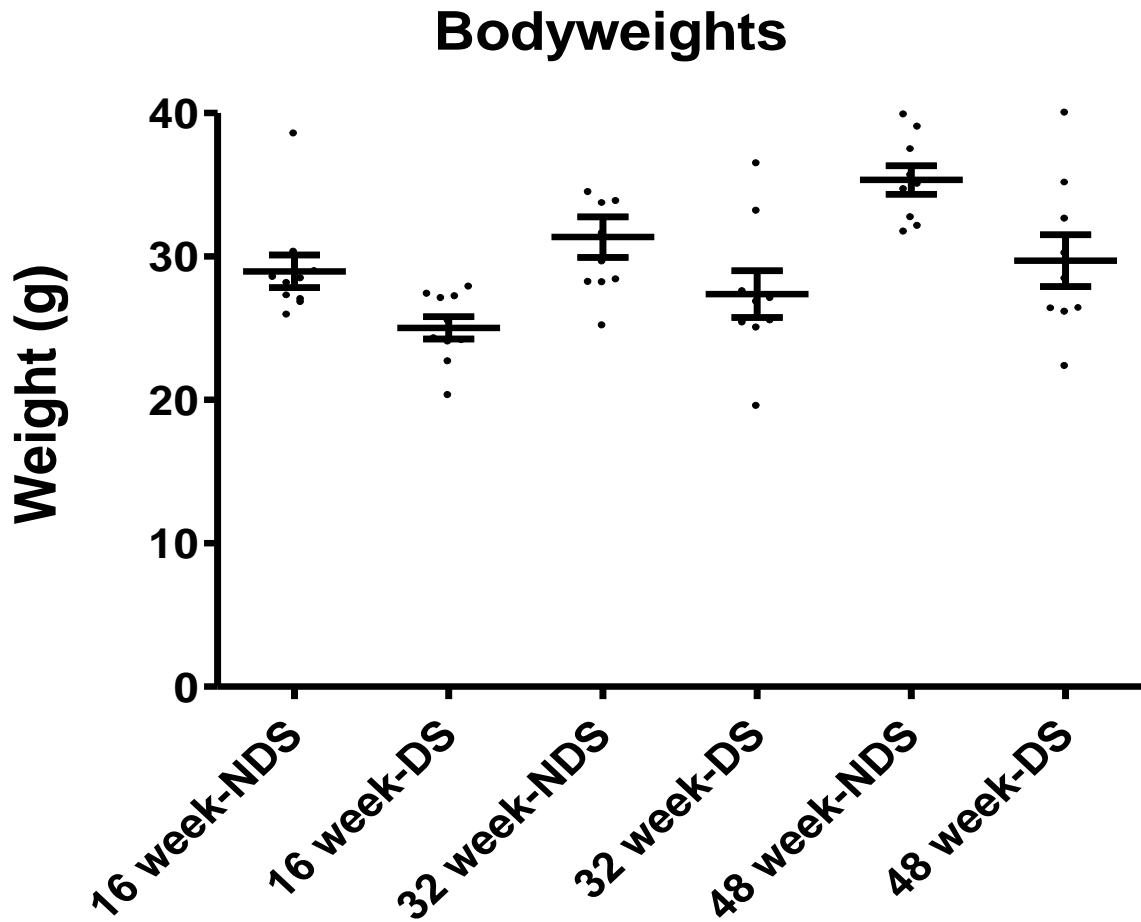


Figure 4.6.0: Ts65Dn are smaller than WT controls. These data were obtained from three cohorts (16, 32, and 48 week) Ts65Dn and WT mice. Body weight ($n = 23, 20,$ and 20 for 16, 32 and 48 week mice, respectively). The data shown is from male mice only. The data are expressed as the mean \pm SEM.

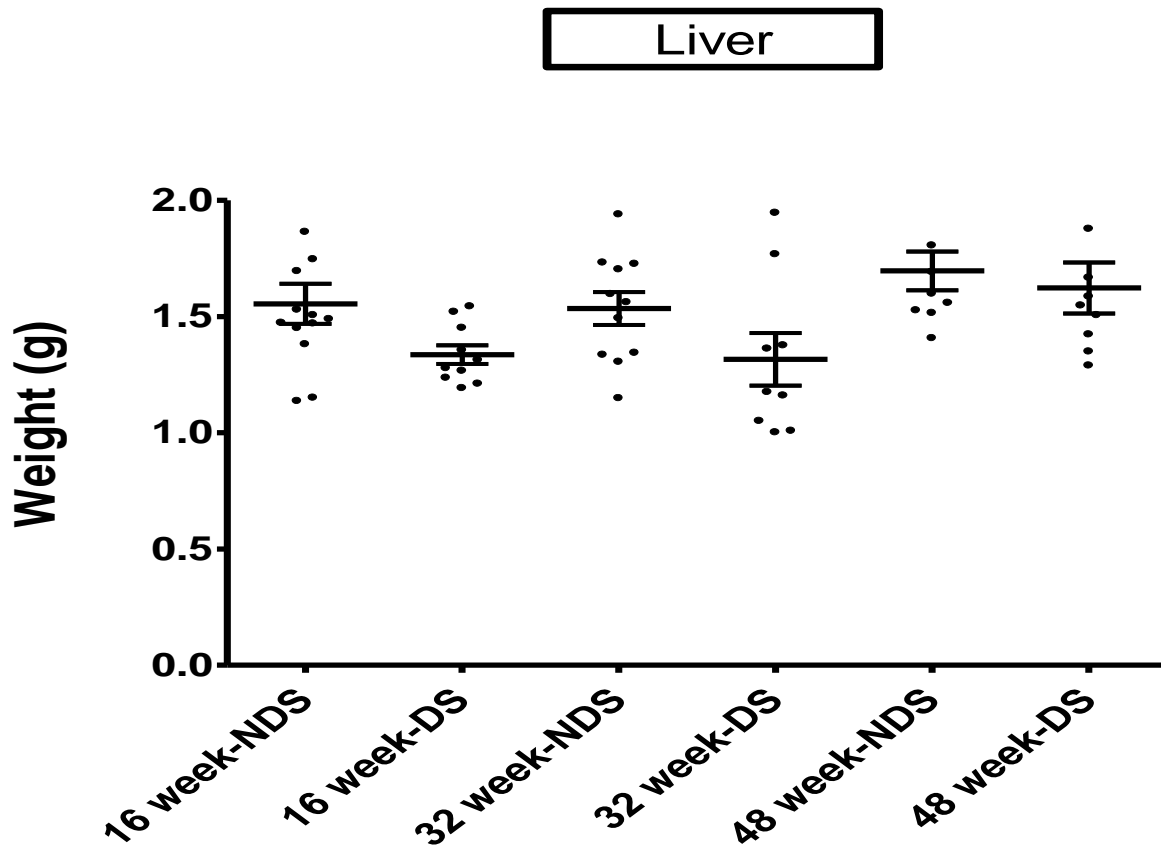


Figure 4.6.1: Liver wet weights in Ts65dn (DS) and WT (NDS). These data were obtained from three cohorts (16, 32, and 48 week) Ts65Dn and WT mice. Body weight and tissue wet weight ($n = 23, 21,$ and 20 for 16, 32 and 48 week mice, respectively). The data shown is from male mice only. The data are expressed as the mean \pm SEM

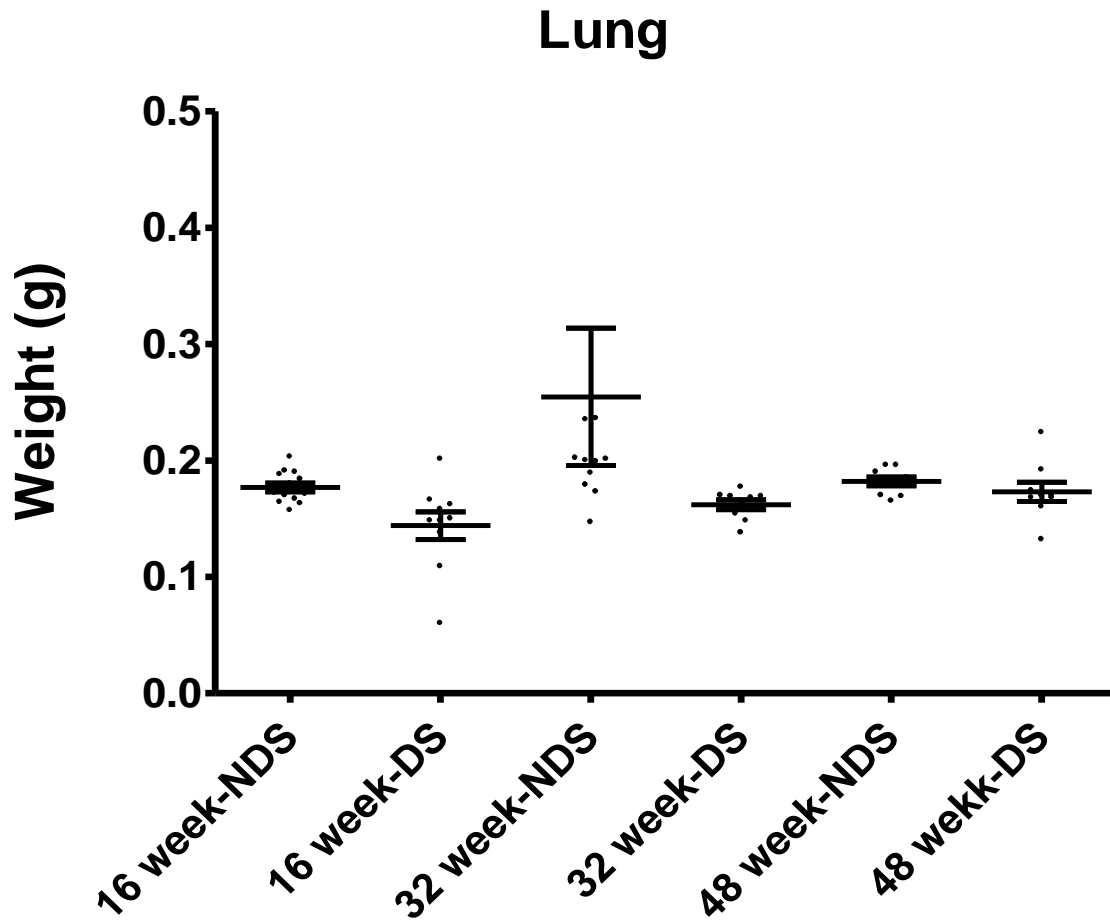


Figure 4.6.2: Weight of Lung in three cohorts. These data were obtained from three cohorts (16, 32, and 48 week) Ts65Dn and WT mice. Tissue wet weight (n = 23, 21, and 20 for 16, 32 and 48 week mice, respectively). The data shown is from male mice only. The data are expressed as the mean \pm SEM

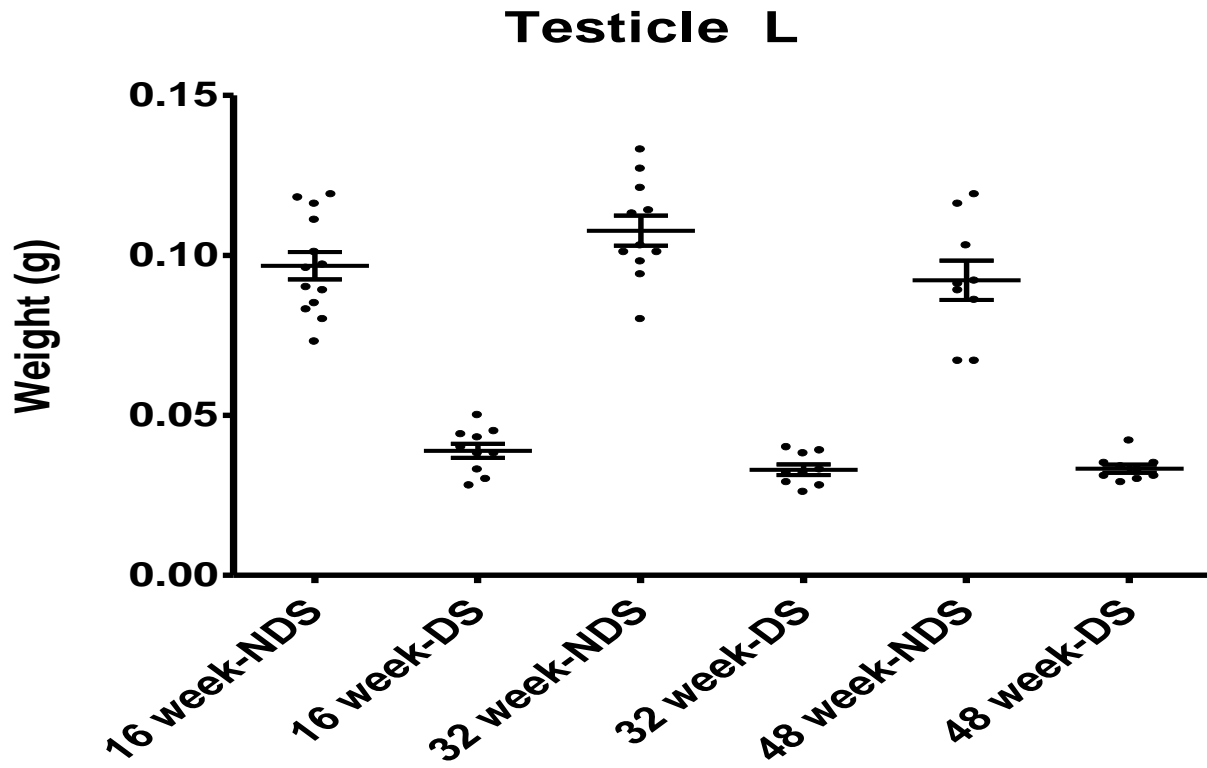


Figure 4.6.3: Ts65Dn mice have underdeveloped Testes. Both left and right testes in Ts65Dn mice were significantly smaller in the three age groups. A total of $n = 23, 21,$ and 20 for 16, 32 and 48 week mice, respectively). The data are expressed as the mean \pm SEM

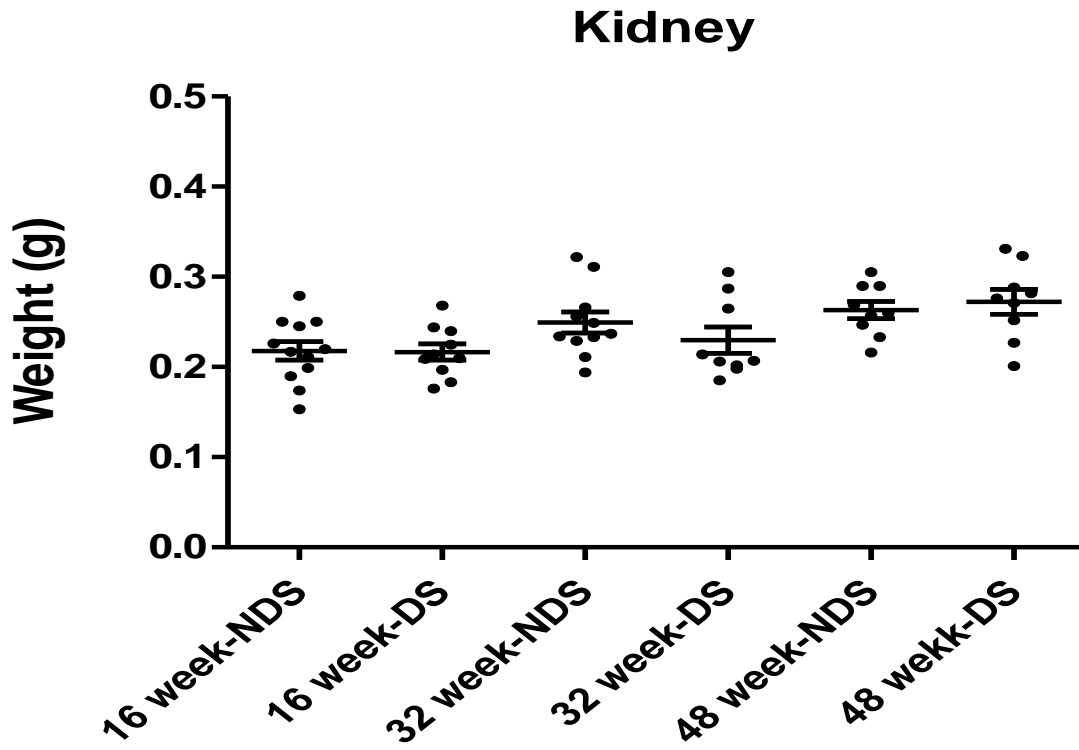


Figure 4.6.4: Kidney weight did not differ. These data were obtained from three cohorts (16, 32, and 48 week) Ts65Dn and WT mice. Body weight and tissue wet weight (n = 23, 21, and 20 for 16, 32 and 48 week mice, respectively). The data shown is from male mice only. The data are expressed as the mean \pm SEM

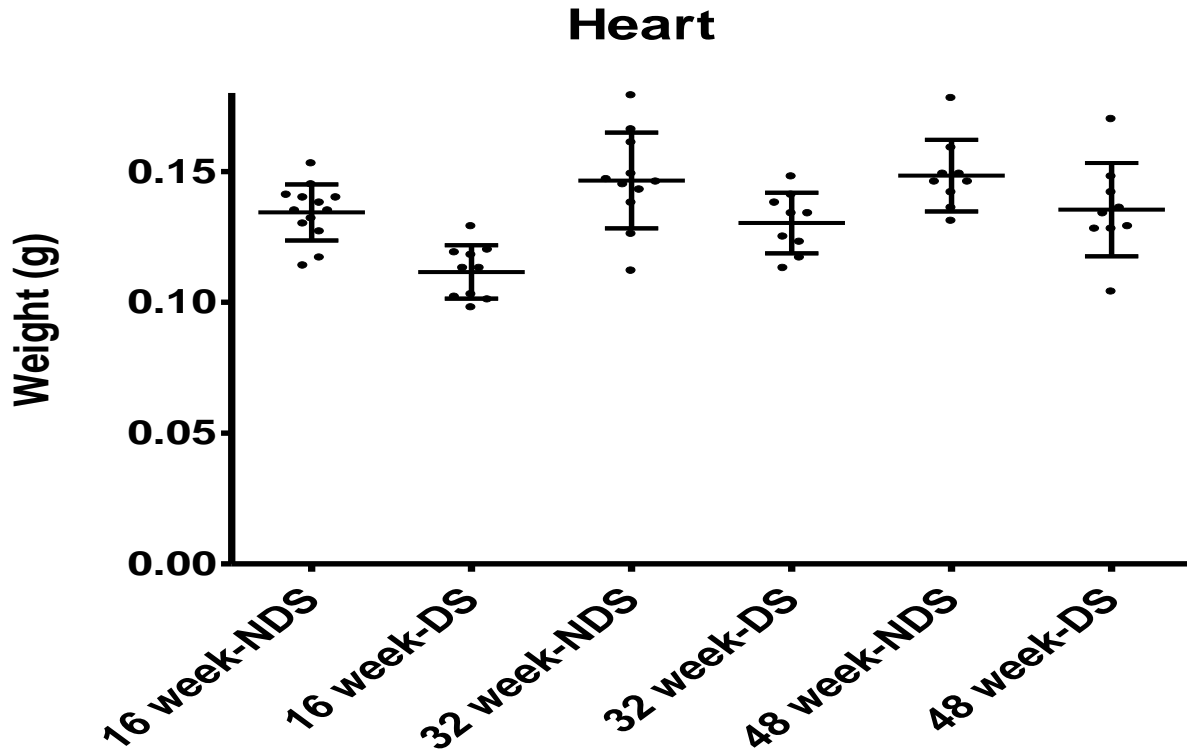


Figure 4.6.5: Ts65Dn heart seems to be smaller than WT. These data were obtained from three cohorts (16, 32, and 48 week) Ts65Dn and WT mice. Body weight and tissue wet weight (n = 23, 21, and 20 for 16, 32 and 48 week mice, respectively). The data shown is from male mice only. The data are expressed as the mean \pm SEM

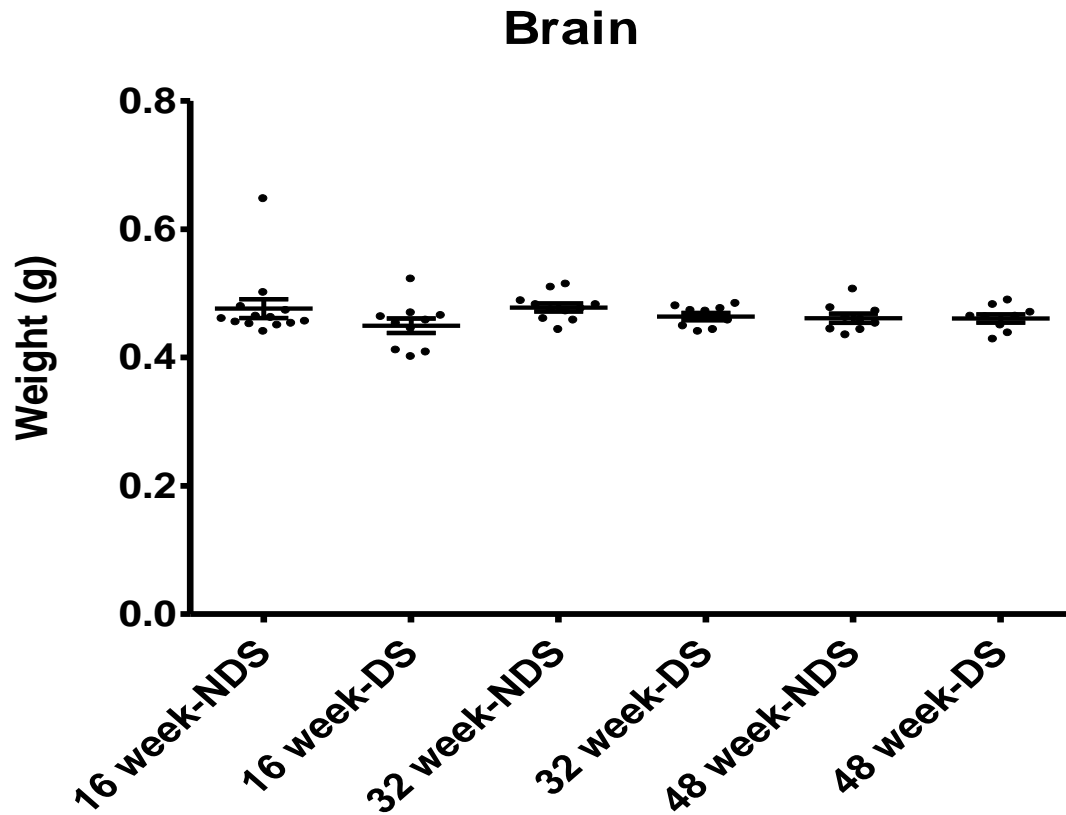


Figure 4.6.6: No difference was seen in Brain weight. These data were obtained from three cohorts (16, 32, and 48 week) Ts65Dn and WT mice. Body weight and tissue wet weight (n = 23, 21, and 20 for 16, 32 and 48 week mice, respectively). The data shown is from male mice only. The data are expressed as the mean \pm SEM

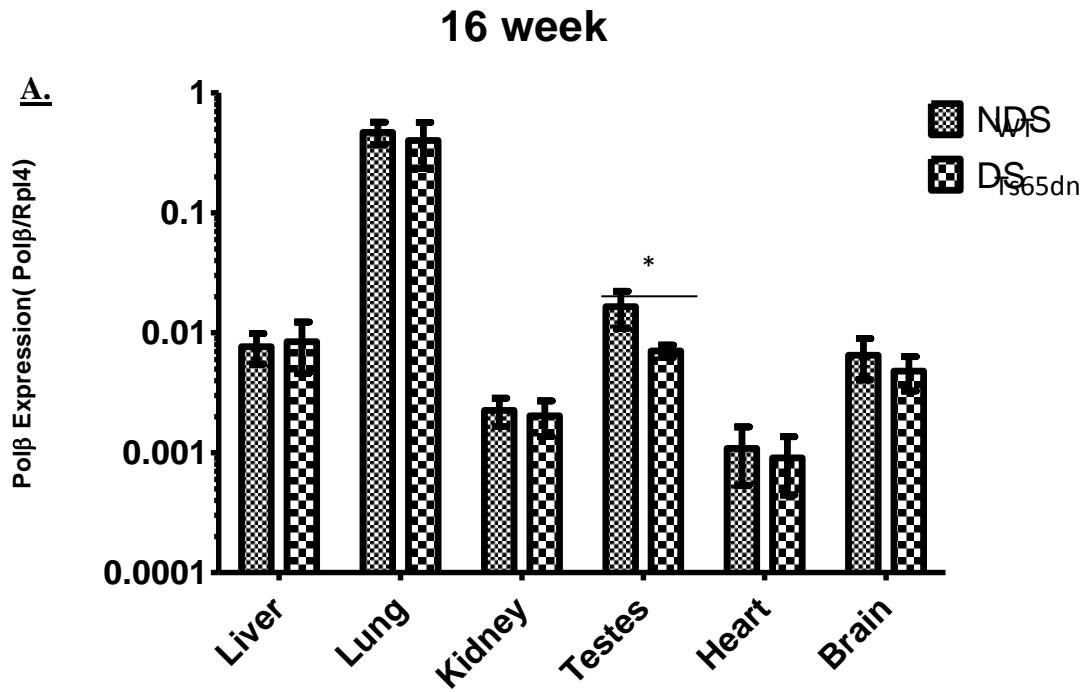


Figure 4.6.7: PolB expression in Ts65Dn mice tissues at 16 week. cDNAs were prepared from RNA isolated from tissues of Ts65Dn (n=10) and control littermates (n=13) mice from three cohorts (16, 32, and 48 week). PolB transcript levels were determined by real time RT-PCR analysis and normalized to Rpl4. Data are presented as mean \pm SEM.

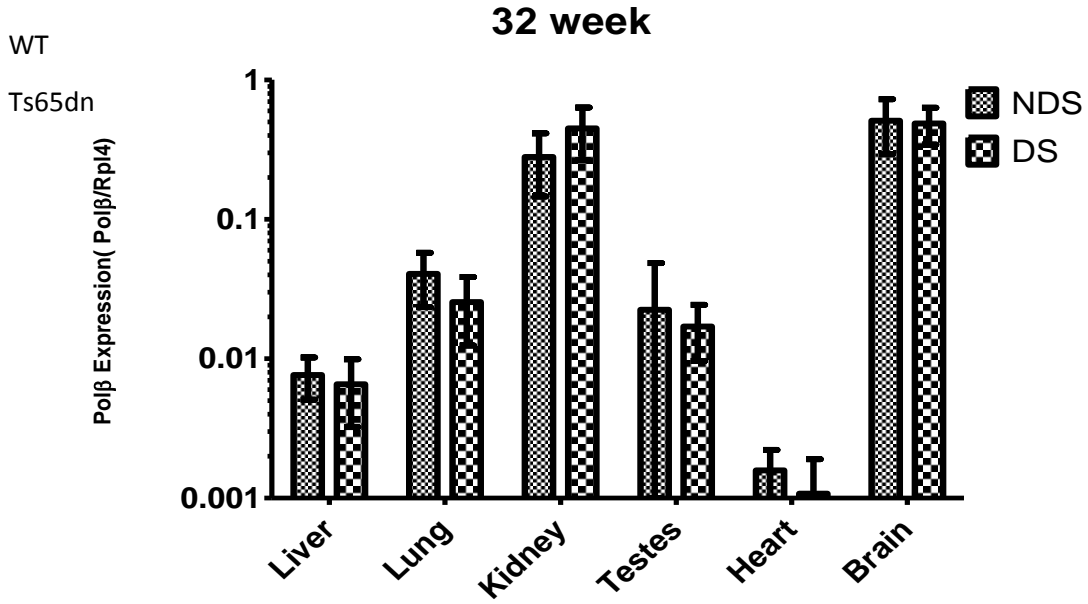


Figure 4.6.8: PolB expression in Ts65Dn mice tissues. cDNAs were prepared from RNA isolated from tissues of Ts65Dn (n=10) and control littermates (n=10). PolB transcript levels were determined by real time RT-PCR analysis and normalized to Rpl4. Data are presented as mean \pm SEM.

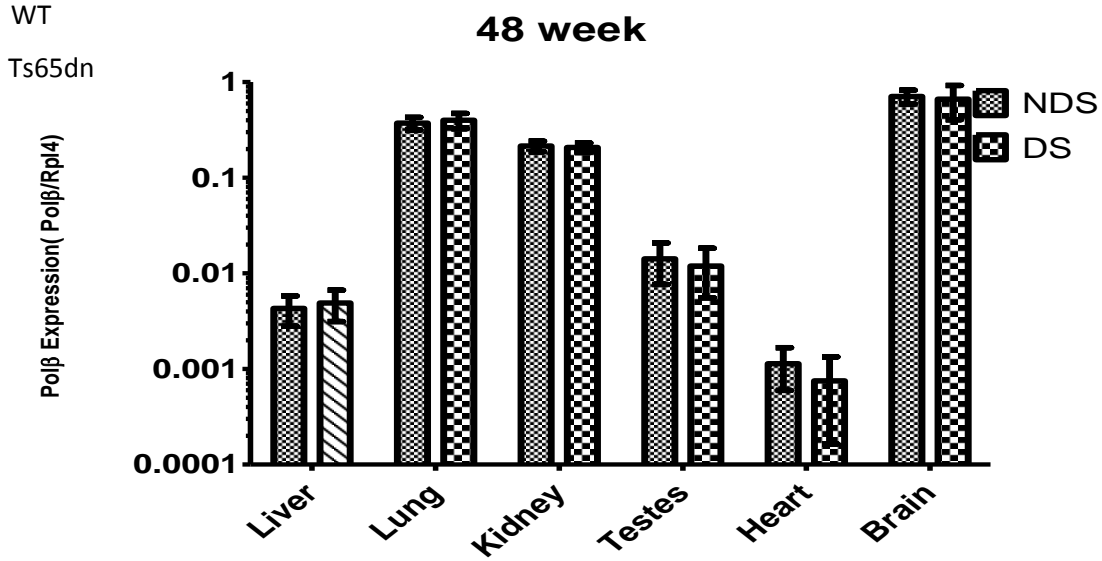


Figure 4.6.9: PolB expression in Ts65Dn mice tissues. cDNAs were prepared from RNA isolated from tissues of Ts65Dn (n=9) and control littermates (n=10) mice from three cohorts (16, 32, and 48 week). PolB transcript levels were determined by real time RT-PCR analysis and normalized to Rpl4. Data are presented as mean \pm SEM

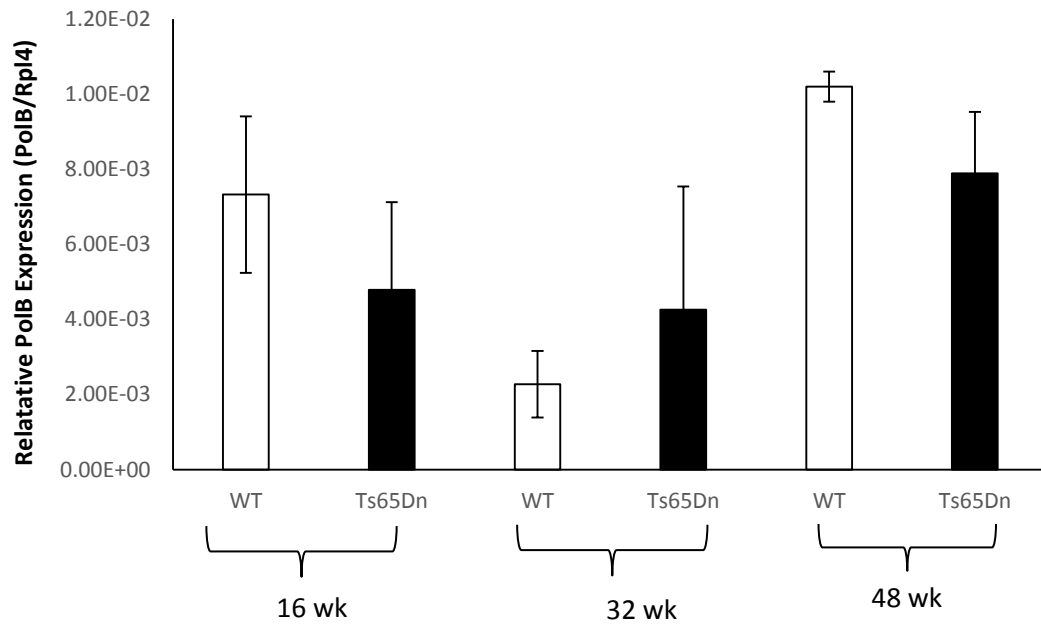


Figure 4.6.10: PolB expression in Ts65Dn Skin. cDNAs were prepared from RNA isolated from tissues of Ts65Dn (n=9) and control littermates (n=10) mice from three cohorts (16, 32, and 48 week). PolB transcript levels were determined by real time RT-PCR analysis and normalized to Rpl4. Data are presented as mean \pm SEM

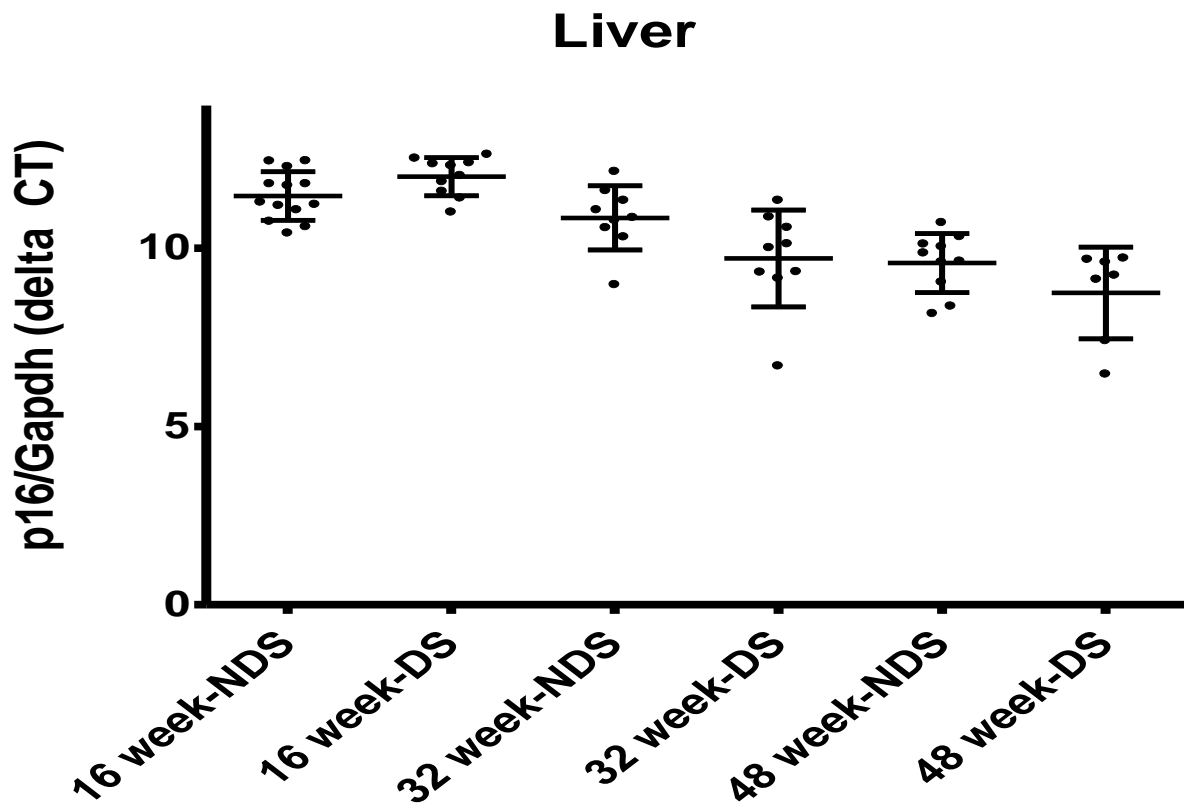


Figure 4.6.11: p16 expression in Ts65Dn Tissues: cDNAs were prepared from RNA isolated from tissues of Ts65Dn (9-11 mice in each group) and control littermates mice from three cohorts(16, 32, and 48 week). PolB transcript levels were determined by real time RT-PCR analysis and normalized to Rpl4. Data are presented as mean \pm SEM.

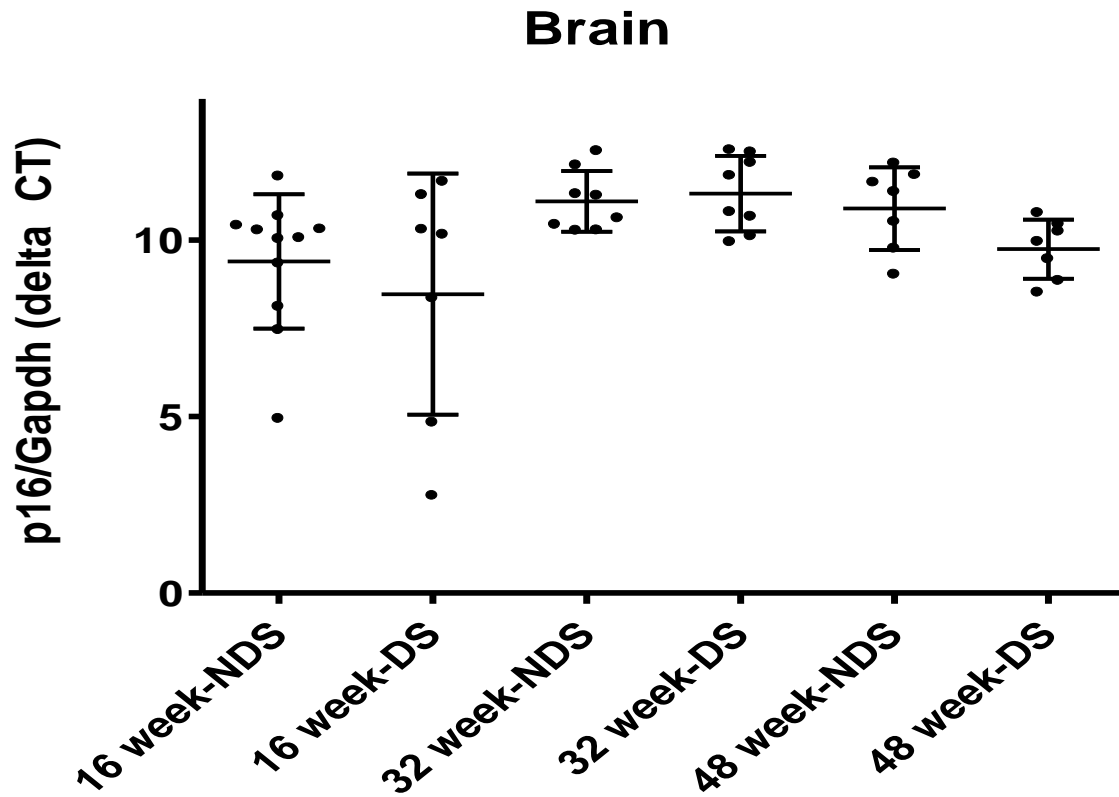


Figure 4.6.12 : p16 expression in Ts65Dn Tissues: cDNAs were prepared from RNA isolated from tissues of Ts65Dn (9-11 mice in each group) and control littermates mice from three cohorts(16, 32, and 48 week). PolB transcript levels were determined by real time RT-PCR analysis and normalized to Rpl4. Data are presented as mean \pm SEM.

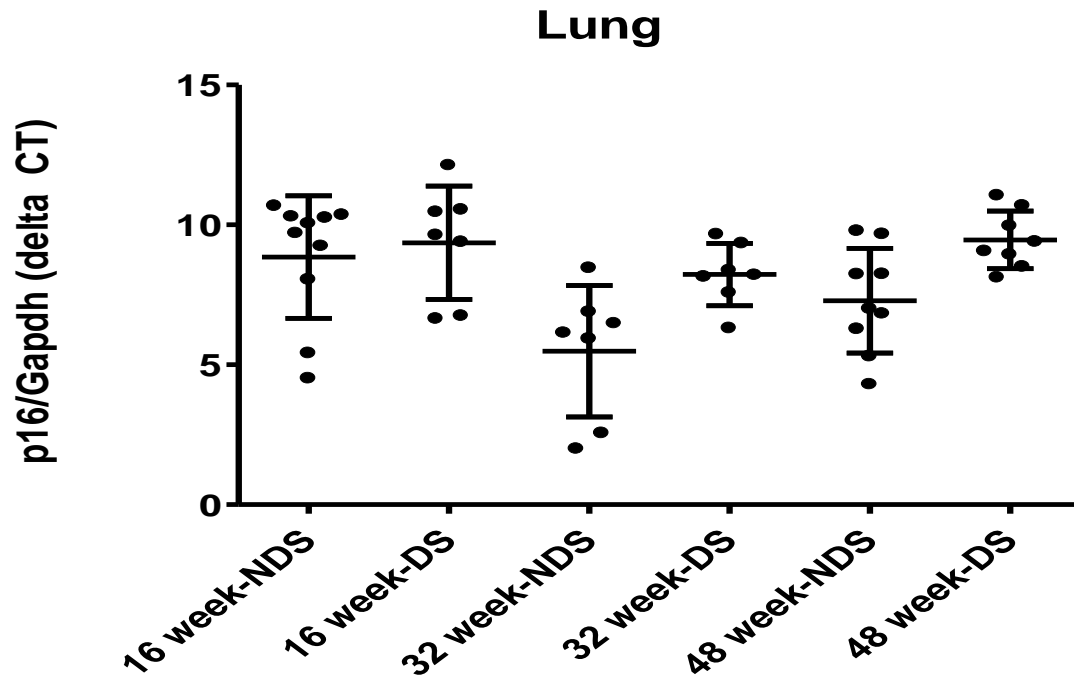


Figure 4.6.13: p16 expression in Ts65Dn Tissues: cDNAs were prepared from RNA isolated from tissues of Ts65Dn (9-11 mice in each group) and control littermates mice from three cohorts(16, 32, and 48 week). PolB transcript levels were determined by real time RT-PCR analysis and normalized to Rpl4. Data are presented as mean \pm SEM.

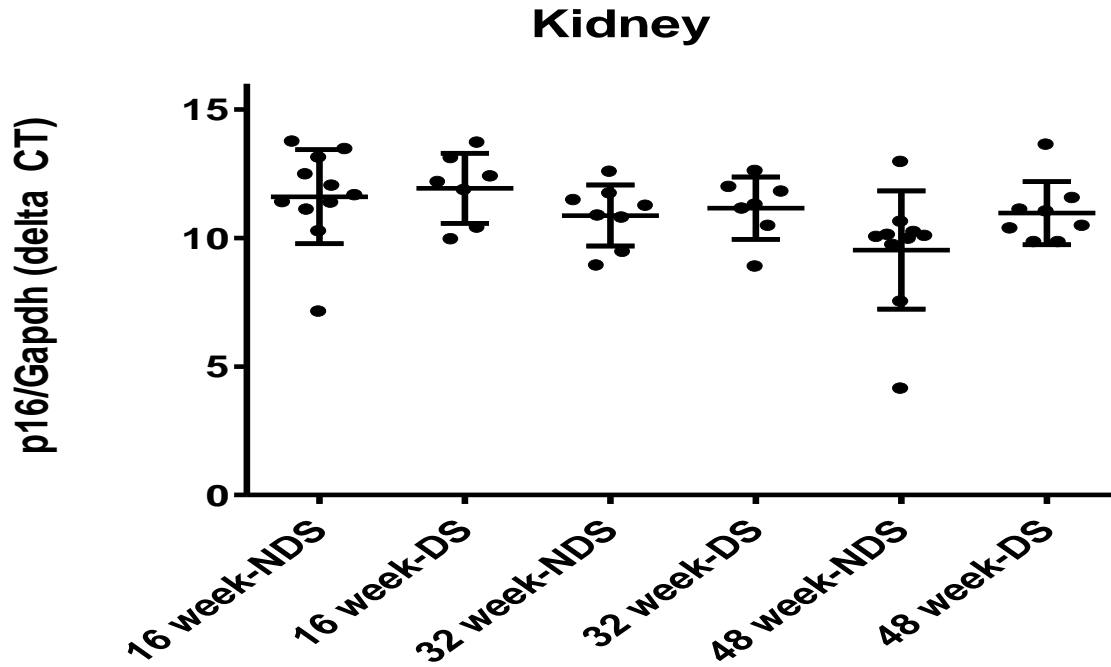


Figure 4.6.14: p16 expression in Ts65Dn Tissues: cDNAs were prepared from RNA isolated from tissues of Ts65Dn (9-11 mice in each group) and control littermate mice from three cohorts (16, 32, and 48 week). PolB transcript levels were determined by real time RT-PCR analysis and normalized to Rpl4. Data are presented as mean \pm SEM.

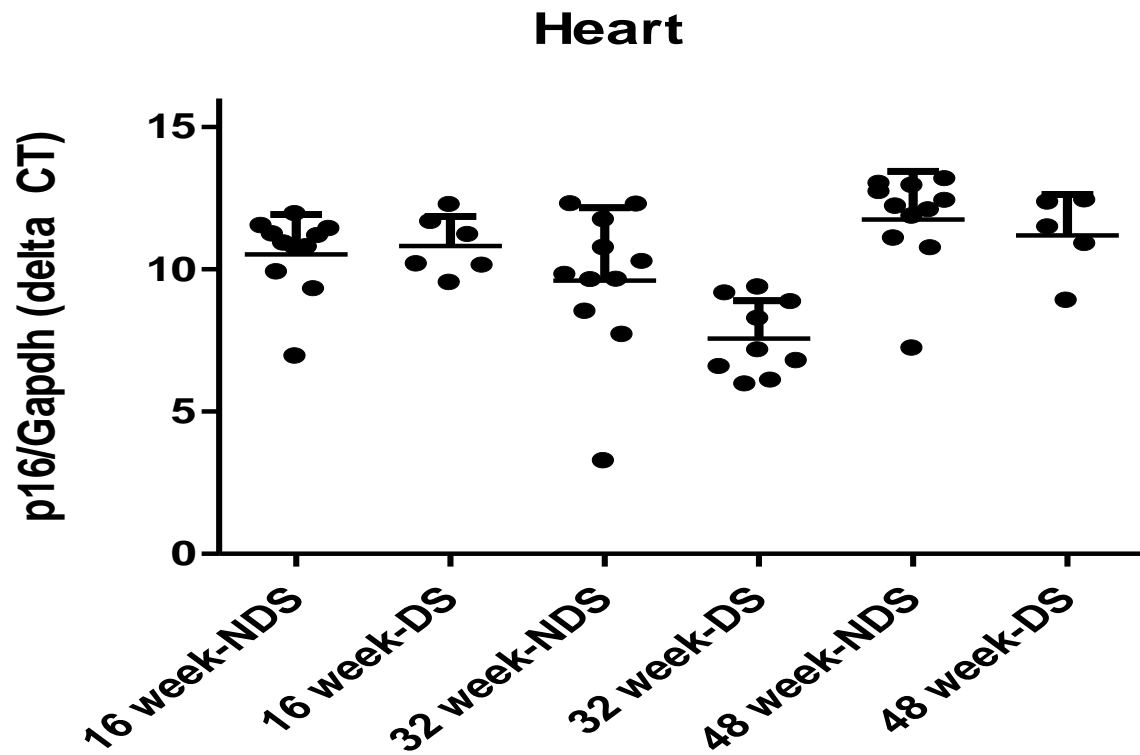


Figure 4.6.15: p16 expression in Ts65Dn Tissues: cDNAs were prepared from RNA isolated from tissues of Ts65Dn (9-11 mice in each group) and control littermates mice from three cohorts(16, 32, and 48 week). PolB transcript levels were determined by real time RT-PCR analysis and normalized to Rpl4. Data are presented as mean \pm SEM.

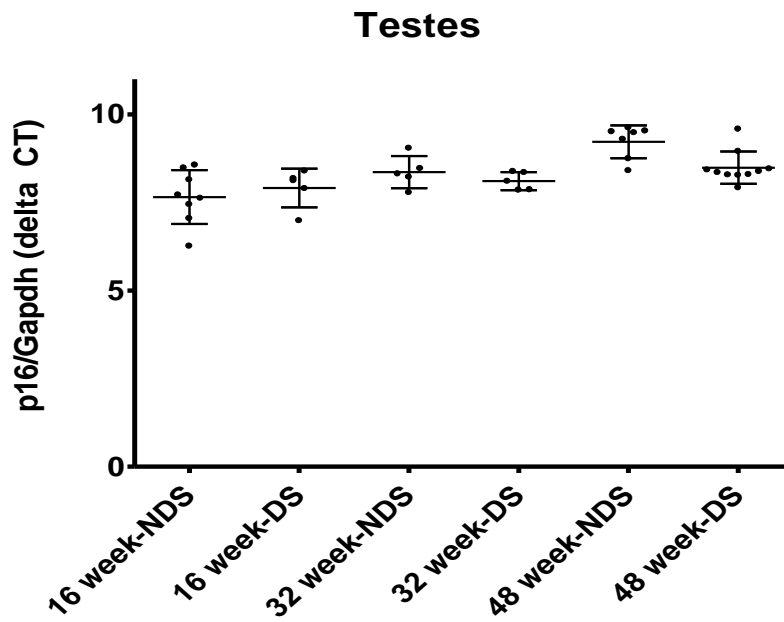


Figure 4.6.16: p16 expression in Ts65Dn Tissues: cDNAs were prepared from RNA isolated from tissues of Ts65Dn (9-11 mice in each group) and control littermates mice from three cohorts(16, 32, and 48 week). PolB transcript levels were determined by real time RT-PCR analysis and normalized to Rpl4. Data are presented as mean \pm SEM.

Age (weeks)	Minimal Invasive Lymphoma	Mild Invasive Lymphoma
16		
WT (n=13)	39%	0%
Ts65dn (n=10)	80%	0%
32		
WT (n=10)	55%	0%
Ts65dn (n=9)	67%	11%
48		
WT (n=10)	60%	20%
Ts65dn (n=10)	80%	0%

Figure 4.6.17: Incidence of lymphoma in Ts65Dn Spleen. Spleen was excised and preserved in 10% buffered formalin. It was sectioned at 5 μ m, and stained with H&E. Grading of neoplastic lesions as described in Materials and Methods was completed on Ts65Dn and WT mice terminated at each of the three time points (16, 32 and 48 weeks). Percentage of mice with lesions is presented.

Age (weeks)	Minimal Invasive Lymphoma	lymphocytic infiltrate	alveolar/bronchiolar adenoma
16			
WT (n=13)	0%	0%	0%
Ts65dn (n=10)	20%	0%	0%
32			
WT (n=10)	9%	9%	0%
Ts65dn (n=9)	11%	11%	0%
48			
WT (n=10)	10%	0%	0%
Ts65dn (n=10)	20%	20%	10%

Figure 4.6.18: Incidence of malignant tumors of lung in Ts65Dn. Lung was excised and preserved in 10% buffered formalin. It was sectioned at 5 μ m, and stained with H&E. Grading of neoplastic lesions as described in Materials and Methods was completed on Ts65Dn and WT mice terminated at each of the three time points (16, 32 and 48 weeks). Percentage of mice with lesions is presented

Age (weeks)	Minimal Invasive Lymphoma	Mild Invasive Lymphoma
16		
WT (n=13)	0%	0%
Ts65dn (n=10)	10%	20%
32		
WT (n=10)	0%	0%
Ts65dn (n=9)	0%	0%
48		
WT (n=10)	0%	0%
Ts65dn (n=10)	0%	20%

Figure 4.6.19: Incidence of lymphoma in stomach of Ts65Dn. Stomach was excised and preserved in 10% buffered formalin. It was sectioned at 5 μ m, and stained with H&E. Grading of neoplastic lesions as described in Materials and Methods was completed on Ts65Dn and WT mice terminated at each of the three time points (16, 32 and 48 weeks). Percentage of mice with lesions is presented

Age (weeks)	Minimal Invasive Lymphoma	Mild Invasive Lymphoma	Minimal lymphocytic infiltrate	Mild lymphocytic infiltrate
16				
WT (n=13)	46%	15%	15%	0%
Ts65dn (n=10)	40%	30%	0%	0%
32				
WT (n=10)	45%	0%	18%	9%
Ts65dn (n=9)	56%	0%	0%	0%
48				
WT (n=10)	20%	10%	10%	0%
Ts65dn (n=10)	40%	0%	20%	0%

Figure 4.6.20: Incidence of lymphoma in Intestine of Ts65Dn. Intestine was excised and preserved in 10% buffered formalin. It was sectioned at 5 μ m, and stained with H&E. Grading of neoplastic lesions as described in Materials and Methods was completed on Ts65Dn and WT mice terminated at each of the three time points (16, 32 and 48 weeks). Percentage of mice with lesions is presented

	DS	Ts65Dn	Ts1Cje	Ms1Ts65	Ts1Rhr
Motor skills	Delayed acquisition	Delayed acquisition			
Motor coordination	Impaired	Impaired			
Activity and attention	Reduced attention	Hyperactivity and reduced attention	Normal activity	Normal activity	Normal activity
Spatial learning and memory	Impaired	Impaired	Impaired	Impaired	
Working and reference memory	Impaired	Impaired			
Novel object recognition		Impaired			Impaired
Brain volume			Reduced during the embryonic period	Reduced during the embryonic period	Reduced
Neuronal density			Reduced	Not affected	
Cerebellar volume				Reduced	Not affected
Cerebellar neuronal density				Reduced	Reduced
Proliferation	Impaired	Impaired			
DNA repair capacity	Defective to extent evaluated	Defective to extent evaluated			

Table 4.7.1: Summary of Segmental trisomy Mouse models.

CHAPTER 5: Conclusion and Future directions

The study sought to answer the following two questions:

- Is the premature aging seen in DS a result of PolB downregulation?
- Is miR-155 involved in down regulations of POLB and DNA repair capacity overall?

The study set out to explore the possible role of POLB in the premature aging phenotype of Down syndrome. Premature aging has been demonstrated in numerous biological systems in individuals with DS: cognition, the auditory system, the skin, brain structure, the immune system, and the olfactory system. Additionally, it has been reported that persons with DS show an earlier appearance of various biomarkers associated with aging, including DNA damage accumulation and chromosomal sensitivity to mutagens. Nearly all premature aging syndromes (including Werner, Rothmund-Thomson and Cockayne syndromes, trichothiodystrophy and ataxia telangiectasia) have been characterized by DNA repair defects, yet no specific defect has yet been identified for DS (Brosh & Bohr, 2007).

The problem with firmly establishing the role of a DNA repair defect, in particular POLB, in premature aging of DS is that DS is a chromosomal disorder and not monogenic. Still, several studies have shown that POLB is downregulated in DS, while other polymerases are unaffected (Cabelof et al., 2009; Raji & Rao, 1998). POLB is the best candidate for the premature aging phenotype of DS, since it has been shown to be reduced with age in multiple models (Krishna et al., 2005; Patterson & Cabelof, 2012; Raji & Rao, 1998; Rao, 2007; Rao et al., 2001; Swain & Rao, 2012; Sykora et al., 2015; Vyjayanti et al., 2012).

Studies conducted in this thesis further provide support that reduced POLB is causative in premature aging. My data from chapter 2 clearly demonstrated that reduced POLB expression results in premature senescence as well as an accumulation of DSBs. To confirm the role of POLB in premature senescence, I show primary PolB null MEFs also have an increase in senescence markers, p16 expression and SA-beta gal staining, as well as DSBs. Another observation generated by the work on PolB null cells is that PolB is upregulated by hydroxyurea and that DSBs generate in these cells in the absence of any exogenous damage. It would be very interesting to investigate the mechanism of hydroxyurea-mediated upregulation of PolB since POLB upregulation is common in several cancers and hydroxyurea is a common antineoplastic chemotherapy drug (Illuzzi & Wilson, 2012).

Establishing that POLB downregulation correlates with premature aging was the primary objective of this thesis. My data in chapter 2 led to further exploration of the cause of POLB downregulation in DS. The hypothesis was that PolB inhibition was induced by chromosome 21-linked miRNA overexpression. miR-155 is one of five microRNAs on HSA21, which has been shown to modulate DNA repair. miR-155's role in mismatch repair is well established and has been linked to an increase in genomic instability in several cancers. However, the mutational specificities of both 155 overexpression and Down syndrome are not consistent with a MMR defect (Cabelof et al., 2009; Czocho et al., 2016; Finette et al., 1998).

My search for a possible link between miR-155 and PolB revealed that Creb1, a transcription factor of PolB, is a direct target of miR-155. Validation studies demonstrated that miR-155 overexpression results in reduced protein levels of creb1 and its targets. Researchers have long established the importance of Creb1 in PolB expression. With that, I anticipated that miR-155 would cause low creb1 resulting in low PolB. Data from chapter 3 demonstrated just that. PolB

promoter activity in cells that I engineered to over express miR-155 was significantly decrease compared to empty (null) vector. Creb1 and PolB protein levels were also significantly reduced. My work only shows the effect of miR-155 in the absence of any damaging agents. It would be interesting to investigate the promoter inducibility by cytotoxic agents such as: MMS, H₂O₂, and methotrexate. An alternative hypothesis to explain PolB downregulation is accumulation of loss of function mutations on the promoter. Sequencing the PolB promoter in DS samples of varying ages would also be interesting. My data shows that DS cells have an attenuated response in presence of DNA damaging agents.

Over the years, studies have emerged to show that miR-155 is an important regulator of DNA repair. On the other hand, DNA damage response (DDR) in DS, has not been extensively studied and thus provides obvious issue when studying DNA repair in these individuals. Is the defect in DS a response or repair impairment? The research thus far does not present a clear answer. A recent study suggests that miR-155 is regulating both the DDR and several repair pathways (Czochor et al., 2016). Interestingly, they show that regulation is via the transcription factor, FOXO3a. They show that miR-155 overexpression results in reduced FOXO3a protein and this leads to inhibition of HR and an upregulation of the error prone NHEJ. But FOXO3a is also a potent activator of BER via Gadd45a. These findings provide an alternative mechanism of how miR-155 could be inhibiting PolB independently of Creb1. Typically FOXO3a would activate Gadd45a in response to DNA damage, and in turn Gadd45a activates recruitment of BER genes via PCNA (Gutierrez-Mariscal et al., 2014). As described in diagram 5.1.1, I'm proposing that miR-155 may be regulating POLB by two distinct mechanisms: directly, via Creb1-induced inhibition, and indirectly via FOXO3a. My proteomic data in chapter 3 provides support or both proposed models, as we found that several BER genes are affected by miR-155.

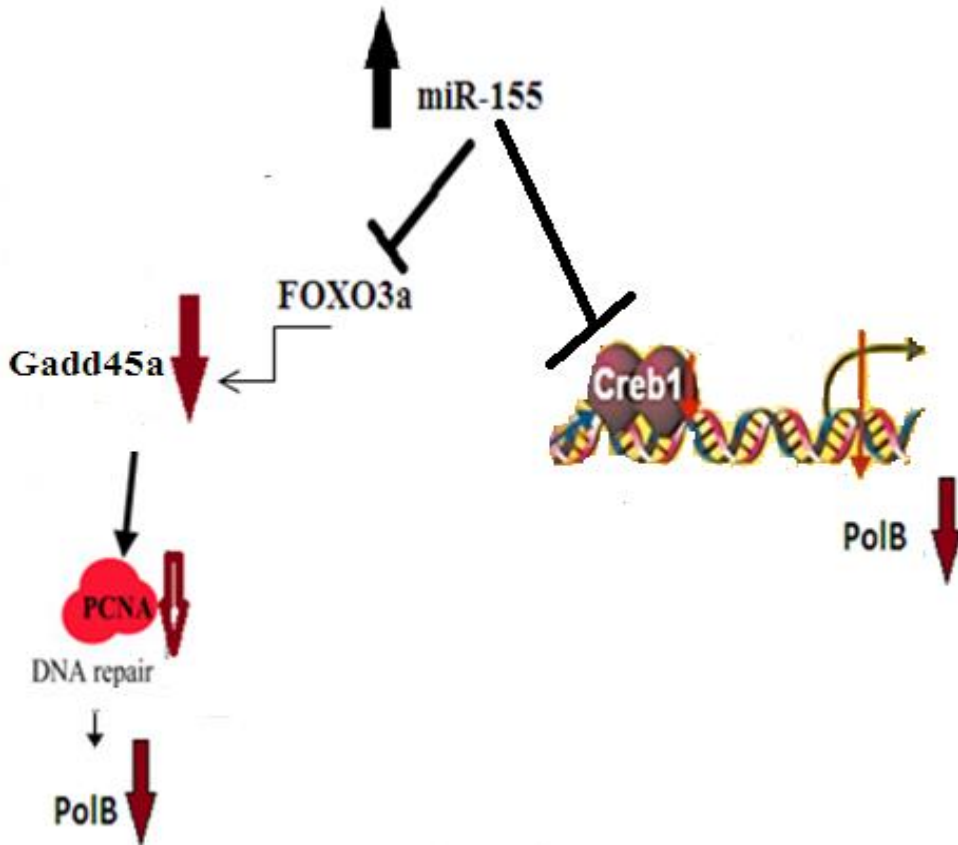


Figure 5.1.1: The proposed mechanism by which miR-155 regulates PolB.

The primary consequence of premature cellular senescence is the loss of proliferation which can be detrimental to survival of the organism. Another important effect is secretion of factors that alter the tissue microenvironment. Recent studies on senescence-associated secretory phenotype (SASP) of senescent cells revealed the potential of these secretions to initiate tumorigenesis (Taguchi et al., 2000). High Mobility Group Box 1 protein (HMGB1) is one of many markers in the SASP. Exocytosis of HMGB1 enhances pro-inflammatory activity of cytokines. Several studies showed that senescent cells secrete HMGB1, which in turn recruits and activates cells from the innate immune system to clear senescent cells (Schlueter et al., 2005). Others showed that extracellular HMGB1 protein may be promoting cancer and metastatic progression (Sha, Zmijewski, Xu, & Abraham, 2008). HMGB1 is being used in

combination with p16 and SA-beta gal staining as markers of in vitro senescence. I have preliminary data on HMGB1 in our DS and PolB null cells that suggest that under both conditions, HMGB1 is extruded from the nucleus: an indicator of senescence. Early passage PolB cells show HMGB1 localized to nucleus while late passage cells show cytoplasmic localization. The DS fibroblast show the delocalization from nucleus to cytoplasm is occurring at earlier passage than their matched controls. Further investigation into the SASP both in the absence of POLB and in DS could provide great insight into the role of these factors in the DS phenotype. One can speculate that the increase in age dependent lymphomas in DS might be due to SASP of the senescent cells that provide the proper microenvironment for tumorigenesis.

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ABSTRACT**INVESTIGATING THE ROLE OF DNA POLYMERASE BETA IN THE AGING PHENOTYPE OF DOWN SYNDROME**

by

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Down syndrome (DS) is a chromosomal condition characterized by accelerated aging that has yet to be directly linked to a DNA repair defect. Reduced PolB and unrepaired damage from oxidative stress observed in DS, point toward defective base excision repair (BER). In this study, we report that low PolB transcript correlates with increased markers of senescence. The gene dosage effect of Trisomy 21 is likely the source for PolB downregulation. We show that the HSA21-localized miR-155 overexpression correlates with a decrease in Creb1 and PolB, thus establishing a putative regulatory pathway. Data from the DS mouse model, Ts65Dn, reveal low incidence of solid tumors consistent with clinical observations. Our findings establish Pol β as a causative factor of senescence, suggesting that base excision repair is one of the processes driving aging in Down syndrome.

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