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Targeting the Redox System to Overcome Mechanisms of Drug Resistance in Chronic Lymphocytic Leukemia

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TARGETING THE REDOX SYSTEM TO OVERCOME MECHANISMS OF DRUG RESITANCE IN CHRONIC LYMPHOCYTIC LEUKEMIA

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by
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Dedication

To mom and dad

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To my family: You've taught me to never give up and that hard work will pay off. Thank you.

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To the CLL patients: Without you, this project would not have been possible. Thank you for your commitment in helping us find a cure.

TARGETING THE REDOX SYSTEM TO OVERCOME MECHANISMS OF DRUG RESITANCE IN CHRONIC LYMPHOCYTIC LEUKEMIA

Marcia Azusa Ogasawara, B.S.

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Chronic Lymphocytic Leukemia (CLL) is the most common form of leukemia diagnosed in Western countries and is characterized by clonal expansion of B cells. The clinical course of CLL is diverse and nearly 50% of patients present with chromosomal abnormalities. Deletion of the short arm on chromosome 17 (del17p) occurs in 5-7% of cases and presents with the shortest median survival time and often respond poorly to therapy. The tumor suppressor gene, TP53 is located on this region and it is well established that the p53 protein regulates multiple functions including: mitochondria biogenesis, response to DNA damage and redox balance. The objectives of the study were to determine the status of mitochondria in the mouse TCL1/p53$^{-/-}$ CLL model, role of the enhancer of zeste homolog 2 (EZH2) in drug resistance, and whether use of Auranofin has any therapeutic potential in CLL. This study determined that TCL1/p53$^{-/-}$ had elevated expression of transcription factors which regulate mitochondria biogenesis and increase in mitochondria DNA copy number. Second, TCL1/p53$^{-/-}$ mice had significantly elevated expression of EZH2 protein relative to TCL1/p53$^{+/+}$ cells while CLL patients had no distinct pattern of expression. CLL patients responded to two EZH2 inhibitors with increased Histone 3 Lysine 27 trimethylation, which is contrary to what has been published. Further studies to determine whether CLL patients carry EZH2 mutations which influence the response to these inhibitors are needed. Lastly, CLL cells showed significant apoptosis induction upon treatment with Auranofin despite stromal co-culture protection. Increased expression of Thioredoxin Reductase 1 (TxnR1), the target of Auranofin was seen in CLL cells upon stromal co-culture. High apoptotic induction in CLL and minimal cytotoxicity towards stromal

cells warrant further examination of Auranofin for CLL therapy. The results of this study identified two relatively unexplored targets, EZH2 and TxnR1, in CLL. Identifying the mechanism for the contradictory response of the EZH2 inhibitors by CLL cells may reveal a new prognostic indicator/biomarker. Lastly, Auranofin may prove to be useful in treatment of CLL patients despite any chromosomal abnormalities or prior treatment, as majority of CLL cells co-cultured with stromal cells responded to Auranofin with significant apoptosis.

Table of Contents

Dedication iii
Acknowledgements iv
Table of Contents vii
List of Illustrations ix
List of Tables xi
Abbreviations xii
Chapter 1 1
Introduction 1
1.1 Chronic Lymphocytic Leukemia 1
1.2 Genetic Aberrations Affect CLL Response to Therapy and Survival 2
1.3 p53 and Mitochondria Dysfunction in Leukemia 7
1.4 EZH2 and its Role in Cancer 10
1.5 Redox Regulation in the Cell 13
1.6 Targeting the Redox System for Cancer Therapy 16
1.7 Auranofin 17
1.8 Hypothesis and Specific Aims 19
Chapter 2 21
Materials and Methods 21
Chapter 3 27
Mitochondria of TCL1/p53$^{-/-}$ Mice 27
3.1 Rationale and Background 27
3.2 Results 27
3.3. Discussion 35
3.3 Future Directions 37
Chapter 4 39
Role of EZH2 in CLL 39
4.1 Background and Rationale 39
4.2 Results 39
4.3 Discussion 53
4.4 Future Directions 54

Chapter 5 56
Therapeutic Potential of Auranofin for CLL Therapy 56
5.1 Background and Rationale 56
5.2 Results 56
5.3 Discussion 82
5.4 Future Directions 86
Chapter 6 88
Overall Discussion and Conclusions 88
Chapter 7 90
Bibliography 90
Chapter 8 107
Vita 107

List of Illustrations

Figure 1: Probability of survival from the date of diagnosis among the patients in the five genetic categories. 6

Figure 2 Mechanism of redox regulation of proteins by Thioredoxin and Thioredoxin Reductase. 15

Figure 3. Comparison of free radical and mitochondria mass in TCL1/p53$^{+/+}$ and TCL1/p53$^{-/-}$ mice. 28

Figure 4. Loss of p53 leads to alterations in mitochondria and nuclear encoded genes in TCL1/p53$^{-/-}$ mice.. 30

Figure 5. Increased TFAM and mitochondria DNA copy number inTCL1/p53$^{-/-}$ mice.. 32

Figure 6. Comparison of mitochondrial respiration between TCL1/p53$^{+/+}$ and TCL1/p53$^{-/-}$ mice.. 33

Figure 7. Upregulation of mitochondria biogenesis regulators in CLL patients with 17p deletion. 34

Figure 8. Loss of p53 leads to increased expression of EZH2 in TCL1/p53 mice. 40

Figure 9. Diverse EZH2 expression level in CLL. 41

Figure 10. CLL response to GSK126. 43

Figure 11. Response to combination treatment of GSK126 with F-Ara-A or OXP in CLL. 45

Figure 12. Increased H3K27(Me)3 in response to GSK126 and DZNep. 47

Figure 13. Inhibition of H3K27(Me)3 by GSK126 non-CLL cells.. 49

Figure 14 Increased trimethylation is maintained in CLL. 50

Figure 15. GSK126 does not affect EZH2 expression. 51

Figure 16. EZH2 knockdown does not affect sensitivity to OXP.. 52

Figure 17. Increased sensitivity to Auranofin in cells with dysfunctional mitochondria.. 57

Figure 18. Impact of Auranofin on CLL survival. 59

Figure 19. Selective sensitivity sensitivity to Auranofin by CLL cells.....61
Figure 20. Increased sensitivity of CLL towards Auranofin when co-cultured on HK stroma. 63
Figure 21. Auranofin triggers ROS production in CLL cells.....65
Figure 22. NAC diminishes Auranofin induced ROS production. 67
Figure 23. Auranofin leads to GSH depletion and increased NADPH/NADP ratios.....69
Figure 24. Stromal co-culture increases GSH in CLL cells.. 70
Figure 25. Stromal co-culture leads to increased expression of Thioredoxin Reductase 1 in CLL cells. 71
Figure 26. Direct contact with stroma is not required for increased sensitivity to Auranofin for CLL. 74
Figure 27. Auranofin activates caspases in CLL.....74
Figure 28. Catalase protects Auranofin induced apoptosis on CLL cells co-cultured on stromal cells. 78
Figure 29. Neutralization of Auranofin by antioxidants. assessing for cell death (n=2). 79
Figure 30. Structure of metal containing drugs. 81
Figure 31. Apoptosis induction by metal containing drugs. 81

List of Tables

Table 1 Incidence of Chromosomal Abnormalities in 325 Patients with Chronic Lymphocytic Leukemia 4

Table 2. Hierarchical Model of Chromosomal Abnormalities in Chronic Lymphocytic Leukemia 5

Table 3 Mouse and Human Primer Sequences 23

Abbreviations

As_2O_3	Arsenic trioxide
BSO	Buthionine sulfoximine
CAT	Catalase
CLL	Chronic Lymphocytic Leukemia
DZNep	3-Deazaneplanocin A
EZH2	Enhancer of zeste homolog 2
F-Ara-A	Fludarabine
GCL	Glutamate cysteine ligase
Grx	Glutaredoxins
GSH	Glutathione
GPx	Glutathione Peroxidase
H3	Histone 3
$H3K27(Me)_3$	Histone 3 Lysine 27 trimethylation
H_2O_2	Hydrogen peroxide
IL-6	Interleukin 6
NAC	n-Acetyl Cysteine
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOX	NADPH Oxidase
NRF-1	Nuclear respiratory factor-1
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
PEITC	beta-Phenylethyl isothiocyanate
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
Prx	Peroxiredoxins
PYR	Sodium Pyruvate
RA	Rheumatoid arthritis
SOD1	Cu-Zn Superoxide Dismutase
SOD2	Manganese Superoxide Dismutase
TFAM	Mitochondria transcription factor A
TNFα	Tumor necrosis factor alpha
Trx	Thioredoxin
TxnR	Thioredoxin Reductase

Chapter 1

Introduction

1.1 Chronic Lymphocytic Leukemia

CLL is the most common form of leukemia and is characterized by clonal B-cell expansion. Not every patient will experience symptoms but those who do, commonly experience generalized lymphadenopathy, fatigue and weight loss. Median age of diagnosis is 65 and affects males twice as often than in females[1]. According to the revised National Cancer Institute-sponsored Working Group guidelines for CLL, diagnosis requires that there be at least 5000 B-cells/μL with cell surface antigen expression of CD5, a T-cell marker, and B-cell markers: CD19, CD20, CD23 as well as low expression of surface immunoglobulin, CD79b, and either kappa or lambda immunoglobulin light chains[1-3]. CLL cells accumulate not only in the blood but in bone marrow, lymph nodes and spleen. The clinical course differs for each patient as some may go years before requiring treatment while others may need to enter immediate treatment. Currently, there is no curative treatment for CLL, although progress has been made in the last decade in treatment course. CLL was previously treated with Chlorambucil, an alkylating agent as the main chemotherapeutic agents for over several decades[4]. Fludarabine (F-ara-A) was added to the treatment regimen as it yielded a higher response rate and longer duration of remission and progression-free survival when used as the first treatment in comparison to chlorambucil[5]. This was followed by combining F-ara-A and another alkylating agent, cyclophosphamide (C). Combination of F-ara-A + C improved the rate of clinical response, complete remission and progression free survival; however, patients still acquire resistance to treatment and can experience recurrence [6].

Currently, a CD20 monoclonal antibody called Rituximab (R), originally developed for the treatment of lymphomas, has been added to the regimen. The combination of F-ara-A, cyclophosphamide and Rituximab, designated FCR, has been shown to be effective in treatment naïve, relapsed and refractory patients[6-9]. Depending on several factors such as clinical stage of disease, patient fitness, genetic risk of the leukemia and any prior treatment, a treatment regimen of FC, FR or FCR may be initiated[2]. Despite the improvements seen with FCR for patient naïve and those who have had prior therapy, not all patients complete response and resistance to this regimen have been documented[10, 11].

1.2 Genetic Aberrations Affect CLL Response to Therapy and Survival

Identifying cellular and molecular markers which can aide in determining the prognosis and best mode of treatment action is constantly being evaluated for CLL. It is well established that CLL patients who have undergone somatic mutations in the Ig heavy H chain variable region V genes (IgHV) account for approximately 50% of cases[12, 13]. Whether patients have undergone this mutation is of prognostic significance as those who have undergone IgHV mutation have a relatively indolent disease requiring minimal to no chemotherapy and longer survival. Oppositely, unmutated IgHV patients tended to respond poorly to chemotherapy and have shorter survival times[12, 14]. Differential expression of zeta-chain associated protein of 70 kDa (ZAP-70) was found between mutated and unmutated IgHV CLL patients. Upon further comparision between IgHV and ZAP-70, the latter was found to be an even stronger prognostic indicator than IgHV mutational status[15, 16]. In addition to these two markers, cytogenetic studies reveal that nearly 50 percent of patients have genetic abnormalities; which can influence and predict disease aggressiveness, response to therapy and overall survival[17, 18]. Fluorescence in situ hybridization (FISH) analyses of hundreds of patient samples revealed varying incidences of chromosomal abnormalities in CLL[19]. **Table 1** indicates in descending incidence the observed genetic

alterations[19]. The top four alterations observed were: deletion of the long arm of chromosome 13 (del13q) was the most prevalent at 55%, followed by deletion of the long arm of chromosome 11 (del11q) at 18%, chromosome 12 trisomy (16%), and short arm of chromosome 17 (del17p). The survival times for the patients with the presented chromosomal abnormalities are presented in ascending hierarchical order in **Table 2**[19]. Furthermore, **Figure 1** indicates that del17p had the overall shortest median survival time at 32 months followed by del11q (79 months), 12q trisomy (114 months), normal karyotype (111 months) and del13q (133 months). This indicates that FISH analyses of CLL patients are an important diagnostic tool and that molecular alterations can significantly affect patient survival[20]. Patients with del17p have the worst prognosis and shortest median survival time and optimal treatment for these patients continues to be a hurdle in the clinic. A contributing factor to this dismal statistic is that the TP53 tumor suppressor gene is located in this region. Enumerable research has shown that loss of this gene, or its mutation, results in altered response to DNA damage, mitochondria function and oxidative stress.

Table 1 Incidence of Chromosomal Abnormalities in 325 Patients with Chronic Lymphocytic Leukemia

TABLE 1. INCIDENCE OF CHROMOSOMAL ABNORMALITIES IN 325 PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKEMIA.

ABERRATION	NO. OF PATIENTS (%)*
13q deletion	178 (55)
11q deletion	58 (18)
12q trisomy	53 (16)
17p deletion	23 (7)
6q deletion	21 (6)
8q trisomy	16 (5)
t(14q32)	12 (4)
3q trisomy	9 (3)
Clonal abnormalities	268 (82)
Normal karyotype	57 (18)

*One hundred seventy-five patients had one aberration, 67 had two aberrations, and 26 had more than two aberrations.

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Table 2. Hierarchical Model of Chromosomal Abnormalities in Chronic Lymphocytic Leukemia

TABLE 3. HIERARCHICAL MODEL OF CHROMOSOMAL ABNORMALITIES IN CHRONIC LYMPHOCYTIC LEUKEMIA.*

KARYOTYPE†	NO. OF PATIENTS (%)
17p deletion	23 (7)
11q deletion	56 (17)
12q trisomy	47 (14)
Normal karyotype	57 (18)
13q deletion as sole abnormality	117 (36)
Various abnormalities	25 (8)

*The model was constructed on the basis of the regression analysis.

†The five major categories are defined as follows: patients with a 17p deletion; patients with an 11q deletion but not a 17p deletion; patients with 12q trisomy but not a 17p or 11q deletion; patients with a normal karyotype; and patients with a 13q deletion as the sole aberration. Twenty-five of the 325 patients with various chromosomal abnormalities could not be assigned to one of these five major categories.

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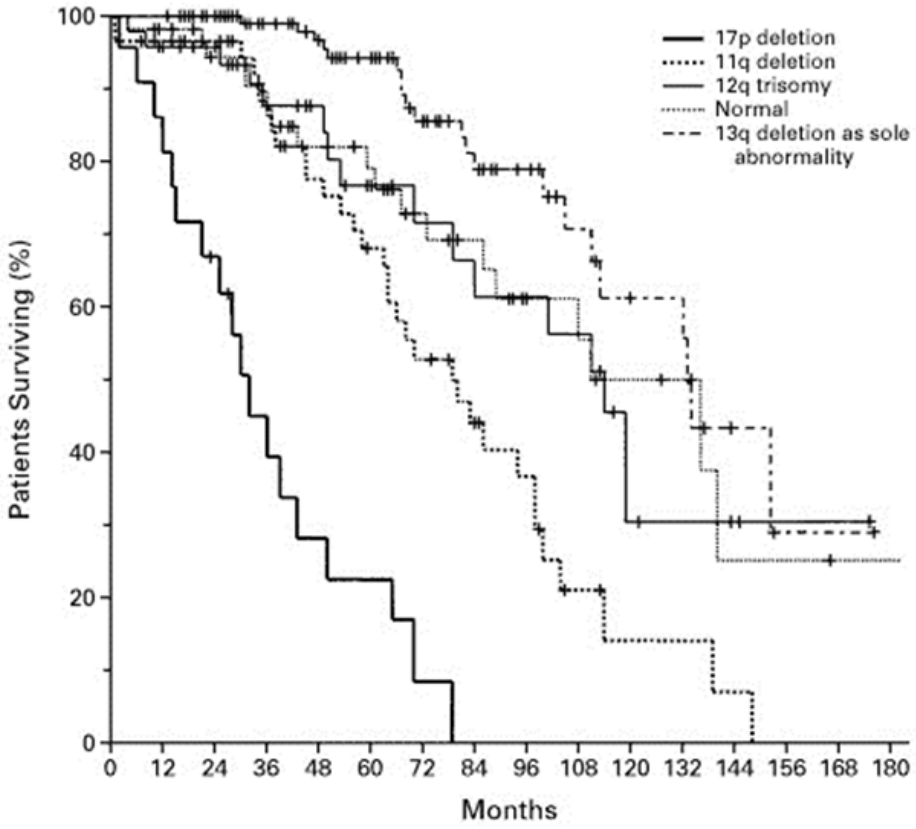


No. at Risk																
17p deletion	23	18	13	8	5	4	1	0	0	0	0	0	0	0	0	0
11q deletion	56	53	47	43	33	27	20	15	10	4	2	2	1	0	0	0
12q trisomy	47	44	41	29	24	17	14	13	12	11	4	3	2	1	1	0
Normal	57	51	45	37	30	27	20	17	12	11	6	5	2	2	1	1
13q deletion as sole abnormality	117	117	106	91	80	63	45	36	24	16	12	11	3	1	1	0

Figure 1: Probability of survival from the date of diagnosis among the patients in the five genetic categories. From: New England Journal of Medicine, Dohner, H., Stilgenbauer, S., et al., Genomic Aberrations and Survival in Chronic Lymphocytic Leukemia, 343, 1913. Copyright © (2000) Massachusetts Medical Society. Reprinted with permission

In order to assess the efficacy of new therapeutic agents in CLL as well as analyze molecular alterations, the importance of patient consent to donate their blood to research is immeasurable. This is supported by the fact that there is a limitation of well-established CLL cell lines for research. However, there are several CLL mouse models by which in vivo studies can be performed. The TCL1 transgenic mouse, along with several others, has been shown to mimic human CLL disease and is a useful model to perform in vivo studies of potential therapeutic agents and further understand the biology of this disease[21]. CLL is initiated in these mice through driving the expression of TCL1 in immature and mature B cells through the control of a VH promoter-IgH-Eμ enhancer[21]. A limitation of the TCL1 transgenic mice is that it retains functional wild-type TP53 and therefore it is difficult to assess whether new therapeutic agents designed for patients with del17p CLL will see benefit. To this end, we recently developed the TCL1/p53$^{-/-}$ mice colony by crossing of TCL1 transgenic with p53$^{-/-}$ mice[22]. Similar to the TCL1 transgenic mouse, TCL1/p53$^{-/-}$ mice showed expansion of the CD5^{+}/IgM^{+} B-cells but with significantly earlier onset. In addition, TCL1/p53$^{-/-}$ mice showed significantly shorter survival time, and were more resistant to clinically used DNA damaging agents[22]. Significantly, these alterations were comparable to what was observed in patient CLL samples with del17p.

1.3 p53 and Mitochondria Dysfunction in Leukemia

Alterations in metabolism has now been established to be one of the hallmarks in cancer; a discovery made by Otto Warburg that tumor cells have a preference to glycolysis instead of aerobic respiration[23, 24]. Mitochondria are an important organelle as it is the core for energy generation as well as execution of cell death. p53 has been shown to be actively involved in mitochondria biogenesis and function either through direct regulation of mitochondrial genes by transcriptional activation/repression or through influencing other signaling pathways such as NFκB[25, 26]. Aerobic respiration (aka oxidative phosphorylation)

is carried out by five multi-subunit enzymes including: complex I, II, III, IV and F_1-F_O-ATP synthase). Loss of p53 has been shown to result in decreased aerobic respiratory capacity in part through Synthesis of Cytochrome c Oxidase 2 (SCO2) which aids in assembly of cytochrome c oxidase (COX)[27].

Mitochondria are unique in that it possesses its own mitochondrial DNA (mtDNA) that encode for components of the electron transport chain and replication machinery. P53 was shown to directly interact with the mitochondria polymerase gamma (DNA Pol γ) upon mtDNA damage; resulting in enhancing the replicative activity of DNA Pol γ. Further, loss of p53 significantly elevated the susceptibility of mtDNA to damage and thus susceptibility to mutations[28]. Fludarabine treated CLL patients had a significant increase in the frequency of heteroplasmic mitochondrial DNA mutations. Acquisition of these mutations seemed to associate with elevation in superoxide production[29]. Relative to normal lymphocytes, alterations in CLL mitochondria have also been observed. Elevation of free radicals can be a direct indicator of mitochondria damage; as evidenced by a tetracycline inducible system for a dominant negative DNA Polymerase Gamma (POLG-dn). Induction of POLG-dn revealed increased ROS production through the NADPH Oxidase (NOX) system, reduced mitochondria respiration, and selective sensitivity when inhibited[30]. Free radicals such as superoxide are normally produced as a result of electron leakage from the electron transport chain.

Regulation of mitochondria biogenesis occurs through nuclear encoded transcription factors including: p53, mitochondria transcription factor A (TFAM), nuclear respiratory factor-1 (NRF-1), and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α)[26, 31-34]. Increased number of mitochondria was observed in CLL patients compared to normal lymphocytes and was found to be related to the level of endogenous nitric oxide[35]. Similarly, CLL patients showed greater mRNA expression for mitochondria biogenesis regulators NRF-1 and TFAM compared to normal lymphocytes. TFAM not only

regulates mtDNA copy number and protect its stability, but can also interact directly with p53; which aids in maintenance of mtDNA[36]. Studies on skeletal muscle fibers of p53 knockout mice showed reduced mitochondria content and PGC1α protein expression in gastrocnemius muscle while decreased mitochondria respiration and elevated ROS in intermyofibrillar mitochondria[37].

Increased expression of anti-apoptosis regulators, Bcl-2 and MCL-1, which are located on the mitochondria have been observed in CLL[38, 39]. Alterations of mitochondrial apoptosis regulators were also seen in TCL1/p53$^{-/-}$ mice. Increased expression of anti-apoptotic proteins: Mcl-1 and Bcl-X_L were elevated in TCL1/p53$^{-/-}$ mice compared to the p53 wildtype counterpart. Importantly, this pattern was also observed upon immunoblotting of patient CLL samples with and without del17p. As this was the first report of the TCL1/p53$^{-/-}$ mouse model, the status of the mitochondria is yet to be investigated. Determining whether there are any alterations in the mitochondria in TCL1/p53$^{-/-}$ mice has implications in disease aggressiveness, survival and therapy. Impaired mitochondria functions in cancer cells result in a preferential switch to glycolysis for energy requirement. This response to impaired mitochondria results in an increase in glycolytic enzymes which can be targeted for therapy. Oncogenic K-Ras induction in a tetracycline-inducible system showed prolonged ROS production resulted in impaired mitochondria function (decreased mitochondria respiration) and an increase in glycolysis as measured by increased glucose uptake and lactate production[40]. Hexokinase II, which catalyzes the first step in glycolysis, is just one of the numerous glycolytic enzymes that have been reported to be elevated in tumor cells with impaired mitochondria[41]. Use of inhibitors such as 3-bromopyruvate to which covalently modifies hexokinase II was shown to induce apoptosis in leukemic, HL-60 cells[41]. Therefore, significant impairment of TCL1/p53$^{-/-}$ mitochondria function can be exploited for therapy.

1.4 EZH2 and its Role in Cancer

In addition to regulating mitochondria biogenesis and metabolism, p53 is central in responding to DNA damage. Chemotherapeutic agents used in CLL such as F-Ara-A, Cyclophosphamide and Chlorambucil function by either directly damaging DNA or inhibiting a process in DNA synthesis. Upon DNA damage initiated by UV radiation, activated p53 has been shown to transcriptional repression of enhancer of zeste homolog 2 (EZH2), a protein which is a component of the Polycomb repressive complex 2 (PRC2)[42]. The PRC2 functions as an epigenetic modifier through its ability to methylate lysine 27 (K27) on histone 3 (H3)[43]. Methylation of H3K27 is successive as kinetic studies of the rates of mono-, di- and tri- methylated residue formation progressively slowed[44]. The tri-methylated product of the reaction, H3K27$(Me)_3$, results in chromatin repression. The PRC2 is composed of several core components including: EZH2, embryonic ectoderm development (EED), Suppressor of zeste 12 homolog (SUZ12) and RbAp46/48 [45]. EZH2 contains a SET domain which is required for the enzymatic activity of the PRC2. Alterations in EZH2 have been reported in solid tumors (breast, ovarian, prostate) and found to influence tumor progression, migration and invasion, and drug resistance[46-49]. EZH2 and the PRC2 regulate numerous cellular processes based on their repressive functions. Overexpression of EZH2 in mammary epithelial cells resulted in repression (mRNA and protein) of five DNA damage response components: RAD51L1, RAD51L2, RAD51L3, XRCC2, and XRCC3[47]. These five Rad51 family members are required in response to perform homologous repair in response to DNA double strand breaks[50].

Elevated expression of EZH2 was observed in cisplatin-resistant A2780/DDP ovarian cancer cells in comparison to the parental A2780 cells. Knockdown of EZH2 decreased H3K27$(Me)_3$ levels as well as proliferation rates of the A2780/DDP cells. Further examination of cell cycle distribution revealed a significantly increased percentage of G2/M-phase cells while decreased G_0/G_1-phase cell; suggesting the reduced proliferation was

due to arrest at G_2/M[49]. EZH2 aberrations have also been observed in numerous hematopoietic malignancies. Overexpression of EZH2 has been detected in Hodgkin's disease and non-Hodgkin's lymphoma[51, 52]. High co-expression patterns of EZH2 along with BMI, another PcG protein, in the nucleus of Reed-Sternberg cells were observed for patients of Hodgkin's disease; with generally no EZH2 detection of surrounding infiltrating lymphocytes[51]. The significance of these observations was the co-expression of EZH2 and BMI in these cells, as generally, expression of these two proteins are mutually exclusive in follicular B cells[51, 52].

Whether EHZ2 functions as a tumor suppressor or oncogene in leukemia is still not well understood. Loss of function studies in the MLL-AF9 acute myeloid leukemia (AML) mouse model where conditional deletions of either EZH2 or EED investigated the role of the PRC2 complex in disease development and progression[53]. EZH2-null mice showed significantly decreased H3K27$(Me)_3$, but showed no survival difference upon transplantation of cells into lethally irradiated donor mice. However, a difference was observed upon isolation of EZH2-null leukemic cells from recipient mice and transplanted into secondary donor mice. When the same experiment was performed on EED-null cells, mice showed significantly prolonged survival and complete inhibition of H3K27$(Me)_3$ upon isolation of the leukemic cells following mouse sacrifice[53].

Whole genome sequencing analyses of patient samples have also revealed genetic aberrations of EZH2 and other components of the PRC2. Inactivating mutations were found for EZH2, EED and SUZ12 in 42% of Early T-cell precursor acute lymphoblastic leukemia (ETP ALL) cases compared to 11.9% in non-ETP ALL[54]. Structural modelling suggested the deletions and sequence mutations observed, majority in the SET domain, would result in loss of function[54]. A larger study analyzing various myeloid disorders, two of which were: myelodysplastic syndrome (MDS) and myelodysplastic-myeloproliferative neoplasms (MDS/MPN) also revealed inactivating mutations of EZH2. Univariate analyses of patients

with and without EZH2 mutations revealed a poorer prognosis for those with the genetic alterations[55]. Opposite to loss of function mutations, several studies have shown gain of function alterations, specifically, tyrosine 641 (Y641), in follicular and diffuse large B-cell lymphomas (DLBCL)[56-58]. The location of this mutation is within the SET domain of EZH2 and leads to altering the enzymatic activity of the PRC2. Expression analyses of lymphoma cell lines with mutant EZH2-Y641 resulted in increased basal H3K27$(Me)_3$ levels compared to EZH2-wild type cell lines[57].

With evidence that alterations in EZH2 are found in both solid and hematologic malignancies, design of EZH2 inhibitors as a potential therapeutic strategy is rational. Studies of EZH2 inhibitors have shown therapeutic efficacy in the context of inhibiting proliferation, inducing apoptosis and inhibiting migration. Use of EI1, which competes with s-adenosyl methionine (SAM), a cofactor used by EZH2 for methylation, on DLBCL cell lines showed selectivity towards cell lines with Y641 mutations. DLBCL-Y641 cells showed proliferation and colony formation inhibition in a dose dependent manner while this was not observed in EZH2-WT cell lines [59]. GSK126, an specific EZH2 inhibitor was also found to be effective in lymphoma cell lines with Y641 and A677 mutations; leading to decreased H3K27$(Me)_3$ levels, cell cycle arrest, and reactivation of PRC2 silenced genes. Gene ontology enrichment analyses revealed common processes were affected among the responsive cell lines, including: cell cycle regulation, cell death and biological/cellular processes[59]. One study which profiled 33 microRNAs in CLL patients based on whether these microRNAs could influence immunologic subsets, revealed microRNA-101 down-regulation was associated with EZH2 over-expression. Observations of EZH2 alterations in the described lymphomas suggest other hematopoietic malignancies may also have mutations, overexpression or deletions. As EZH2 has been found to regulate response to DNA damage, it is still unknown what role(s) EZH2 has in affecting response to therapy in CLL with and without del17p.

1.5 Redox Regulation in the Cell

Free radicals are constantly being produced within the cell as byproducts of metabolism, fighting off foreign agents and as signaling molecules. As described earlier, the mitochondria are a primary source of free radical production; specifically $O_2^{\bullet -}$, as a result of oxidative phosphorylation. Loss of p53, oncogene activation and tumor cells with previous chemotherapy have been observed to elevate ROS production[60-63],[64]. Antioxidant mechanisms are in place to ensure proper elimination of these oxidative molecules, as they have the potential to be damaging to cellular components such as DNA and proteins. Maintaining homeostasis between oxidative molecules and antioxidants and the processes which encompass this within a cell is described as 'redox regulation' and it is a matter of life or death for a cell. Another source of $O_2^{\bullet -}$ is the plasma membrane NAPDH oxidase (NOX) which are found to have increased activity for cells which have been oncogenically transformed[30, 40]. Other radical reactive oxygen/nitrogenous species (ROS/RNS) include the hydroxyl anion ($HO^{\bullet}$), nitric oxide (NOS) and peroxynitrite ($OONO^-$). A non-radical ROS is H_2O_2. Elimination of $O_2^{\bullet -}$ is performed by superoxide dismutases (SOD) in the reaction of:

$$O_2^{\bullet -} \longrightarrow H_2O_2 + O_2$$

There are three SODs in humans and are differentially located. SOD1 is located in the cytoplasm, SOD2 the mitochondria and SOD3 is secreted to the extracellular space[65, 66]. Elimination of H_2O_2 can be performed by catalase (CAT) through the reaction of:

$$H_2O_2 \longrightarrow H_2O + O_2$$

ROS can also be neutralized by interaction with antioxidant peptides such as glutathione (GSH) or antioxidant proteins: glutaredoxins (Grx), thioredoxins (Trx), peroxidredoxins (Prx)[67]. GSH is the most abundant antioxidant in the cell as concentrations range from 1-

10mM. GSH functions not only as an antioxidant but also detoxifies xenobiotics, regulates cell growth, and acts as a store for cysteine. Upon interaction of ROS with GSH:

$$GSH + Oxidant \rightarrow GSSG$$

enzymes such as GPx will utilize NADPH as the reducing equivalent to reduce GSSG → GSH. In addition, ROS can attack proteins with redox sensitive, thiol containing proteins[67]. Oxidized proteins which require to be in a reduced state for function can be returned to this by GSH, where:

$$Protein\text{-}SSG + GSH \rightarrow Protein\text{-}SH + GSSG$$

Transcription factors which contain cysteine residues that are sensitive to oxidation include: p53, NFκB, and Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Maillet,2012,Gloire 2006,Lee 2003). In addition, transcriptional response elements for p53, NFκB, and Nrf2 are found on the promoter regions for genes such as GPX, SOD, and, GCL respectively[68-70]. In cases of high oxidative content within a cell, severe GSH depletion can lead to cell death. Synthesis of GSH occurs through a two-step reaction of the following:

(1) *l*-glutamate + *l*-cysteine + ATP ⟶ γ-glutamyl-*l*-cysteine + ADP +Pi

(2) γ-glutamyl-*l*-cysteine + *l*-glycine + ATP ⟶ GSH + ADP + Pi

In step (1) glutamate cysteine ligase (GCL) ligates l-glutamate to l-cysteine under an ATP dependent reaction to yield γ-glutamyl-*l*-cysteine. The final step is catalyzed by GSH synthase (GS) where γ-glutamyl-*l*-cysteine is conjugated with *l*-glycine to yield GSH.

Another important group of antioxidants are the thioredoxins (Trx) and Thioredoxin reductase (TrxnR) enzymes which help maintain the redox status of proteins. Synonymous to GSH, ROS-oxidized proteins on their cysteine residues can be reduced back by reduced Trx ($Trx\text{-}SH_2$). Upon oxidation, oxidized Trx ($Trx\text{-}S_2$) will be reduced back by TxnR which uses NADPH as an electron donor (**Figure 2**). Redox regulation of proteins are important

as oxidation of sulfhydryl residues on proteins will lead to disulfide formation which can affect its function. Two Trx forms are found in a mammalian cell: Trx1is localized in the cytosol while Trx2 is in the mitochondria[71]. TxnR are also found in the cytosol (TxnR1), mitochondria (TxnR2) and a testis-specific thioredoxin glutathione reductase (TGR)[71].

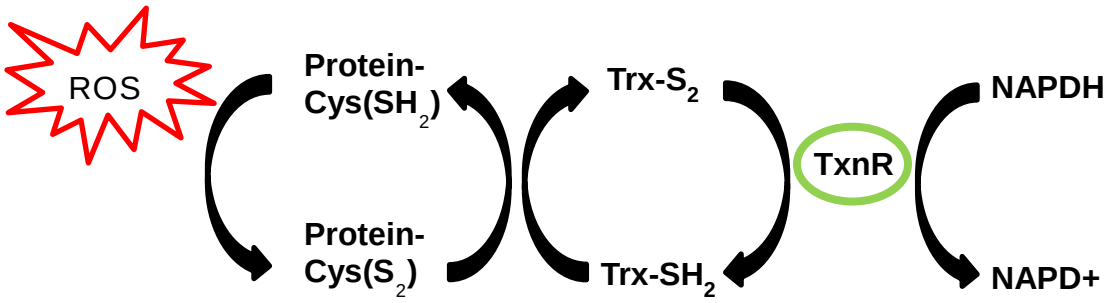


Figure 2 Mechanism of redox regulation of proteins by Thioredoxin and Thioredoxin Reductase.

Trx can regulate apoptosis as well as activate downstream regulators through interactions that are redox regulated. Reduction of cysteine residue on by Trx enhanced its DNA binding activity which allows induction of gene transcription[72]. The NFκB pathway, in addition to regulating mitochondria transcription factors as described above, regulates genes involved in cell survival. Constitutive activation of the NFκB pathway is often seen in CLL and implicated for promoting cell survival signals[73]. Trx also regulates apoptosis in cells through its interactions with apoptosis signal-regulating kinase (ASK-1)[74]. Reduced Trx is able to bind with ASK-1 and signal it for degradation; however, oxidation of the residue by ROS can lead to its dissociation. Activated ASK-1can lead to induction of apoptosis as ectopic expression of ASK-1 in multiple myeloma cell lines increased apoptosis following days after transduction of the vectors. Similar results were seen when a tetracycline-inducible system for ASK-1 was introduced into in these cells[75].

1.6 Targeting the Redox System for Cancer Therapy

Targeting the redox system of cancer cells is a therapeutic strategy that is an ongoing endeavor[63, 76]. It has been shown in numerous instances that cancer cells have altered redox states which often result in increased basal levels of ROS. CLL has been shown to produce abundant ROS as a result of mitochondria metabolism[77]. Basal $O_2^{\bullet-}$ mean levels were significantly higher in CLLs when compared with peripheral normal lymphocytes[78]. CLL cells analyzed from patients who have had prior chemotherapy produced significantly higher levels of $O_2^{\bullet-}$. Furthermore, measurement of $O_2^{\bullet-}$ from a CLL patient before and after chemotherapy of F-Ara-A and cyclophosphamide showed an increase in basal $O_2^{\bullet-}$ levels[29, 35]. Several compounds which trigger oxidative stress through depletion of GSH has been shown to initiate apoptosis in CLL. Use of beta-phenylethyl isothiocyanate (PEITC), a natural compound found in cruciferous vegetables was shown to deplete GSH in a time dependent manner and lead to ROS accumulation[63]. This mechanism by PEITC was able to take advantage of the intrinsic oxidative stress often found in cancer cells and lead to selective elimination of oncogenic RAS-transformed cells. PEITC was also able to induce apoptosis in CLL cells who were resistant to F-Ara-A[62]. F-Ara-A sensitive CLL cells had a mean IC50 (μM) of 3.4 ±0.8 and 5.1 ± 0.5 for F-Ara-A and PEITC, respectively; while F-Ara-A-resistant CLL had an IC50 of 38.6 ± 13.1 and 5.4 ± 0.5 for F-Ara-A and PEITC, respectively[62]. A study with arsenic trioxide (As_2O_3) on CLL showed a dose dependent reduction in viability after 24hr of treatment. Supplementation with CAT, GSH and n-Acetyl Cysteine (NAC) reduced the cytotoxicity of As_2O_3[79]. Addition of ascorbic acid with As_2O_3 triggered $O_2^{\bullet-}$ production as no production was found individually. Ascorbic acid enhanced the cytotoxicity of As_2O_3 in both CLL and multiple myeloma cells through depletion of GSH[80]. The cytotoxicity of As_2O_3 has been attributed through its abilities to inhibit the functions of GPx, TrnxR as well as mitochondrial respiration[30, 81, 82]. Inhibition of antioxidant enzymes has been another strategy investigated for cancer therapy. Use of 2-

methoxyestrodial (2-ME), an inhibitor of SOD was shown to selectively induce apoptosis in CLL but not normal lymphocytes[83].

1.7 Auranofin

Auranofin (Ridaura) is an FDA approved drug prescribed for the treatment of rheumatoid arthritis. Rheumatoid arthritis (RA) is an auto-immune disease characterized by accumulation of cells in the synovial joint which leads to inflammation, joint pain, and damage. Infiltration of various immune cells (i.e macrophages, T-cells) release both pro- and anti-inflammatory cytokines, although the former type predominates[84]. One of the mechanisms by which Auranofin showed efficacy towards RA is through its cytotoxicity towards immune cells. However, there is a bimodal effect to this drug as low dose Auranofin showed prolonging the survival of neutrophils and maintained its ability to express adhesion molecules[85]. Higher concentrations of Auranofin was reported to suppress chemotaxis and shorten the survival of neutrophils through inhibition of Protein Kinase C enzymatic activity[85, 86]. Auranofin inhibited IL-6 mediated phosphorylation of STAT in myeloma cells as well as inhibit NFκB binding activity in a dose dependent manner[87]. Apoptosis induction by Auranofin has been shown to be activated by both the extrinsic and intrinsic pathways as evidenced by cleavage products of caspase-8 (extrinsic) and 9 (intrinsic)[87, 88]. Auranofin not only has anti-inflammatory properties but with its ability to conjugate with thiol-containing molecules, it is a redox modifiying drug as well. Auranofin has been shown to inhibit TxnR activity in Jurkat T-lymphoma, and HL-60 leuekmia cell lines in a dose-dependent manner[89, 90].

As redox alterations are observed in cancers, targeting the redox system is a rational therapeutic approach. Inhibition of thioredoxin reductase and conjugation to thiol-containing molecules by Auranofin rationalize the investigation of its and other gold-

containing phosphine complexes for use as anti-cancer drugs[91]. Indeed, Auranofin induced apoptosis in multiple leukemia and solid tumor models *in vitro*[85, 87, 92]. While the studies of Auranofin in CLL were ongoing for this thesis, the first preclinical report of Auranofin's efficacy in CLL was published[93]. The study reported Auranofin to induce endoplasmic reticulum stress, elevate ROS production and induce apoptosis in CLL[93]. This is the first preclinical assessment of Auranofin's action on CLL. In addition, a Phase I and II study of Auranofin in CLL has been ongoing under trial: NCT01419691 which can be found at www.ClinicalTrials.gov.

1.8 Hypothesis and Specific Aims

The median survival time for CLL patients with del17p is the shortest relative to other chromosomal abnormalities. Loss of p53 affects multiple parameters including mitochondria biogenesis and function, apoptosis, response to DNA damage and redox balance. Development of the TCL1/p53$^{-/-}$ mouse model has provided a unique tool to further investigate the mechanisms by which del17p CLL patients have an aggressive disease phenotype. Alterations in apoptotic regulators have been observed in these mice, yet, the status of the mitochondria in TCL1/p53$^{-/-}$ mice is yet to be determined. Continued search for novel targets in CLL, not only for those with del17p, are needed. EZH2 alterations: mutations, over-expression, loss of function, has been observed in numerous types of cancers. Whether EZH2 can serve as a therapeutic target in CLL is unknown. On the other hand, targeting the redox system of CLL cells has shown promising results. Auranofin is a known inhibitor for TxnR and has already shown preclinical promise in one study while the studies for this thesis were ongoing.

I **hypothesize** that loss of TP53 in Chronic Lymphocytic Leukemia contributes to drug resistance by altering mitochondrial biogenesis and EZH2 expression but can be overcome by the redox inhibitor, Auranofin. Specifically, the **major goals** of this study were to: determine mitochondria DNA copy number, expression of mitochondria biogenesis genes, EZH2 status in TCL1/p53$^{-/-}$ mice relative to TCL1/p53$^{+/+}$ mice as well as between CLL patients with and without del17p, and test if Auranofin shows any therapeutic potential for treatment of CLL.

The **significance** of this study is supported by evidence that loss of p53 results in alterations affecting the survival and response to therapy in CLL cells. This study aims at further characterizing the TCL1/p53$^{-/-}$ model, determining the role of EZH2 in CLL which is yet unknown, and whether use of Auranofin, an FDA approved drug, can be a potential therapeutic agent. In order to test my hypothesis, my specific aims were:

Specific Aim 1: Determine the effects of p53 deletion on the mitochondria of TCL1/p53$^{-/-}$ CLL cells

Specific Aim 2: Determine if EZH2 promotes disease progression by affecting drug resistance in TCL1/p53$^{-/-}$ and del17p CLL

Specific Aim 3: Determine if redox inhibition by Auranofin CLL can be a therapeutic strategy in CLL

Chapter 2

Materials and Methods

Mice

The generation of TCL1 transgenic and TCL1/p53$^{-/-}$ mice and their maintenance were described previously [21, 22]. All mice were housed in the conventional barrier animal facility at the University of Texas MD Anderson Cancer Center, and the animal study was carried out under a research protocol approved by the Institutional Animal Care and Use Committee (IACUC).

Reagents

Cells were maintained in RPMI-1640 (SigmaAldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (SigmaAldrich, St. Louis, MO), penicillin (100 units/ml) + streptomycin (100 µg/ml) (P4333, SigmaAldrich, St. Louis, MO) at 37 °C in a humidified 5% CO2 incubator. Auranofin (A6733, Sigma-Aldrich, St. Louis, MO), Catalase (C1345, Sigma-Aldrich), N-acetylcysteine (A5099, Sigma-Aldrich), Glutathione (G6013, Sigma-Aldrich), trans-Platinum(II)diammine dichloride (P1525, Sigma-Aldrich), Chloro[diphenyl(o-tolyl)phosphine]gold(I) (702749, Sigma-Aldrich), GSK126 (M60071, Xcess Bio, San Diego, CA), 3-Deazaneplanocin A (11102, Cayman Chemical, Ann Arbor, MI).

Isolation of CLL cells

Primary CLL cells were obtained from the peripheral blood samples of B-CLL patients, who were diagnosed according to National Cancer Institute criteria (Zweibel, 1998). Prior to blood collection, informed consent under a research protocol approved by the Institutional Review Board of MD Anderson Cancer Center were obtained from all patients in accordance with the Declaration of Helsinki. In all experiments, CLL cells were isolated

from blood samples by density gradient centrifugation (Huang, 2000) and incubated in RPMI-1640 medium containing 10% FBS and penicillin (100 U/ml1) + streptomycin (100 µg/ml) overnight before experiments. Mice splenocytes were isolated as previously described[22]. CD19 positive selection of CLL cells were performed using CD19 microbeads as directed by the manufacturer (130-050-301, Miltenyi Biotech, San Diego, CA).

Assessment of ROS, superoxide and mitochondrial superoxide Cellular ROS was measured by incubating 1x106 cells for 1 hour with either 0.5µM CM-H_2DCF-DA (C6827, Life Technologies, Grand Island, NY) for ROS, 100ng/mL of dihydroethidium (D11347, Life Technologies, Grand Island, NY) for superoxide or 2.5µM MitoSox Red (M36008, Molecular Probes, Grand Island, NY) followed by washing and re-suspension in PBS. Cell fluorescence was measured using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with CellQuest Pro software. Flow cytometric data were analyzed using FlowJo (Tree Star, Ashland, OR).

Real time PCR

RNA was isolated using the RNEasy mini kit (74104, Qiagen, Valencia, CA) with addition of the DNase clean-up step. cDNA was prepared using 1µg of RNA for reverse transcriptase using RevertAid First Strand cDNA Synthesis Kit (K1621, Thermo Scientific, Rockford, IL). mRNA transcript levels were measured by running the reaction mix of cDNA template, primers and SYBR Green PCR Master Mix (4344463, Applied Biosystems, Grand Island, NY) on the Viia7 Real-Time PCR System (Life Technologies, Grand Island, NY). Fold changes were determined by normalizing to β–actin as an internal control and presented as $2^{-\Delta CT}$. Gene primers can be found in **Table 1**.

Table 3 Mouse and Human Primer Sequences

Gene	Sequence	Reference
Mouse		
Ms smMito F13597	CCCAGCTACTACCATCATTCAAGT	[94]
Ms smMito R13688	GATGGTTTGGGAGATTGGTTGATGT	[94]
Ms HistoneH2A-For-1606	AGTCTGTTGTCGCTCTTTGG	[95]
Ms HistoneH2A-Rev-1755	AGTTGAGTCTTAGTCCTGGG	[95]
Ms PolgA For-2025	CCTAAGCTCATGGCACTGAC	[95]
Ms PolgA Rev-2208	TGCTGCTTCCCCTGTTCAAG	[95]
Ms NRF1 For- 696	TCTCACCCTCCAAACCCAAC	[95]
Ms NRF1 Rev- 931	CCCGACCTGTGGAATACTTG	[95]
Ms Polrmt For-3051	GTCTACAGGAGATGTTCACC	[95]
Ms Polrmt Rev-3295	CAGGGAGTGGATGAAGTTG	[95]
Ms Tfb1m For- 665	AAGTTGATGTAGGAGTGGTG	[95]
Ms Tfb1m Rev-838	ATGTCTGCCAACTGTAACAG	[95]
Ms COX1-For	TGGAGGCTTTGGAAACTGAC	[96]
Ms COX1-Rev	TCCTGCATGGGCT AGATTTC	[96]
Ms PGC1a-For	CGGAAATCATATCCAACCAG	[96]
Ms PGC1a-Rev	TGAGGACCGCTAGCAAGTTTG	[96]
Ms TFAM For-590	CATTTATGTATCTGAAAGCTTCC	[95]
Ms TFAM Rev-761	CTCTTCCCAAGACTTCATTTC	[95]
Ms CytB-For	AGACAAAGCCACCTTGACCCGAT	[97]
Ms CytB-Rev	ACGATTGCTAGGGCCGCGAT	[97]
Ms Twinkle-For	CATCCCCCGGCCTGG TGTCT	[97]
Ms Twinkle-Rev	GCCCCG TCGACTGGCTCAAG	[97]
Ms PolG2-For	TCTGGGAAACTACGGGCGACTCT	[97]
Ms PolG2-Rev	TGCTGGGTGTCTGATTGCTGTTC	[97]
Ms ActinB For-16	CACCCGCGAGCACAGCTTCTT	
Ms ActinB Rev-133	TTTGCACATGCCGGAGCCGTT	
Ms EZH2 For-767	TGCCTCCTGAATGTACTCCAA	[98]
Ms EZH2 Rev-895	AGGGATGTAGGAAGCAGTCATAC	[98]
Ms SUZ12 For-588	CCAGAACAAGCATCAAAAGGAA	
Ms SUZ12 Rev-782	GCAGGACTTCCAGGGTAACAG	
Ms EED For-515	AAGTTGAGCAGCGACGAGAA	
Ms EED Rev-643	TCCTGGTGCATTTGGCGTAT	
Human		
Hu Twinkle-For1646	AACTGAACCCCAAACGATGC	
Hu Twinkle-Rev1874	CGATTGAGGTCTGGAAAGCG	
Hu GAPDH-For	AAGGTGAAGGTCGGAGTCAAC	
Hu GAPDH-Rev	GGGGTCATTGATGGCAACAATA	
Hu ACTB-For-393	CATGTACGTTGCTATCCAGGC	PrimerBank ID: 4501885a1,[99]

Hu ACTB-Rev-642	CTCCTTAATGTCACGCACGAT	PrimerBank ID : 4501885a1,[99]
Hu NRF1-For-21	CCGAGGACACCTCTTACGATG	
Hu NRF1-Rev-288	TACATGAGGCCGTTTCCGTTT	

Mitochondria DNA copy number

DNA was extracted using the DNeasy Blood & Tissue Kit (69504, Qiagen, Valencia, CA). 100ng of DNA was used to measure mitochondria DNA (mtDNA) copy number using nuclear DNA (ActinB, **Table 3**) primers and mitochondrial DNA primers (Ms CytB, **Table 3**). The reaction mix of DNA, primers and SYBR Green was run using conditions as previously described (Venegas, 2012) using a 7900HT Fast Real-time PCR system (Applied Biosystems, Grand Island, NY).

Western blot analysis

Cell lysates were prepared by addition of ice-cold RIPA buffer (50mM Tris-HCl pH 8, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 1x cOmplete ULTRA protease inhibitor cocktail (05892970001, Roche, Indianapolis, IN) and 1 x PhosSTOP phosphatase inhibitor cocktail (04906837001, Roche, Indianapolis, IN) on ice for 30 minutes. Samples were centrifuged at 20,000 x g for 15 minutes and protein concentration measured with the BCA protein assay (23225, Thermo Scientific, Rockford, lL). 20µg of protein was separated on a SDS-PAGE gel, transferred onto a nitrocellulose membrane and analyzed with various antibodies. Antibodies are as follows: Rabbit anti-PGC1α (SC-13067, Santa Cruz Biotechnology, Dallas, TX), rabbit anti-TFAM (ARP31400_P050, Aviva Systems Biology, San Diego, CA), mouse anti-MitoProfile Total OXPHOS Rodent WB Antibody Cocktail (ab110413, AbCam, Cambridge, MA), mouse anti-MitoProfile® Total OXPHOS Human WB Antibody Cocktail (ab110411, AbCam, Cambridge, MA), mouse anti-β-actin (A5441, SigmaAldrich, St. Louis, MO), mouse anti-

EZH2 (3147, Cell Signaling, Danvers, MA), rabbit anti-Histone H3 (4499, Cell Signaling), rabbit anti-Tri methyl Histone 3 (Lys 27) (9733, Cell Signaling), Thioredoxin reductase 1 (ab16840, AbCam, Cambridge, MA)

Measurement of mitochondria respiration

To measure mitochondria oxygen consumption, $7x10^7$ cells were centrifuged at 400xg for 5 minutes at room temperature. The supernatant was removed and cells were resuspended in 1mL of respiration buffer (25mM mannitol, 75mM sucrose, 10mM KCl, 10mM Tris-HCl, 5mM KH_2PO_4, pH 7.2). Cells were then loaded into a temperature regulated chamber with a computer-controlled Clark-type O_2 electrode (Oxygraph; Hansatech, Norfolk, UK).

AnnexinV Propidium Iodide apoptosis assay

CLL cells (1×10^6 cells ml^{-1}) were plated either alone or atop stromal cells ($5x10^4$) and treated with Auranofin for 24h. Following incubation, cells were collected and washed once with cold 1× Annexin V binding buffer (556454, BD Biosciences, San Jose, CA). Cells were stained with Annexin V-FITC (cat. no. 556420, BD Biosciences) for 15min, washed once with binding buffer followed by addition of propidium iodide (287075, Sigma-Aldrich). Stained cells were washed with room temperature PBS and analyzed immediately by flow cytometry using a FACSCalibur equipped with CellQuest Pro software.

Glutathione analysis from cell extracts

CLL cells (1×10^7 cells ml^{-1}) were treated for 3 and 5h with Auranofin, collected and washed once with PBS. Cells were sonicated with 1xMES buffer prior to deproteination with 10% metaphosphoric acid (239275, Sigma-Aldrich St. Louis, MO). Precipitated proteins were removed by centrifugation at 10,000 × *g* at 4 °C for 15min. The extracts were then

neutralized and measured for GSH according to the procedures directed by the manufacturer (703002, Cayman Chemical, Ann Arbor, MI).

NADPH/NADP$^+$ analysis of CLL cells

CLL cells (4×10^7 cells ml^{-1}) were treated for 1 hour with Auranofin followed by collection and washing one time with cold PBS. Cell extracts were prepared by addition of either NADP or NADPH extraction buffer along with lysis buffer and heated for 30min at 60 °C. Samples were cooled on ice followed by immediate addition of reaction buffer and the opposite extraction buffer while vortexing. Samples were then centrifuged at 10,000 × *g* at 4 °C for 10 minutes to clarify the samples. The supernatant was then used to perform the assay as directed by the manufacturer for the Fluoro NADP™: Fluorescent NADP/NADPH Detection Kit (NADPH100, Cell Technology, Mountain View, CA).

Auranofin Neutralization Assay

Culture medium was dispensed into a 24 well plate and supplemented with the following: 1mM N-Acetyl Cysteine (A9165, Sigma-Aldrich, St. Louis, MO), 500U Catalase (C1345, Sigma-Aldrich), 2mM L-Glutathione (G6013, Sigma-Aldrich) or 1mM sodium pyruvate (MT-25-000-CI, Fisher Scientific) followed by 1μM Auranofin. The mixture was incubated at 37 °C for 1h prior to addition of CLL cells (1×10^6 cells ml^{-1}) to each experimental condition. CLL cells were incubated for 24h followed by analyzing for apoptosis by flow cytometry.

Statistical analysis

All experiments were done in CLL cells from at least three different mice and patient samples. Statistical significance was analyzed by Student's t-test, and P values of <0.05 were considered statistically significant. Graph bars and plots were generated using Prizm software (GraphPad, La Jolla, CA)

Chapter 3

Mitochondria of TCL1/p53$^{-/-}$ Mice

3.1 Rationale and Background

It is not yet known whether TCL1/p53$^{-/-}$ mice will exhibit mitochondrial alterations that could help explain its aggressive disease development and reduced survival time. The **aim of this study** was to determine the effects of p53 deletion on the mitochondria of TCL1/p53$^{-/-}$ CLL cells. Given the central role of the mitochondria in regulation and execution of apoptosis, understanding the biological alterations of CLL mitochondria is valid to understand how correlative the TCL1/p53$^{-/-}$ mouse model is to CLL with 17p deletion. Identifying alterations in the mitochondria of TCL1/p53$^{-/-}$ mice may help us better understand disease aggressiveness upon loss of this tumor suppressor and better understand this unique mouse model system.

3.2 Results

We first sought to determine if there was an increase in cytosolic or mitochondrial free radical production. Flow cytometric analysis showed TCL1/p53$^{-/-}$ mice had slightly elevated mitochondrial superoxide levels but did not reach significance (**Fig. 3a**). Similarly, measurement of cellular superoxide levels detected by hydroethidium indicated no significant difference between the two groups (**Fig. 3b**).

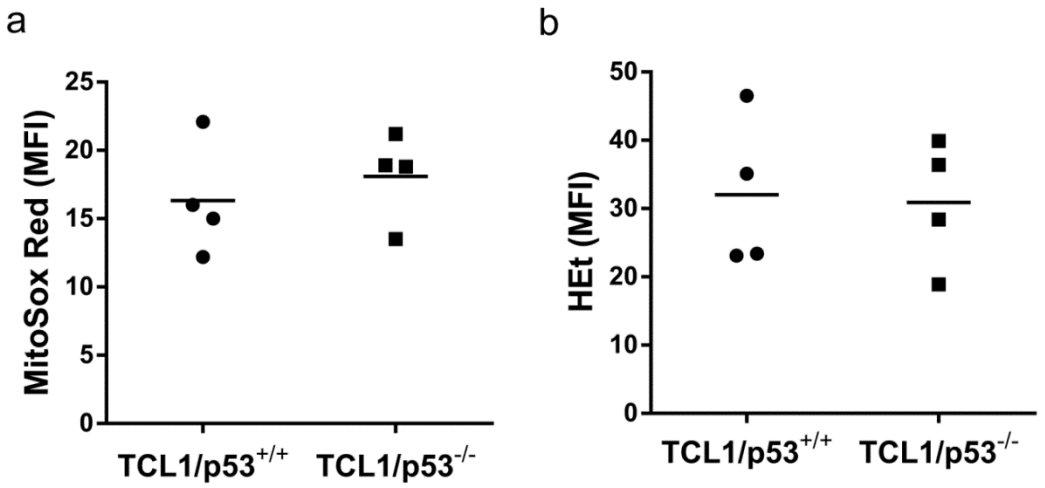


Figure 3. **Comparison of free radical and mitochondria mass in TCL1/p53$^{+/+}$ and TCL1/p53$^{-/-}$ mice.** a, Basal levels of cytosolic and mitochondrial superoxide was measured in the indicated mouse groups by staining of splenocytes with MitoSox Red and b, hydroethidium and analyzed by FACS, (n=4 per group).

Cytochrome c Oxidase I, a component of complex IV in the respiratory chain was used to assess any changes in mitochondria-encoded transcripts. A significant decrease in MT-CO1 transcripts was measured using real-time quantitative PCR (**Fig 4a**). Nuclear encoded transcription factors nuclear respiratory factor 1 (NRF-1) and mitochondria transcription factor A (TFAM) were both elevated in TCL1/p53$^{-/-}$ mice, however, only NRF-1 reached significance (**Fig 4b**). To further determine which alterations, with respect to mitochondria biogenesis, occurred, genes involved in mitochondria replication was also measured. DNA polymerase gamma (POLGA, POLG2) is a mitochondria specific DNA polymerase. POLGA encodes for the catalytic subunit of the enzyme while POLG2 encodes for an accessory subunit. Both genes showed an increasing trend in mice without p53 but did not reach significance. Similarly, POLRMT and TFB1M also showed an increasing trend without reaching significance. Polymerase (RNA) Mitochondrial (DNA Directed) (POLRMT) is a mitochondrial DNA-directed RNA polymerase which regulates mitochondria gene expression. Transcription factor B1, mitochondrial (TFB1M) is a mitochondrial

methyltransferase that functions to aid the mitochondrial ribosome. No changes were observed for Twinkle, the mitochondria DNA helicase, as well as for histone 2A (H2A) an additional housekeeping gene (**Fig 4c**). To determine if there was an increase in activated NFκB via p65 phosphorylation, to explain the increase in NRF-1 transcription, Western blot analysis between the two mouse groups was performed. TCL1/p53$^{-/-}$ mice exhibited greater basal phosphorylated p65 than the wild-type P53 group (**Fig 4d**).

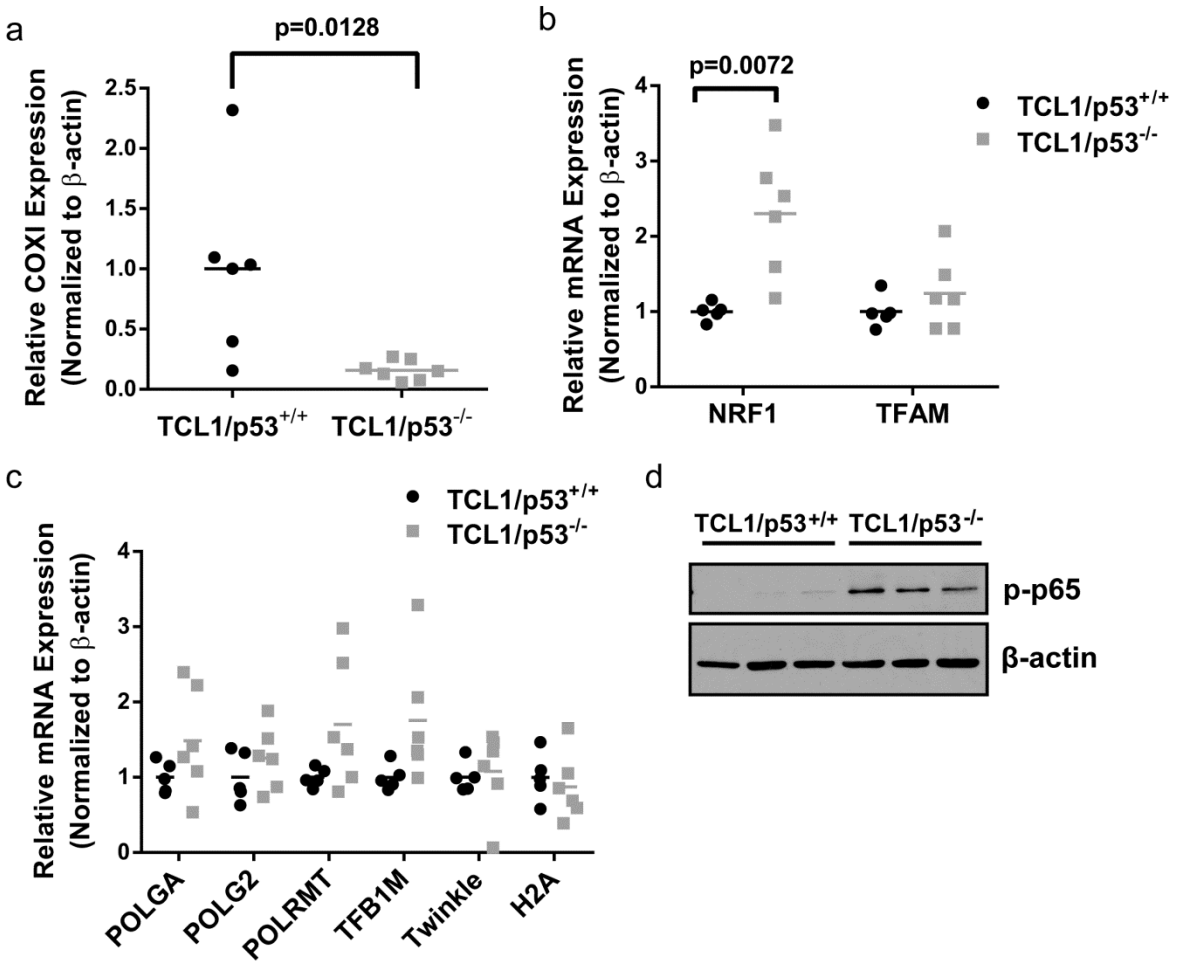


Figure 4. Loss of p53 leads to alterations in mitochondria and nuclear encoded genes in TCL1/p53$^{-/-}$ mice. a, Real time RT-PCR was used to determine differences in gene expression of mitochondria and nuclear encoded genes. Mitochondria-encoded gene expression was compared between mouse groups by measuring COXI (p=0.0128, n=6-7). b, Comparison of mRNA transcript levels for two nuclear encoded transcriptional regulators of mitochondria biogenesis, NRF-1 and TFAM are shown for TCL1/p53$^{+/+}$ and TCL1/p53$^{-/-}$ mice (NRF1, p=0.0072, n=5-6). c, Genes involved in mitochondria DNA transcription and replication: POLGA, POLG2, POLRMT, TFB1M, and Twinkle, along with H2A as a housekeeping gene was measured between the indicated groups (n=5-6). d, Western blot analysis of phosphorylated p65 between TCL1/p53$^{+/+}$ and TCL1/p53$^{-/-}$ mice, β-actin served as internal loading control (n=3 per group).

Mitochondria DNA copy number is tightly regulated by TFAM. Western blot analysis revealed increased expression of TFAM in TCL1/p53$^{-/-}$ mice (n=4) (**Fig 5a**). From this observation, we sought to determine whether this increase lead to changes in mtDNA copy number. qPCR using mitochondria and nuclear DNA specific primers was performed on isolated DNA. Relative to TCL1/p53$^{+/+}$ mice, a significant increase in mtDNA copy was observed in TCL1/p53$^{-/-}$ mice (p=0.0229, n=5-7) (**Fig 5b**). To determine if this increase resulted in changes in mitochondrial mass, splenocytes were stained with MitoTracker Green and analyzed by FACS. TCL1/p53$^{-/-}$ mice exhibited a trend of elevated fluorescence but did not reach significance (p=0.151, n=4) (**Fig 5c**). Loss of p53 has been shown to reduce optimal mitochondrial respiration via Synthesis of Cytochrome c Oxidase 2 (SCO2). SCO2, which is required for assembling the COXII subunit to the COX complex, was shown to increase mitochondria respiration upon expression in p53$^{-/-}$ HCT116 colon cancer cells[27]. To determine if mitochondria respiration is compromised in TCL1/p53$^{-/-}$, we used a Clark-type electrode to measure oxygen consumption of isolated cells (**Fig 6a**). Comparison of the two mice groups revealed no significant difference in respiration rate (**Fig. 6b**).

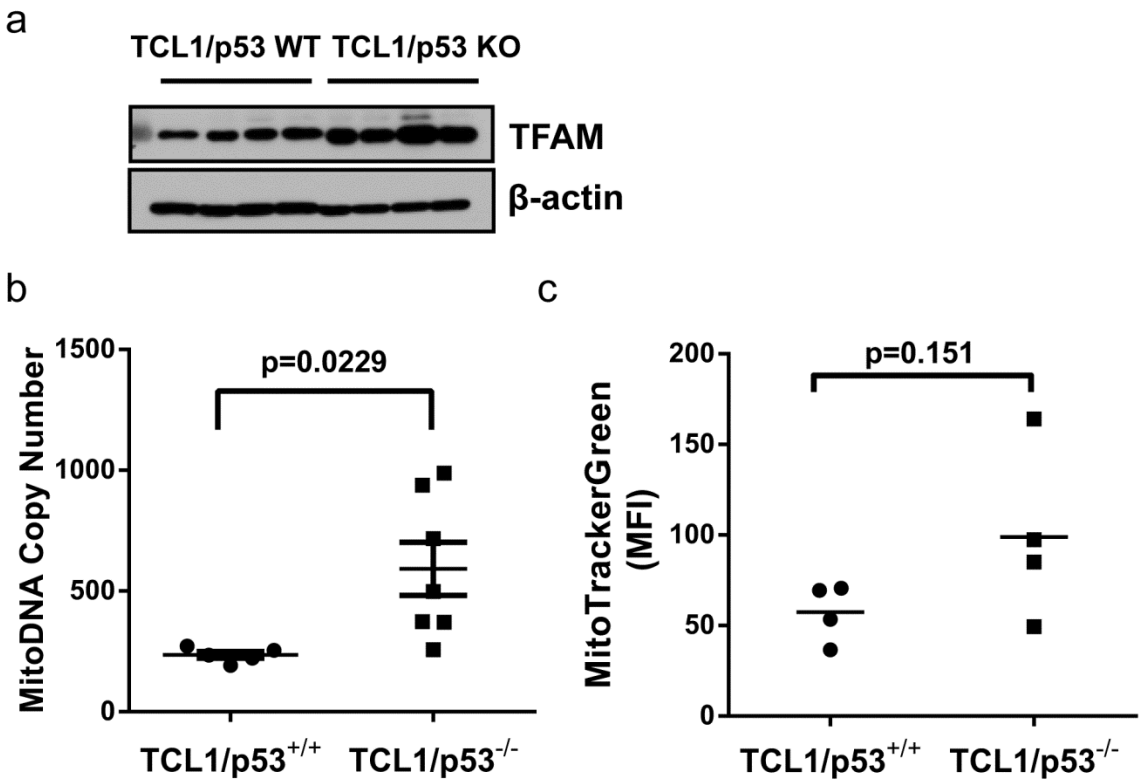


Figure 5. Increased TFAM and mitochondria DNA copy number inTCL1/p53$^{-/-}$ mice. a, TFAM protein expression was shown between TCL1/p53$^{+/+}$ and TCL1/p53$^{-/-}$ mice by Western blot analysis (n=4). b, Mitochondria DNA copy number was determined by DNA isolation from splenocytes and comparing the ratio of mitochondria to nuclear DNA by qPCR (p=0.0229, n=5-7). c, To determine changes in mitochondrial mass, splenocytes were stained with MitoTracker Green and analyzed by FACS.TCL1/p53$^{-/-}$ mice exhibited a trend of elevated fluorescence but did not reach significance (p=0.151, n=4). Student's t test was used to determine p values.

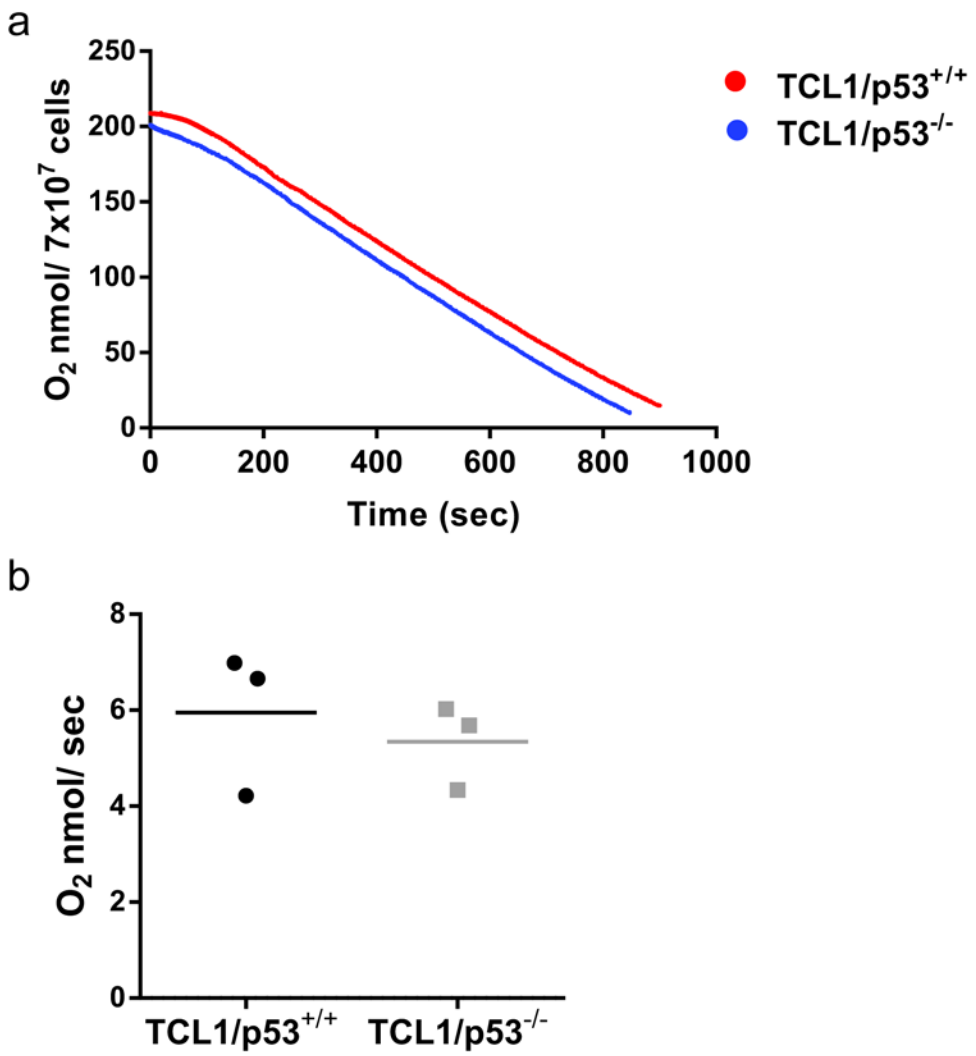


Figure 6. Comparison of mitochondrial respiration between TCL1/p53$^{+/+}$ and TCL1/p53$^{-/-}$ mice. a, Basal mitochondria respiration rates were determined using a Clark-type O_2 electrode. Equal number of splenocytes were collected per group, re-suspended in a respiration buffer, and loaded into a temperature-regulated chamber (n=3). b, Respiration rate was determine by the slope and presented as nmol of O_2 /sec.

To determine if alterations seen in the mice would also be found in human CLL samples, we measured the expression levels of TFAM and PGC1α by Western blot. CLL patients with 17p deletion showed significantly elevated NRF-1 mRNA transcript levels but not for TFAM. However, protein expression levels for TFAM and PGC1α were elevated in 17p deleted patients (**Fig 7a**). When components of the oxidative phosphorylation complex

were probed, a heterogeneous expression between non-17p deleted and 17p deleted CLL was observed. All CLL samples expressed relatively comparable levels of complex V (CV) and complex II (CII). Complex III (CIII) was diminished in CLL #1, 2 and 4, while highest expression was in CLL #5 and 6. Complex I (CI) also varied in expression with the lowest in CLL #1 while highest in # 3, 5 and 6 (**Fig 7b**).

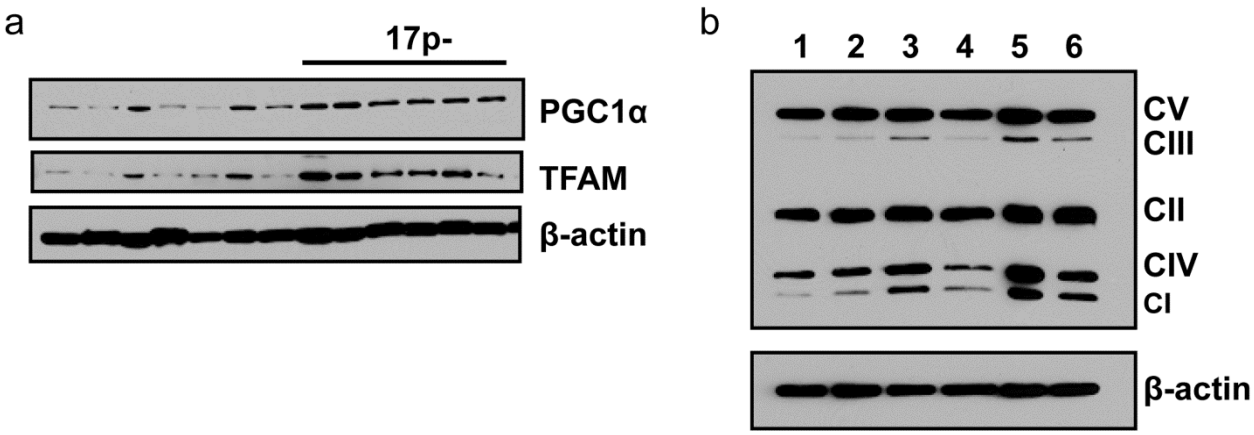


Figure 7. Upregulation of mitochondria biogenesis regulators in CLL patients with 17p deletion. a, Comparison of protein expression levels between non 17p del and 17p del CLL patients were performed using Western blot analysis. Expression of oxidative pho phosphorylation chain components (Complex I-V) was determined on 6 CLL samples. CLL 2-4 are non-17p deletion and CLL 1, 5-6 are 17p deleted. b, Mitochondria biogenesis regulators TFAM and PGC1α expression levels between the indicated subtypes are shown.

3.3. Discussion

It has been well established that p53 is an important regulator of apoptosis and metabolism in part, by way of the mitochondria. Alterations in mitochondria function and mutations in mitochondria DNA have been observed in CLL patients who received chemotherapy; this intimates a role of mitochondria in disease progression[29]. **In this study, we aimed to determine if alterations in the mitochondria are present in TCL1/p53$^{-/-}$ mice and how this could functionally affect the CLL cells**.

Our results indicate towards a compensatory response in mitochondrial biogenesis upon loss of p53. A decrease in mitochondria-encoded COX1 mRNA expression indicates that transcription of mitochondria encoded genes may be deregulated. A study showed that with loss of p53, mitochondria respiration was decreased as result of it being a direct activator for synthesis of cytochrome c oxidase (SCO2)[27].

Increase in transcripts for TFAM and NRF-1 support this compensatory response. Importantly, the increase in TFAM transcript levels also translated to elevated protein expression and increased mtDNA copy number; which therefore indicates that this was a functional increase. Studies have shown TFAM elevation can overcome mitochondria deficiencies induced by physiological damage such as myocardial infarction[100]. Similarly, overexpression of NRF-1 was shown to minimize chemically induced mitochondria damage. Neuronal cells treated with MPTP, which metabolizes to the complex I inhibitor MPP+, triggered mitochondria damage and elevated ROS production. Transfection of NRF-1 or TFAM minimized the inhibitory effects of MPP+ by significantly reducing the amount of ROS production, degree of mitochondrial membrane potential change and intracellular ATP loss[101].

Despite the loss of p53, it is reasonable to expect to see elevation in NRF-1 and TFAM. Transcription of TFAM can be directly regulated by NRF-1 and NRF-2[33] while NRF-1 contain transcriptional response elements for NFkB on its promoter region. Activation of NFκB by lipopolysaccharide (LPS) induction led to increase in NRF-1 and downstream targets such as TFAM[102]. Despite not having LPS induction in the TCL1/p53$^{-/-}$ mice, aberrant activation of NFκB has been reported in CLL[103]. The TCL1 oncogene has been shown to activate NFκB by its interaction with ATM in addition through increased phosphorylation of IκBα upon treatment with hydroxyurea[104, 105].

No significant increase in free radical production, neither mitochondrial nor cytosolic superoxide was observed in TCL1/p53$^{-/-}$ mice (**Fig 3a, b**). Two explanations for this lack of change are possible. Firstly, there was minimal decrease in the oxygen consumption rate of TCL1/p53$^{-/-}$ mice relative to TCL1/p53$^{+/+}$ mice (**Fig 6b**). Superoxide is generated during oxidative phosphorylation as a consequence of electron leakage from the respiratory complexes. Dysfunctional mitochondria often result in increased ROS production through excess leakage of electrons and are often seen in cancers[30, 35]. Despite not seeing any changes in the oxygen consumption rate, this does not necessarily exclude the possibility that defects in the electron transport chain are not present. It could be that any potential defects are not observable under basal conditions. Future experiments for further metabolic profiling should be performed using metabolic analyzers which now have the ability to measure oxygen consumption, ATP turnover as well as maximal respiration capacity under stressed conditions (i.e. with oxidative phosphorylation inhibitors)[106]. The second explanation may be through the increased expression of SOD2; which is responsible for converting superoxide to hydrogen peroxide. Although transcription for SOD2 is controlled by p53, it may also be activated by NFκB; therefore, a compensatory mechanism to elevate SOD2 was already activated to prevent an over-oxidative environment[107, 108]. Mitochondrial biogenesis regulators itself can function as free radical sensors. It was hypothesized that

NRF-1 may be regulated in a redox-dependent matter; given its role in regulating mitochondria biogenesis and that the mitochondria is the site for where majority of ROS is produced. Exposure of rat hepatoma cells to the oxidant t-butyl hydroperoxide (t-BOOH) increased TFAM mRNA expression and mtDNA copy number while NRF-1 translocation to the nucleus was observed[109]. Despite loss of p53, basal phosphorylation of p65 in the TCL1/p53$^{-/-}$ samples supports the possibility of NFκB taking over regulation of the mitochondria. A study by Mauro et. al showed NFκB's role in regulating energy metabolism by balancing the switch between glycolysis and mitochondrial respiration.

3.3 Future Directions

As stated earlier, the TCL1 oncogene was shown to be an activator of NFκB, however, very little is known whether TCL1 has any influence on the mitochondria. One study reported TCL1 localized to the mitochondria and helped support mitochondria membrane potential and interpretation of their result did not look supportive of this conclusion[77, 110]. As this was the only measurement regarding the mitochondria performed in the study, it is yet unknown whether TCL1 can directly affect mitochondria biogenesis. Therefore, it would be worth transiently silencing TCL1 in TCL1/p53$^{-/-}$ mice and determine if components of OXPHOS would be altered. Should there be any alterations in mitochondria function (respiration capacity) or ROS production, it would supportive of TCL1 having regulatory functions for the mitochondria. In addition, should the increase in NRF-1 and TFAM be due to compensation by the NFκB pathway, knockdown of TCL1 should decrease its basal activity. Immunoblotting for phospho-p65 status as well as expression changes of NRF-1 and TFAM can be performed.

Our study established an important role for mitochondria biogenesis regulators in the TCL1/p53$^{-/-}$ mouse model. Despite not observing basal respiratory changes or significant ROS production between TCL1/p53$^{+/+}$ and TCL1/p53$^{-/-}$ mice, it is possible that

only when the system is perturbed, that differences may be visible. Use of OXPHOS inhibitors such as: rotenone (complex I inhibitor), Thenoyl trifluoro acetone (complex II), antimycin A (complex III), potassium cyanide (complex IV) and oligomycyin (complex V) can be used to determine if there are functional differences in each of the OXPHOS components. Further, use of uncoupling agents such as p-trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP) will abolish the proton gradient of the mitochondria and may reveal whether there are overall differences in maximal respiration capacity between TCL1/p53$^{+/+}$ and TCL1/p53$^{-/-}$ mice.

Chapter 4

Role of EZH2 in CLL

4.1 Background and Rationale

P53 has been shown to directly repress EZH2 transcription upon its activation in response the topoisomerase inhibitor, doxorubicin[42]. EZH2 alterations (mutations, overexpression, and deletions) have been observed in numerous cancers[48, 55-58, 111]. Further, use of the EZH2-specific inhibitor GSK126 in diffuse large B-cell lymphoma (DLBCL) was found to be effective in inhibiting growth of EZH2 mutant xenografts in mice[112]. Despite the study showing EZH2 expression can be regulated through activation of p53, it is yet unknown what role, if any EZH2 has in CLL and whether its expression will be altered in CLL patients with del17p. It is also yet unknown whether EZH2 inhibitors will have any potential therapeutic properties for CLL as what has been observed in solid tumors. The **aim of this study was to determine if EZH2 promotes disease progression by affecting drug resistance in TCL1/p53$^{-/-}$ mice and as well in CLL patient samples with and without del17p.**

4.2 Results

We first sought to determine if there would be any alterations in mRNA expression of PRC2 components in TCL1 mice splenocytes with and without P53. TCL1/p53$^{-/-}$ mice showed a significant increase in EZH2 ($p=0.013$, $n=4$) and SUZ12 ($p=0.0026$, $n=4$) transcript expression (**Fig 8A**). However, there was no change in expression for EED. To see if these transcript changes translate to a protein level, an immunoblot was performed. In comparison to TCL1/p53$^{+/+}$ mice, there was a clear increase in expression of EZH2 in both TCL1 mice with heterozygous and knockout P53 (**Fig 8B**). SUZ12 expression was also elevated but seen only in the TCL1/p53$^{-/-}$ mice (**Fig 8C**).

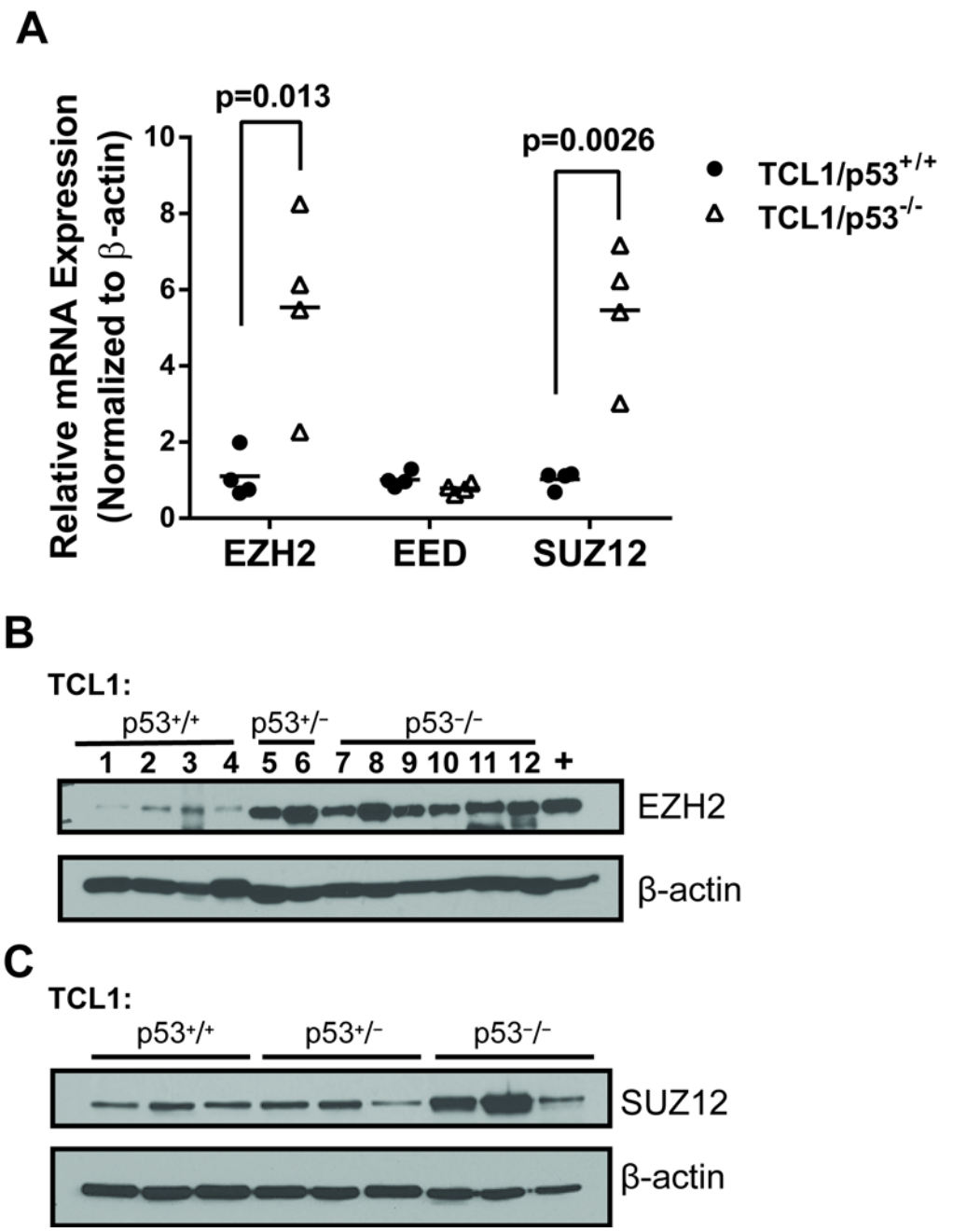
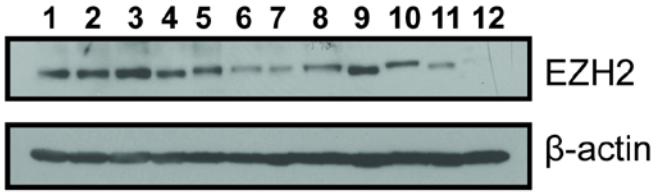


Figure 8. Loss of p53 leads to increased expression of EZH2 in TCL1/p53 mice. A, Real time PCR was performed using RNA extracted from TCL1/p53+/+ and TCL1/p53-/- mice splenocytes to measure relative expression of EZH2, EED, and SUZ12. Samples were run in triplicate with β-actin used as an internal control (n=4 for each group). Data are expressed as relative expression ($2^{-\Delta Ct}$). B, EZH2 expression level was measured by extracting protein from TCL1/p53$^{+/+}$ (n=4), TCL1/p53$^{+/-}$ (n=2), and TCL1/p53$^{-/-}$ (n=6) mice splenocytes. HCT116 p53+/+ cells served as a positive control with β-actin used as a loading control. C, SUZ12 expression was analyzed by immunoblot on protein extracts from TCL1/p53+/+ (n=3), TCL1/p53+/- (n=3), and TCL1/p53-/- (n=3) mice splenocytes with β-actin used as a loading control.

Although this pattern of increase in EZH2 was observed in mice, it was pertinent to determine if this would also be the case in CLL patients. Immunoblot of CLL patient samples with EZH2 revealed an inconsistent pattern of expression (**Fig 9A**). FISH results of CLL samples 6,7, 9-12 classified these as del17p (**Fig 9B**). Expression was inconsistent as several del17p patients had from low to non-detectable levels of EZH2. The remaining 6 CLL patients also had a heterozygous expression pattern of EZH2 levels that could not be explained by their previous treatment history (**Fig 9B**).

A



B

CLL Sample	FISH Results	Treatment at Time of Collection?
1	del11q	Yes
2	NA	No
3	del13q	No
4	del13q	No
5	negative	No
6	del17p	Yes
7	del17p	No
8	negative	No
9	del17p	No
10	del17p	No
11	del17p	No
12	del17p	Yes

Figure 9. Diverse EZH2 expression level in CLL. A, EZH2 expression from CD19+ CLL cells were determined by immunoblot with β-actin used as a loading control. B, Table shows fluorescence in situ hybridization (FISH) results and whether patient were undergoing treatment during time of sample collection. The CLL sample number matches to the EZH2 immunoblot in A.

As there was a differing expression level of EZH2 in CLL patients, we next sought if inhibition of EZH2 would have any effect on the viability of these cells. A 72hr MTT assay was performed using 2-fold serial dilutions of GSK126 on several CLL patients (**Fig 10A**). The 50% inhibitory concentration (IC50) was the lowest for CLL 1, which also happened to be del17p at 0.58μM followed by 2.46 and 5.48μM for CLL 2 and 3, respectively (**Fig 10B**). To determine if this decrease in viability was through induction of apoptosis, an AnnexinV/Propidium iodide assay was performed. Minimal apoptosis was induced by GSK126 at 2μM after 48hr incubation for CLL samples 2-4. An approximately two-fold increase in apoptosis was observed in CLL 1 (from 20 to 40%) at 2μM GSK126. High dose GSK126 (10μM) induced significant cell death for CLL 1 and 2 whereas approximately 50% of both del17p samples remained AnnexinV negative (**Fig 10C**).

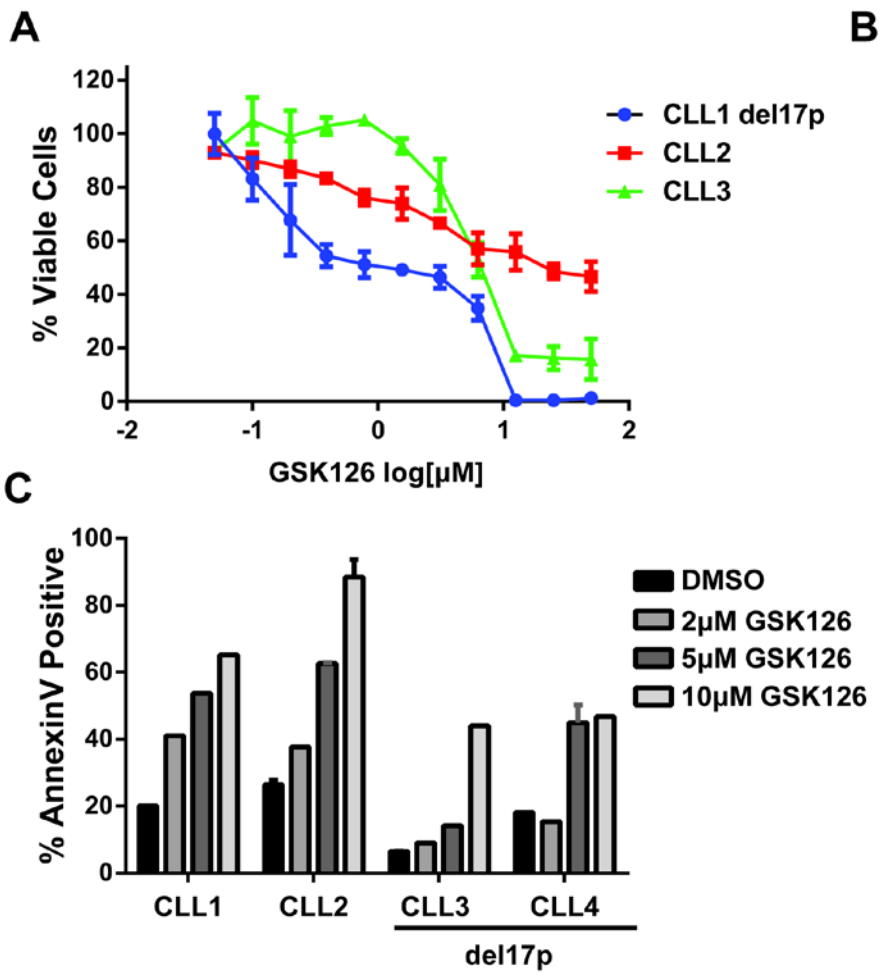


Figure 10. CLL response to GSK126. A, CLL cells were treated with 2-fold serial dilutions of GSK126 for 72hr and measured for cell viability by MTT assay. B, 50% Inhibitory concentrations (IC50) to GSK126 for three CLL patients from A.C, CLL cells were treated with 2, 5 and 10μM GSK126 for 24hr and assessed for apoptosis by AnnexinV/PI positivity through flow cytometry.

As the PRC2 complex targets genes which regulate response towards DNA damage, we next sought whether inhibition of EZH2 would enhance the activity of DNA damaging agents in CLL. To test this, CLL cells were treated either with GSK126, the purine analog Fludarabine (F-Ara-A), or the DNA cross-linker oxaliplatin (OXP) as a single agent or in combination for 24hr. Single agent treatments with 2μM GSK126 exhibited minimal apoptosis induction in CLL 1, 3 and 4. CLL 2 had high basal level of AnnexinV

positivity and 2μM GSK126 further elevated this from 35% to 47%. 5μM F-Ara-A also induced minimal apoptosis in all CLL patient samples while addition of 2μM GSK126 with F-Ara-A resulted in minimal increase in cell death. 5μM OXP was most effective on CLL 3 by inducing AnnexinV+ from 18% to 33% apoptosis; however, addition of 2μM GSK126 reduced this to 27%. Single agent OXP treatment resulted in minimal apoptosis in CLL 1 and 2 while adding GSK126 led to a trend of increasing apoptosis (**Fig 11A**). CLL 4 exhibited minimal response to both single or combination treatments.

The same experiment was performed using 3-deazaneplanocin A (DZNep) to determine if similar results would be attained while using a different EZH2 inhibitor. Similar to GSK126, single agent 2μM DZNep was minimally apoptosis inducing to CLL cells while 10μM OXP lead to significant apoptosis by itself ($p=0.0026$, $n=3$). Combining DZNep and OXP also lead to significant apoptosis relative to the control ($p<0.0001$, $n=3$) but not significantly greater than with OXP alone (**Fig 11B**). OXP was more effective as a single agent than in combination with DZNep. DZNep alone induced about 11.8% apoptosis while 25% with 10μM OXP alone. (**Fig 11C**). Despite having no significant increase between 10μM OXP alone and in combination with DZNep, the latter treatment resulted in a lower percentage of viable cells remaining; 48.9% compared to OXP alone at 60.7%.

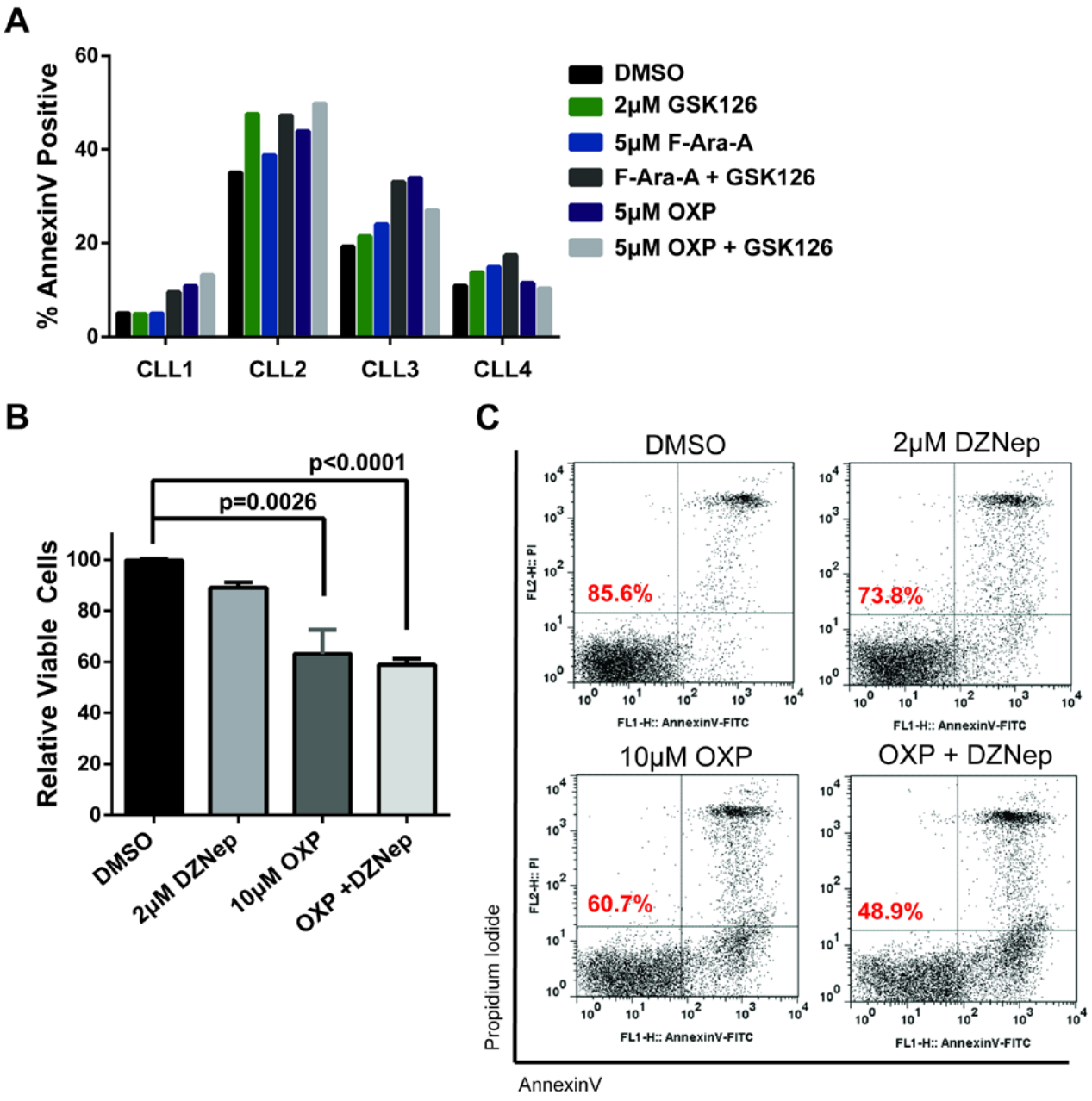


Figure 11. Response to combination treatment of GSK126 with F-Ara-A or OXP in CLL. A, CLL cells were treated with either single agent 2μM GSK126, 5μM F-Ara-A, 5μM OXP or in combination for 24hr. CLL cells were measured for apoptosis induction by AnnexinV/PI. B, CLL cells were analyzed for apoptosis following treatment with single agent 2μM DZNep, 5μM OXP or in combination after 24hr. C, Representative dot plot depicting AnnexinV vs PI of a CLL patient treated with 2μM DZNep, 5μM OXP or in combination after 24hr.

Treatment of lymphoma cells with GSK126 resulted in decreased $H3K27_3$[112].

Despite the expression of EZH2, we wanted to ensure that GSK126 was acting on its target and to see the basal trimethylation status in CLL. To determine these two points, an immunoblot was performed on CLL cells treated with GSK126 at several doses for 24hr and probed for $H3K27(Me)_3$. Both CLL samples exhibited basal $H3K27(Me)_3$ and trimethylation was further increased upon treatment with 0.1, 0.5 and 1μM GSK126 (**Fig 12A, B**). Similarly, treatment with the topoisomerase inhibitor Etoposide (Etopo) also resulted in a dose dependent increase in $H3K27(Me)_3$. To determine if this would also be observed with DZNep, CLL cells were also treated with 0.5 and 1μM DZNep for 24hr. Similarly, although not to the same extent, $H3K27(Me)_3$ was increased at 1μM DZNep (**Fig 12B**).

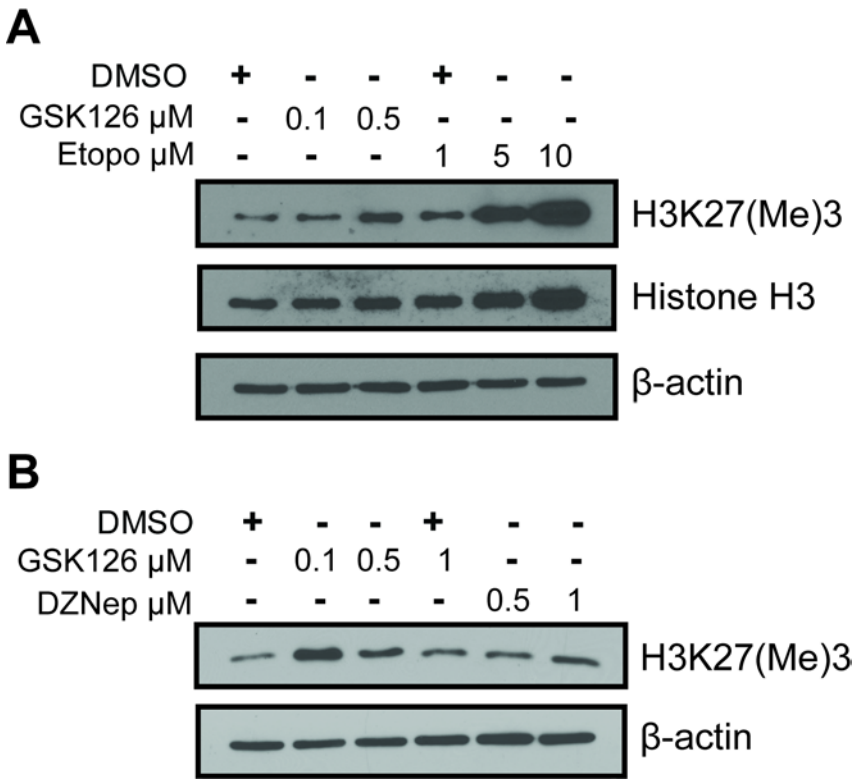


Figure 12. Increased H3K27(Me)3 in response to GSK126 and DZNep. A, Protein extracts of CLL patients treated with 0.1 and 0.5μM GSK126 or 1, 5 and 10μM Etopo for 24hr and immunoblotted for H3K27(Me)3. B, Protein extracts of CLL patients treated with 0.1, 0.5μM, and 1μM GSK126 or 0.5μM, and 1μM DZNep for 24hr and immunoblotted for H3K27(Me)3. Histone H3 and β-actin served as a control and internal loading control, respectively.

To understand why we were observing this result, we wanted to ensure that we were working with the correct compound. To do this, we obtained a second batch of GSK126, treated the mouse B cell line BaF3 with GSK126 at 2.5, 5 and 10μM for 48hr and immunoblotted for H3K27$(Me)_3$. Both stocks resulted in near or complete inhibition of H3K27$(Me)_3$ after 48hr of treatment. This inhibition was not due to an artifact via decrease in Histone H3 expression as its expression did not change (**Fig 13A**). To see if this would also be seen in a human cell line, Daudi cells, a B cell Burkitt's lymphoma cell line was treated with 2μM GSK126 for 40hr from stock 1 and 2. Similarly, Daudi cells had a strong basal H3K27$(Me)_3$ expression level which was depleted to nearly half after treatment (**Fig**

13B). When CLL samples were treated with either stocks of GSK126 at 5 and 10μM, H3K27$(Me)_3$ expression was increased (**Fig 13C**). To see if this was a time sensitive response, as the BaF3 and Daudi were treated for over 24hr, CLL cells were also treated for 24 and 48hr at 5μM from both drug stocks. Opposite to BaF3 and Daudi cells, H3K27$(Me)_3$ was elevated and maintained even after 48hr of treatment (**Fig 13D**).

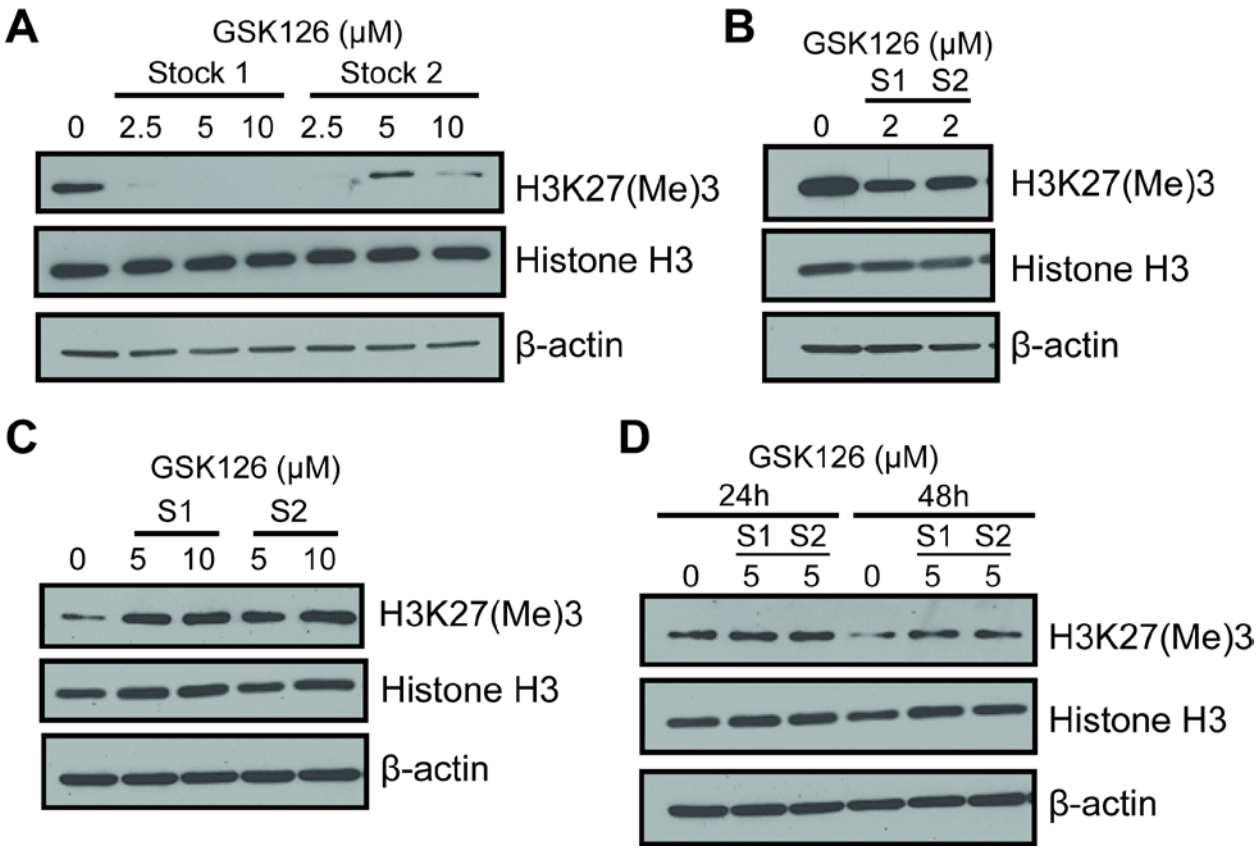


Figure 13. Inhibition of H3K27(Me)3 by GSK126 non-CLL cells. A, Mouse B cell BaF3 cells treated with doses of 2.5, 5 and 10μM GSK126 from stock 1 or stock 2 for 48hr and probed for H3K27(Me)3. B, Burkitt's lymphoma Daudi cell line treated with 2μM GSK126 from stock 1 and stock 2 for 40hr and probed for H3K27(Me)3. C, Protein extract of a CLL patient treated with 5 and 10μM GSK126 from stock 1 or stock 2 for 24hr and immunoblotted for H3K27(Me)3. D, CLL patient treated with 5μM GSK126 from stock 1 or stock 2 for 24 and 48hr before whole cell protein extraction was performed and immunoblotted for H3K27(Me)3. Histone H3 and β-actin served as a control and internal loading control, respectively for all immunoblots.

The response to GSK126 was consistently observed although the degree to increase in H3K27(Me)$_3$ varied as there was a significant difference in basal and GSK126-induced H3K27(Me)$_3$ signal intensity (**Fig 14**). Two images with short and long exposure lengths are presented as CLL 1 required a longer exposure than for CLL 2. CLL 1 had less basal H3K27(Me)$_3$ than CLL 2 but was still responsive to 5μM GSK126 after 24 and 48hr. CLL 2 had intense H3K27(Me)$_3$ signal without GSK126 treatment and was further elevated upon treatment. Increased H3K27(Me)$_3$ was also maintained after 48hr of treatment.

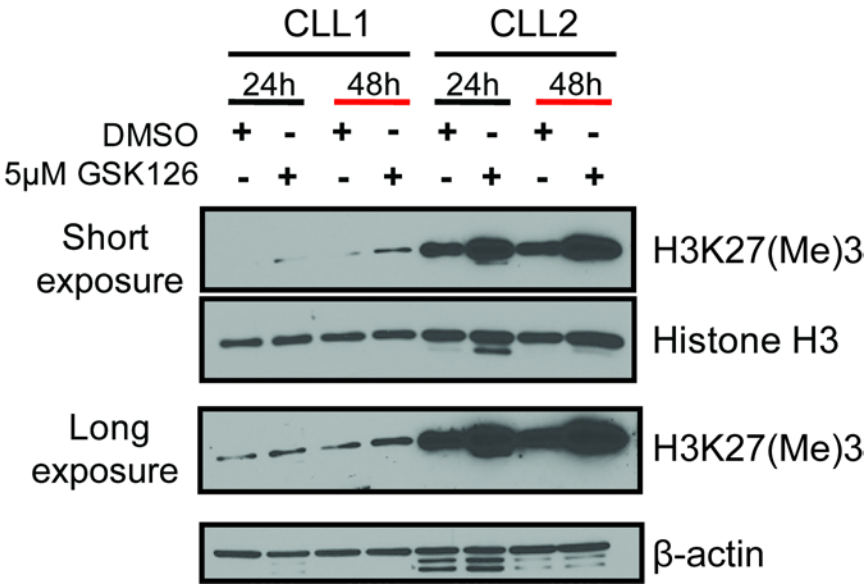


Figure 14 Increased trimethylation is maintained in CLL. A, Two CLL patients were treated with 5μM GSK126 for 24 and 48hr and immunoblotted for H3K27(Me)3.Two exposure times are presented to exhibit changes in trimethylation. Histone H3 and β-actin served as a control and internal loading control, respectively for all immunoblots.

To determine if this increase in H3K27(Me)$_3$ was due to alterations in the expression of EZH2 itself, we treated cells with GSK126 and probed for changes in EZH2. Dose dependent treatment of cells with GSK126 revealed an increase in EZH2 at 10μM (**Fig 15A**). However, because an increase in H3K27(Me)$_3$ was observed even as low as 0.1μM, this change could be a non-specific response. Further examination with CLL samples

showed only CLL 4 increased EZH2 expression after 5μM GSK126 after 24hr (**Fig 15B**). These results also further support that each CLL patient expressed varying basal levels of EZH2.

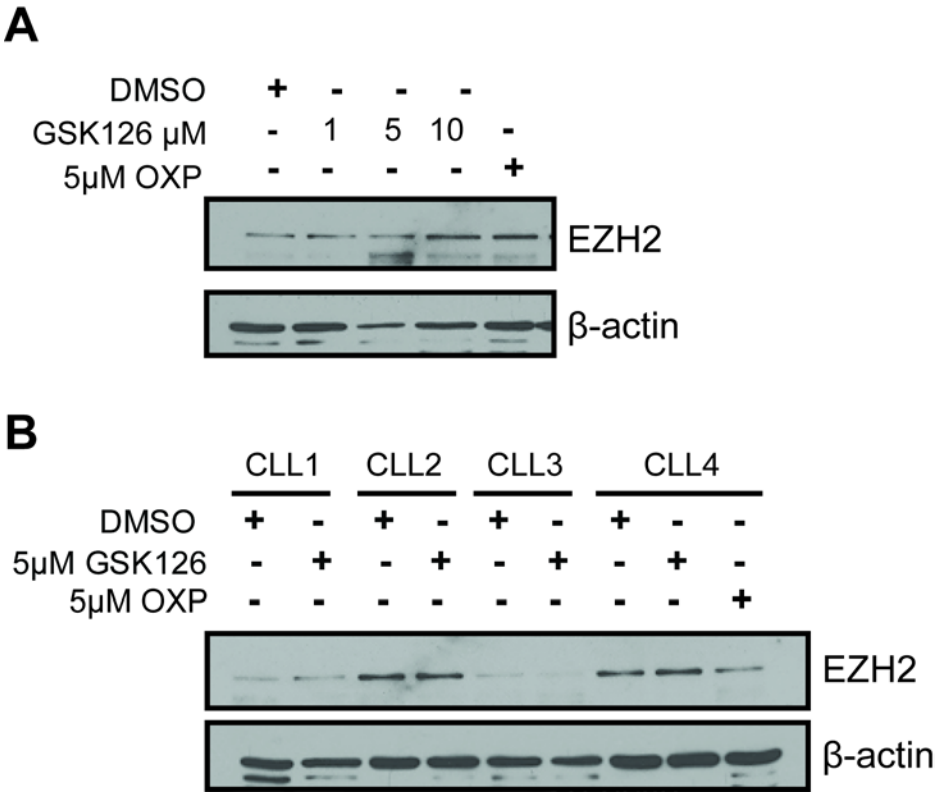


Figure 15. GSK126 does not affect EZH2 expression. A, Protein extracts of a CLL patient treated with 1, 5 and 10μM GSK126 or 5μM OXP for 24hr and immunoblotted for EZH2. B, Four CLL patients treated with 5μM GSK126 or 5μM OXP for 24hr and immunoblotted for EZH2. β-actin served as a loading control for both experiments.

As the PRC2 complex is important for regulation of DNA damage response, we hypothesized that loss of EZH2 would alter its response towards DNA damage. We also wanted to know if loss of EZH2, rather than its pharmacological inhibition would alter its viability. To answer these two questions, BaF3 cells were electroporated with EZH2 siRNA for 48hr and treated with 5 or 10μM OXP for 24hr. Immunoblot analysis was performed on electroporated cells to determine the efficiency of transient knockdown. Relative to control

siRNA, 50nM EZH2 siRNA resulted in nearly complete knockdown of basal expression levels of EZH2 (**Fig 16A**). Despite near complete loss of EZH2, there was no further induction of apoptosis in the DMSO control cells. Further, 5 and 10μM OXP resulted in 60 to 80% AnnexinV positivity, respectively (**Fig 16B**).

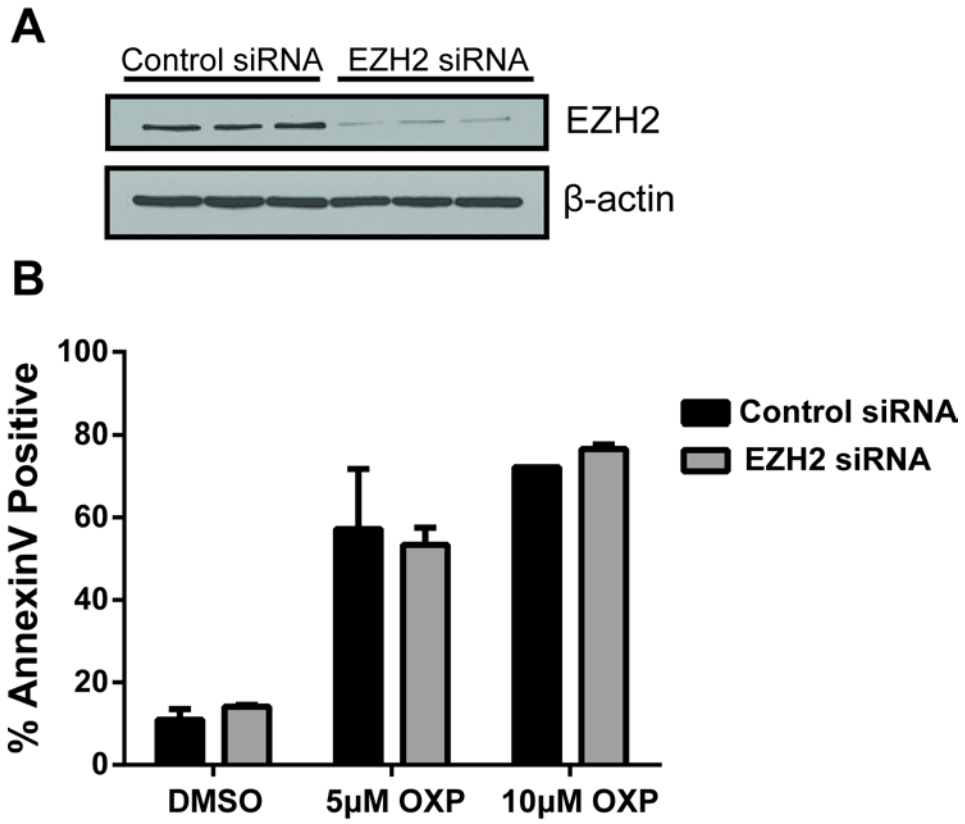


Figure 16. EZH2 knockdown does not affect sensitivity to OXP. A, BaF3 cells were electroporated with 50nM control or EZH2 siRNA and incubated for 72hr. Protein was extracted and immunoblotted for EZH2 while β-actin served as a loading control. B, BaF3 cells with EZH2 knockdown were treated with 5μM and 10μM OXP for 24hr and assessed for apoptosis by AnnexinV/PI flow cytometry assay.

4.3 Discussion

The **aim of this study** was to determine if EZH2 promotes disease progression by affecting drug resistance in TCL1/p53$^{-/-}$ mice.GSK126 was shown to inhibit H3K27(Me)$_3$ in lymphoma cell lines[112]. However, our results suggest that EZH2 inhibition is not a feasible therapeutic strategy; either as a single agent or as an adjuvant to increase sensitivity to currently used chemotherapeutic agents for CLL. We consistently observed CLL cells responding to GSK126 and DZNep with increased H3K27(Me)$_3$.This increase was an unexpected result as prior studies showed both drugs resulted in decreased H3K27(Me)$_3$[59, 112]. To ensure that what we observed was not due to an artifact, two different stocks of GSK126 was tested on two cell lines: BaF3 and Daudi cells. Immunoblotting for H3K27(Me)$_3$ revealed an inhibition upon treatment with GSK126 in both cell lines. These results indicate that both GSK126 drug stocks possessed the activity as reported and that what was being observed in the CLL samples was not due to an error in the compound or its preparation. The increase in H3K27(Me)$_3$ was also maintained after 48hr of treatment which indicates that it is not an acute stress response to GSK126. It is yet unknown as to the exact mechanism(s) for the increased methylation. It is possible that alternate signaling mechanisms may be compensating for the loss of EHZ2. Such was the case where EZH2 knockout embryonic stem cells had residual H3K27(Me)$_3$ which was attributed to EZH1[113].

Methyltransferase activity inhibition for GSK126 is indicated to be in the nanomolar concentrations[59]. Despite inhibiting cell viability as indicated by the MTT assay, two out of the three patients would require concentrations that are significantly higher than required to inhibit the methyltransferase activity. One can infer that the observed inhibition of cell viability was a result of off target effects. Furthermore, siRNA knockdown of EZH2 in BaF3 cells did not alter basal apoptosis levels nor alter sensitivity towards OXP.

Addition of EZH2 inhibitors in combination with F-Ara-A or OXP also failed to further enhance the cytotoxicity of the two chemotherapeutic agents. OXP alone significantly induced apoptosis but addition of DZNep had no significant effect. F-Ara-A and GSK126 was only minimally beneficial for one CLL patient when in combination. These experiments were approached with the hypothesis that inhibition of EZH2 will remove the repressive effect the PRC2 has on genes which regulate DNA damage response. Re-activation of such genes may be beneficial for CLL chemotherapy as significant DNA damage, to the point where repair is not sufficient, will lead to initiation of apoptosis. However, the observed results of minimal increase in drug sensitivity as well as increased H3K27$(Me)_3$ in CLL cells indicate that these cells are not responding to GSK126 or DZNep as has been previously reported.

4.4 Future Directions

In order to determine the mechanism by which increased H3K27$(Me)_3$ was observed upon treatment with EZH2 inhibitors, the following experiments are proposed:

Despite having less trimethylation activity than EZH2, EZH1 expression levels in samples where significant increase in H3K27$(Me)_3$ is observed upon DZNep/GSk126 treatment should be performed. In addition, methyltransferase activity assays, should be performed in parallel to determine whether enzymatic activity of EZH2 is really being inhibited. If EZH1 activity is elevated more-so than EZH2, it is possible that despite GSK126 inhibition, which is primarily selective towards the SET domain within EZH2, will have continued methylation activity. As stated earlier, EZH1 has been shown to have less methylation activity in comparison to EZH2[61]. However, this does not explain the results observed with DZNep, as it is a more general EZH2 inhibitor than GSK126. The primary target of DZNep is S-adenosyl-L-homocysteine hydrolase which is an enzyme involved in metabolizing s-adenosylhomocysteine (SAH)[106]. SAH is important in regulating

methyltransferase activity as its accumulation will inhibit methylation reactions[40],[43]. Thus, DZNep is not as specific as GSK126, yet H3K27$(Me)_3$ elevation was still observed in CLL cells.

Another approach to decipher the reason for what is observed is to perform DNA sequencing of CLL samples for EZH2. As mutations have frequently been observed within the SET domain of EZH2 in lymphomas, it is quite possible that similarly, mutations are observed in CLL[66]. Although, the mutations would most likely be different as GSK126 was able to be more effective in lymphoma cells possessing mutant EZH2 than wildtype[66]. Limitations of understanding the role of EZH2 is due to the diversity observed in each patient, heterogeneity of the leukemia population seen even within one patient, and the constant evolution of tumor cells as the disease progresses or after therapy. Nonetheless, genomic sequencing would be worthwhile for CLL patients to help find an explanation to this increased trimethylation.

Chapter 5

Therapeutic Potential of Auranofin for CLL Therapy

5.1 Background and Rationale

Treatment response in CLL depends on a multitude of factors including genetic abnormalities. Despite the progress made in treating CLL, there is still not curative therapy for this disease. Targeting the redox status of CLL by pro-oxidant agents such as PEITC and 2-ME has been shown to be effective in inducing apoptosis[62, 83, 114]. Auranofin is an FDA approved drug used for the treatment of rheumatoid arthritis and functions through inhibition of the antioxidant, Thioredoxin Reductase (TxnR). **The aim of this study was to determine if Auranofin shows any therapeutic potential for the treatment of CLL.**

5.2 Results

Mitochondria of leukemia cells have been found to have elevated ROS levels when compared to primary normal B cells[29, 35]. Our lab has previously established a tetracycline-inducible dominant negative DNA Polymerase Gamma cell line[30]. To test if Auranofin exhibits any cytotoxicity to cells with dysfunctional mitochondria, an apoptosis assay was performed. Doxycycline induced Tet-POLGdn cells were induced for four days followed by 48hr treatment with Auranofin. Relative to the non-induced 'Off' cells, 1μM Auranofin reduced the viability from 86.1% to 68.2% in the '+Dox' group compared to 92.5% to 80.4% in the 'Off' (**Fig 17A**). Further doxycycline induction to a total of 7 days lead to even greater apoptosis at 1μM Auranofin (**Fig 17B**).

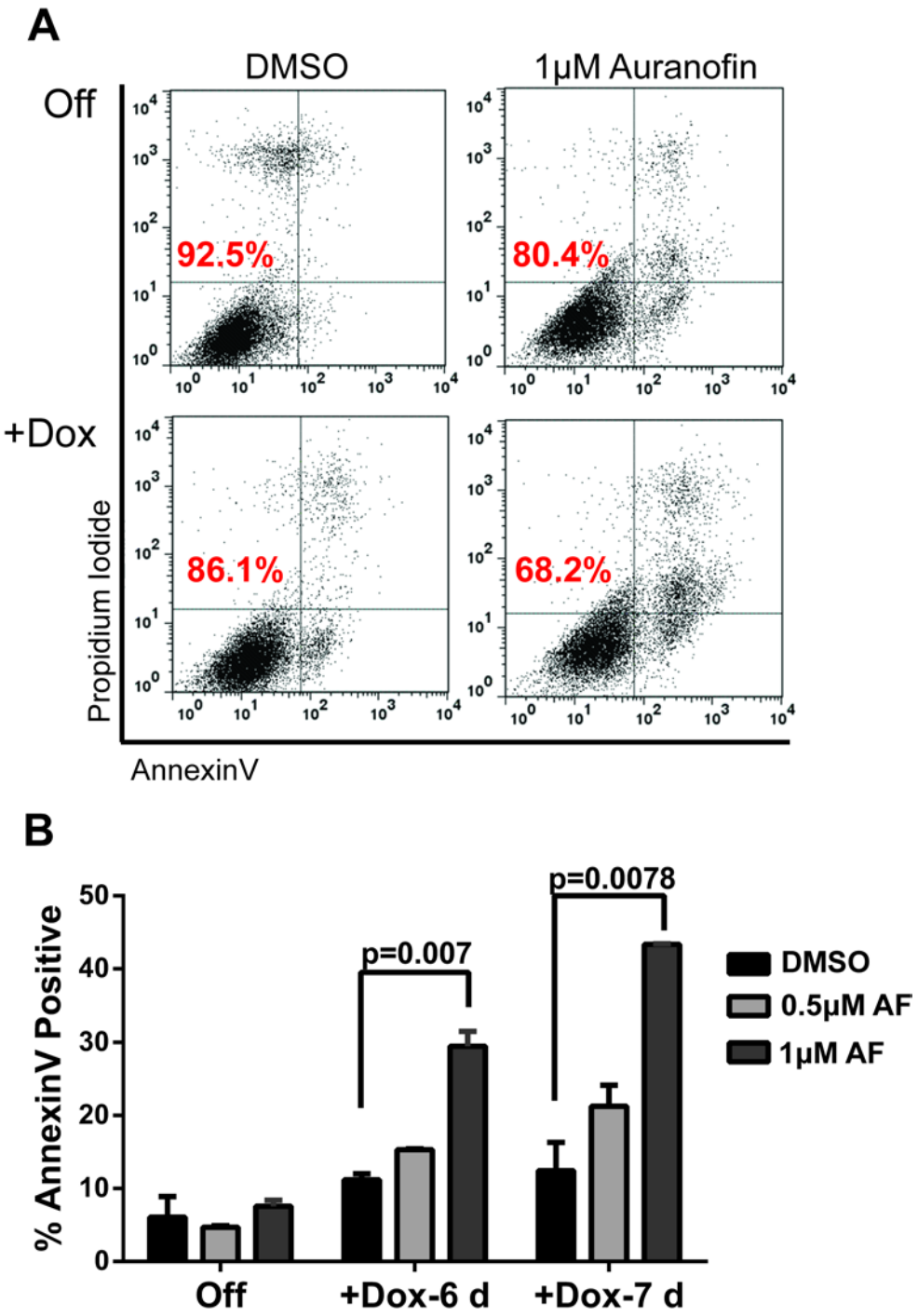
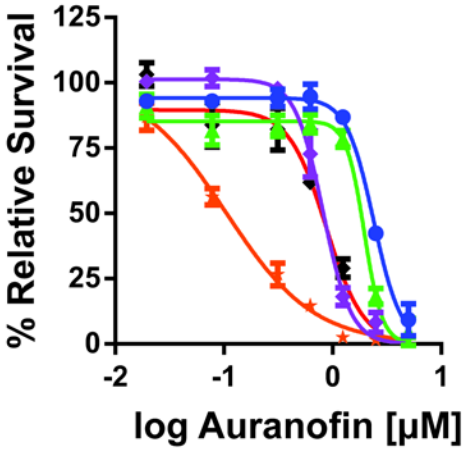


Figure 17. Increased sensitivity to Auranofin in cells with dysfunctional mitochondria. A, Apoptosis induction was compared in Tet-POLGdn cells with and without doxycycline induction. Tet-POLGdn were induced for 4 days followed by 48hr treatment with 1μM Auranofin. Apoptosis was measured by AnnexinV/PI assay.Values depict percentage of cells that were AnnexinV and PI negative. B, Comparison of Tet-POLGdn Off, 6 and 7 day doxycycline induced cells to 0.5 and1μM Auranofin for 48hr. Graphs represent the mean of triplicate ± SD. p value was determined by student's t-test.

To first determine whether there would be any cytotoxicity of Auranofin to CLL cells, an MTT assay was performed. CLL cells were treated in two-fold dilutions of Auranofin for 72 hours (**Fig 18A**). CLL cells had a range in sensitivity, between 0.1 to 2.4μM Auranofin, for 50% inhibition as depicted in the table (**Fig 18B**). As Auranofin targets the TxnR enzyme, we sought to determine if there would be any indication of response to Auranofin with relation to the expression levels of the protein. Western blot analysis of CLL cells revealed a heterogeneous pattern of TxnR1 protein expression (**Fig 18C**).

A



B

CLL#	IC50 (μM)
1	2.39
2	0.81
3	1.96
4	0.87
5	0.10

C

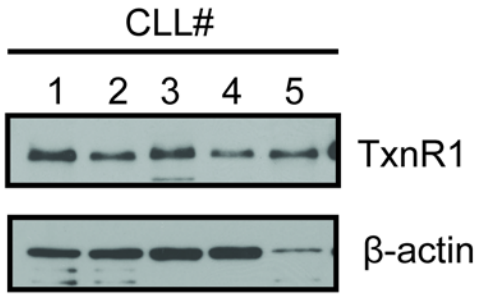


Figure 18. Impact of Auranofin on CLL survival. A, CLL cells were treated in quadruplicates at 2-fold serial dilutions of Auranofin for 72hr and measured for cell viability by MTT assay. B, 50% Inhibitory concentrations (IC50) values (μM) to Auranofin in CLL patients (n=5). C, CLL protein extracts were immunoblotted to assess basal TxnR1 expression levels. β-actin serves as a loading control.

As CLL is described to be a disease of irregular clonal expansion, its pathology is also identified as having defective apoptosis. The latter is supported by the evidence of elevated expression of anti-apoptotic proteins such as Bcl2 and Mcl1[38, 39]. Despite showing responsiveness in the MTT assay, this did not indicate whether Auranofin was able to trigger cell death in CLL cells. At the same time, we also asked whether Auranofin would be cytotoxic to a relatively 'normal' cell line. To answer these questions, an AnnexinV/Propidium Iodide assay was performed. CLL cells and immortalized bone marrow stromal cells (NKTert) were treated for 24 hours and analyzed by flow cytometry. CLL cells showed over 60% cell death at 0.5μM with no further increase at 1μM. In contrast, NKTert showed minimal cell death at these same concentrations (**Fig 19A**). We next sought to determine if co-culturing the CLL cells with NKTert cells will minimize the apoptotic effect of Auranofin. Numerous studies have shown that the CLL microenvironment (i.e. bone marrow, lymph node) affords a protective effect to these cells. CLL cells have been shown to have increased resistance to chemotherapeutic agents upon stromal co-culture[115-117]. It would be important to know if the efficacy of Auranofin would be diminished upon stromal co-culture support. To determine if this would be the case, CLL cells were seeded atop NKTert cells at a CLL:NKTert ratio of 20:1. Stromal cells alone increased the viability of CLL cells from 83.4% to 97%. Interestingly, rather than a decrease in cytotoxicity towards Auranofin, CLL cells showed greater cell death with co-culture (**Fig 19B**). Further tests confirmed significantly increased sensitivity towards 1μM Auranofin upon NKTert co-culture ($p<0.0001$, $n=23$) with CLL samples (**Fig 19C**).

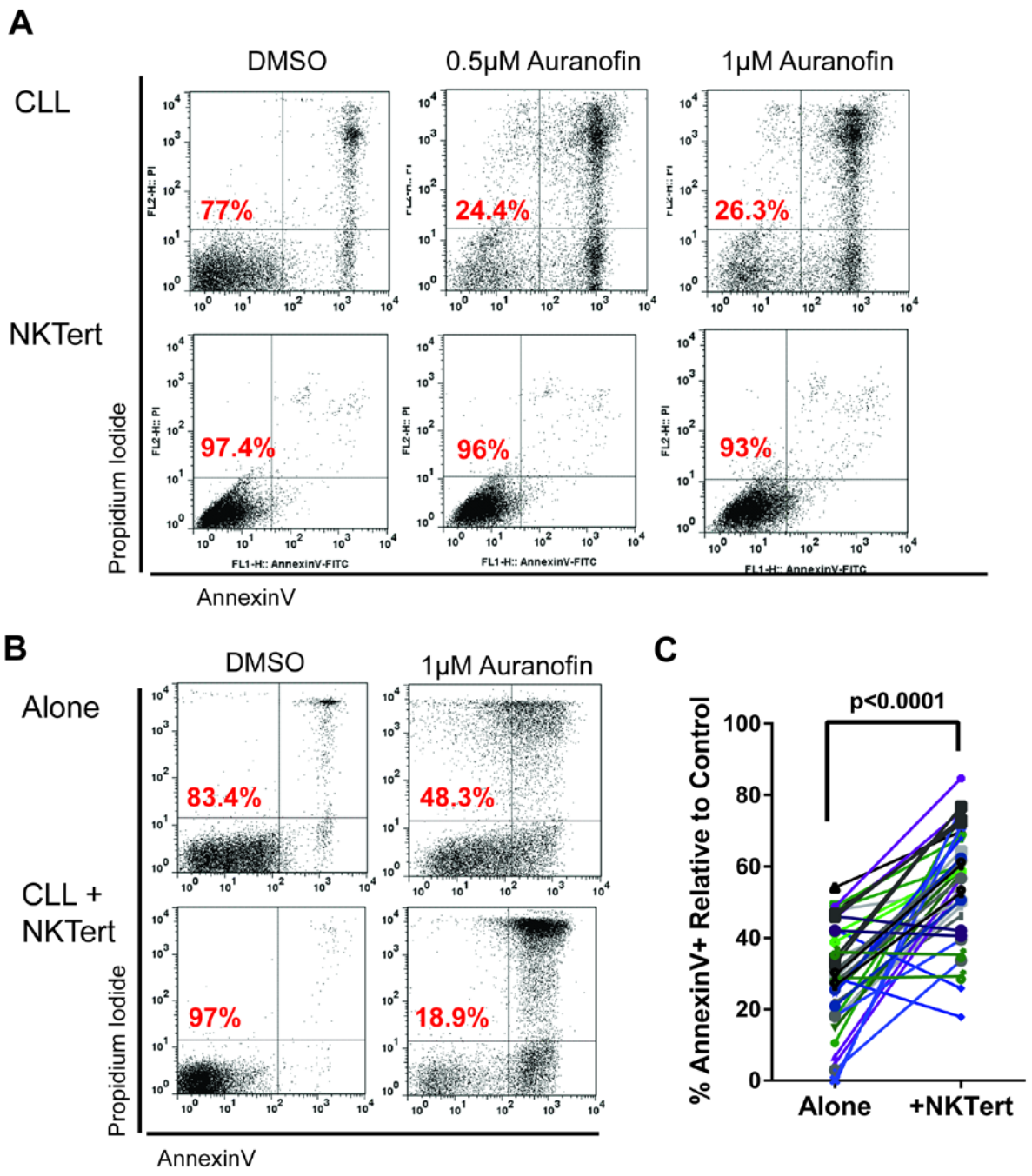


Figure 19. Selective sensitivity sensitivity to Auranofin by CLL cells. A, CLL and NKTert were treated with 0.5 and 1μM Auranofin for 24hr and assessed by AnnexinV/PI assay for apoptosis induction. B, CLL cells either alone or co-cultured atop NKTert cells (CLL:NKTert, 20:1 ratio) were treated with1μM Auranofin for 24hr and assessed by AnnexinV/PI assay. C, Result of total AnnexinV positive CLL cells measured in either duplicate or triplicates treated with 1μM Auranofin for 24hr. Basal apoptosis was subtracted from each group to show Auranofin-induced apoptosis ($p<0.0001$, $n=23$). Values on dot plots depict percentage of cells negative for AnnexinV and PI and p value was determined by student's t test.

To determine if the increased sensitivity of CLL cells to Auranofin was due specifically to the NKTert bone marrow stromal cells, we performed the same experiment on the lymph node stromal cells HK. In comparison to NKTert cells, HK cells did not afford as great a protective effect on the control cells; 66.3% on HK compared to 73.6% alone. There was approximately 54% induction of apoptosis in the CLL alone group at 0.5μM Auranofin versus 24% with the CLL + HK group. However, at 1μM Auranofin, CLL+HK cells showed 79% cell death whereas CLL alone showed only 36% death (**Fig 20A**). Further tests at 1μM Auranofin between CLL alone and CLL+HK showed significant increase ($p=0.0139$, $n=4$) in AnnexinV positivity after 24 hour treatment (**Fig 20B**).

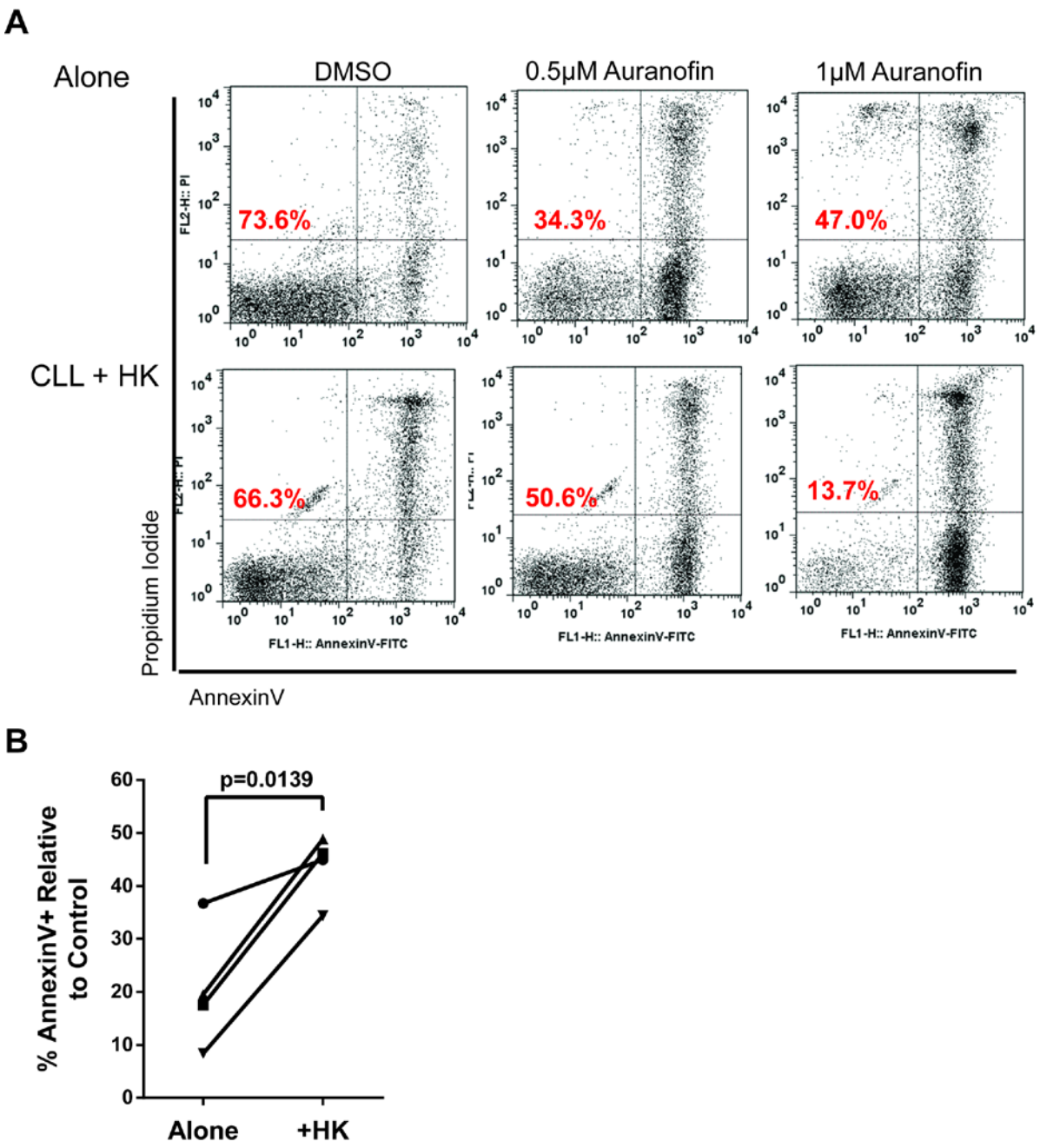


Figure 20. Increased sensitivity of CLL towards Auranofin when co-cultured on HK stroma. A, CLL cells either alone or co-cultured with HK cells (CLL:HK, 20:1 ratio) were treated with1μM Auranofin for 24hr and assessed by AnnexinV/PI assay. B, Results of total AnnexinV positive CLL cells measured in either duplicate or triplicates treated with 1μM Auranofin for 24hr. Basal apoptosis was subtracted from each group to show Auranofin-induced apoptosis (p=0.0139, n=4). Values on dot plots depict percentage of cells negative for AnnexinV and PI and p value was determined by student's t test.

Previous studies have shown CLL have intrinsically higher basal ROS levels and this may be taken advantage of for selective elimination of leukemic cells[35, 62]. Given the importance of TxnR in maintaining redox balance, we sought to determine what redox alterations Auranofin may be causing in CLL cells. Indeed, a dose dependent elevation of intracellular ROS, as detected by DCF-dA, was observed upon one hour Auranofin treatment. ROS increase was observed at 0.5μM (dotted line) with greater elevation at 1μM (solid black) and slightly further at 2μM (dashed line) when compared to DMSO control (filled gray) (**Fig 21A**). Further studies showed a plateau in ROS production after 1μM Auranofin as there appeared to be nearly the same fluorescence intensity at 2μM (**Fig 21B**). To see if this elevation of ROS is an acute oxidative burst or prolonged event, CLL cells were treated at two time points and measured with DCF-dA. Out of four patients, three showed elevation after one hour of Auranofin treatment but with reduction to near basal levels by four hours. However, one patient exhibited a delayed elevation in ROS as an increase was observed only after four hours (**Fig 21C**). To determine if this acute increase in ROS could be inhibited, CLLs were co-treated with the antioxidant, N-acetyl cysteine (NAC). 2mM of NAC was able to prevent/reduce the excess ROS production triggered by Auranofin in all three patients (**Fig 21D**). Further, NAC was able to reduce the basal ROS levels in patient CLL3. Further examination at lower (1mM) and higher (3mM) concentrations of NAC was able to reduce ROS production relative to Auranofin alone (**Fig 22**).

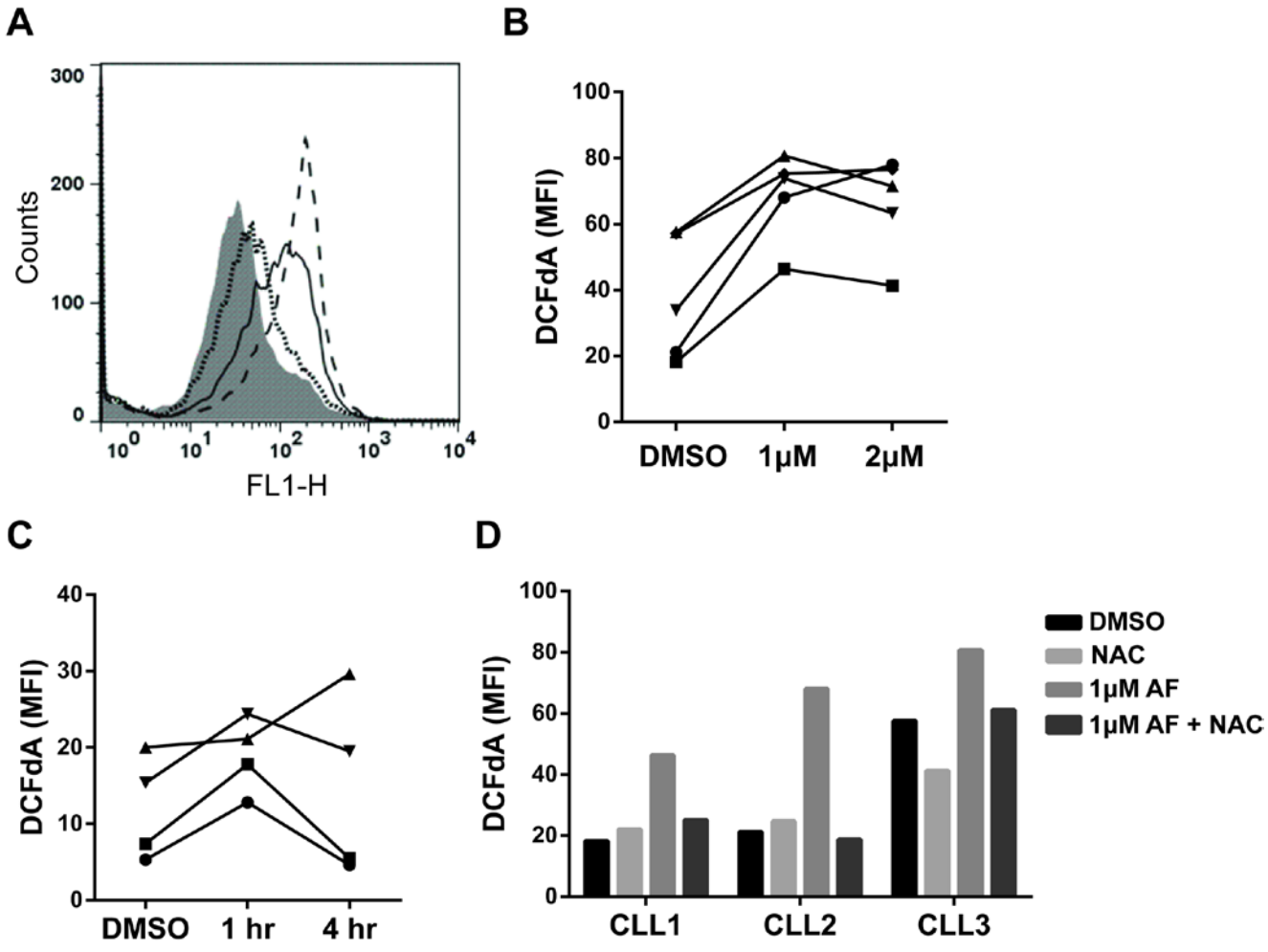


Figure 21. Auranofin triggers ROS production in CLL cells. A, CLL cells were treated with DMSO (filled gray), 0.5μM (dotted line), 1μM (solid black) and 2μM (dashed line) Auranofin for 1hr and measured for ROS production by DCF-dA staining. B, CLL cells were dosed with 1μM and 2μM Auranofin for 1hr and assessed for ROS production by DCF-dA staining (n=4). C, CLL cells were dosed with 1μM Auranofin and incubated for 1 and 4hr prior to measuring ROS production with DCF-dA (n=4). D, CLL cells were treated with DMSO, 2mM NAC, 1μM Auranofin or NAC+ Auranofin for 1hr and measured for ROS production by DCF-dA. Values on graphs (B-D) represent mean fluorescence intensity.

To further determine whether there is a dosage effect of NAC to inhibit ROS produced by Auranofin, a dose study was performed. As expected, Auranofin alone lead to significant production of ROS in both patients at 1 and 2μM. Using 1mM NAC inhibited over 50% of ROS production in CLL1; with complete inhibition at 2 and 3mM NAC. However, CLL2 required the 3mM NAC to prevent the production of ROS by Auranofin. Despite reduction of the degree of Auranofin-induced ROS relative to the no NAC group, ROS was still induced at 1 and 2mM NAC when compared to its control (**Fig 22**).

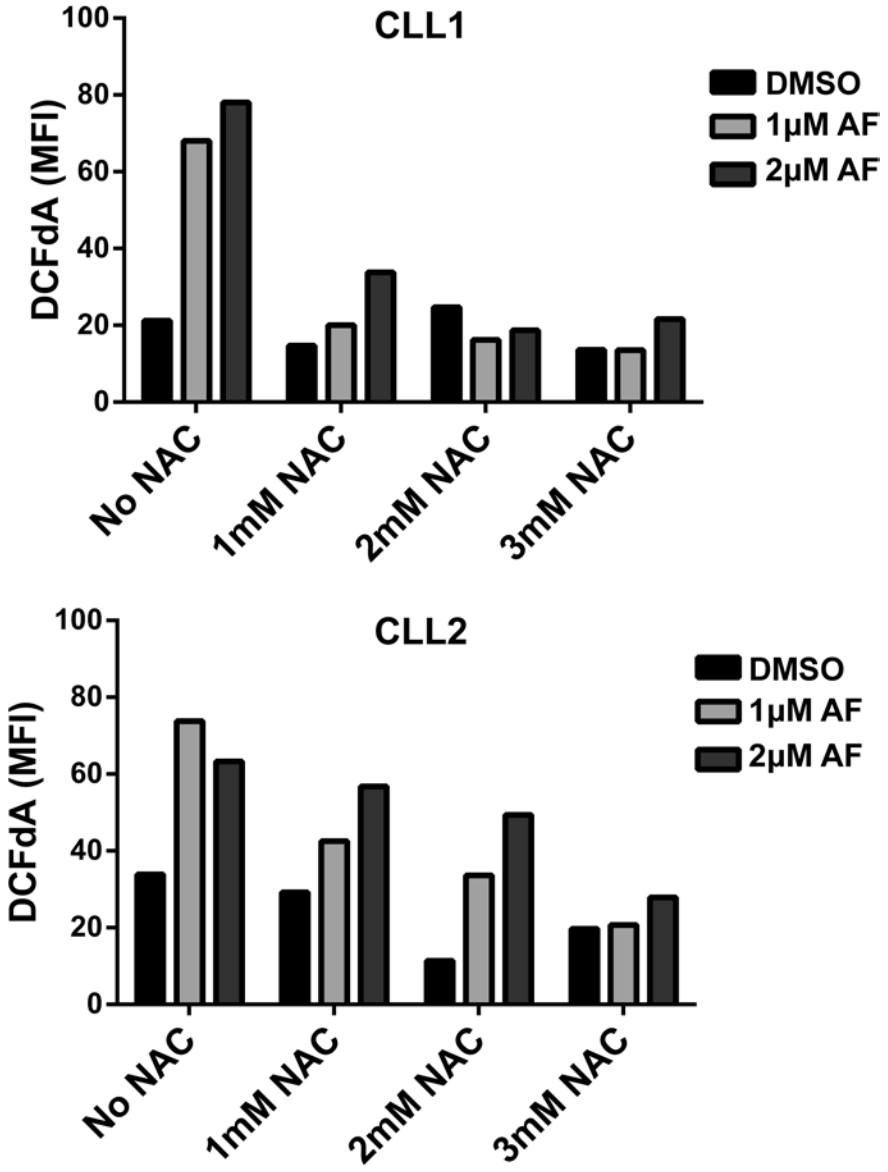


Figure 22. NAC diminishes Auranofin induced ROS production. CLL cells were dosed with no NAC, 1mM, 2mM or 3mM NAC in combination with DMSO, 1μM or 2μM Auranofin for 1hr. Cells were stained with DCF-dA analyzed by flow cytometry. Graph bars represent mean fluorescence intensity.

Oxidative balance within a cell can be a life and death situation. Because cells have various processes by which free radicals can be produced, a competent antioxidant system must be in place. Glutathione (GSH) is the most abundant antioxidant within a cell and may even be found in millimolar concentrations[118]. Depletion of GSH within a cell can trigger apoptosis and has been found to be a potent and selective mechanism for eliminating CLL cells[62, 117]. Because significant ROS was observed by Auranofin, we wanted to determine if GSH was depleted in CLL cells. CLL cells treated with 1μM Auranofin showed significant depletion at both three ($p=0.031$, $n=8$) and five hours ($p=0.0078$, $n=8$) (**Fig 23A**). We can also observe that each patient had heterogeneous basal GSH concentrations. GSH and thioredoxin (Trx) undergo an oxidation and reduction reaction to maintain the redox balance in the cell. This is performed by their respective oxidoreductase enzymes, Glutathione Reductase (GR) and TxnR, respectively. In order to carry this reaction out, NADPH is required as the reducing equivalent. Because Auranofin is known to inhibit TxnR, we hypothesized that the NADPH/$NADP^+$ ratios would be altered. As expected, treating CLL cells for one hour with 1μM Auranofin lead to an increase in the NADPH/$NADP^+$ ratio in all four CLL patients (**Fig 23B**).

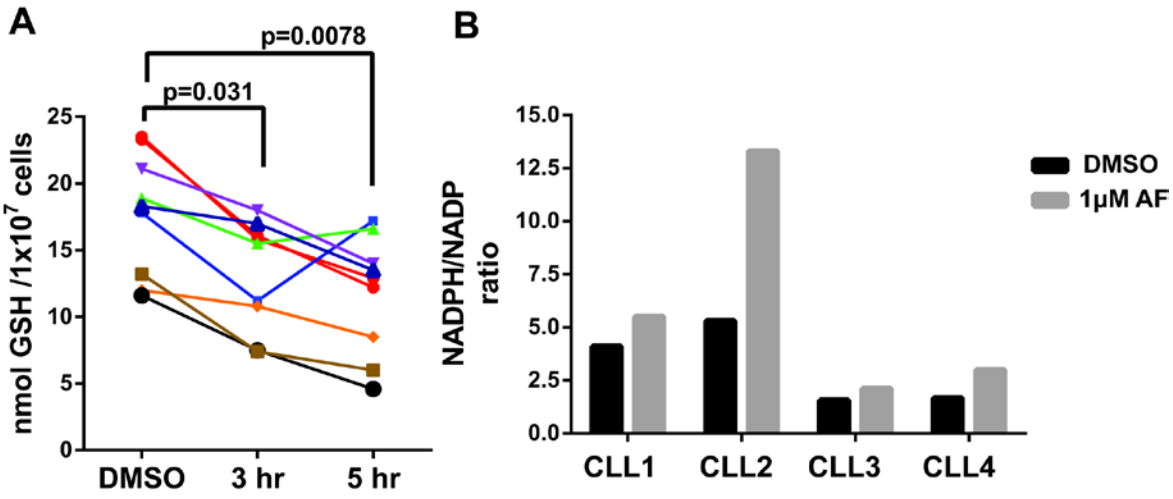


Figure 23. Auranofin leads to GSH depletion and increased NADPH/NADP ratios. A, CLL cells were treated with 1μM Auranofin for 3 and 5hr prior to collection and measured for total GSH concentration (n= 8). B, CLL cells were treated with 1μM Auranofin for 1hr before extracting for NADPH and NADP+. Bar graphs represent the ratio of NADPH/NADP+ concentrations.

CLL cells treated with Auranofin showed significant production of ROS after one hour of treatment. However, it is not known whether stromal co-culture will prevent ROS production. Previous studies showed that stromal co-culture can decrease basal ROS levels and elevate GSH levels[117]. Zhang et al. showed CLL:stromal co-culture significantly elevated GSH after 72 hours. To determine if GSH levels can be altered at an even shorter co-culture period, we seeded CLL cells on NKTert cells for the indicated times and measured for GSH levels. We were able to observe a 151% and 254% elevation in GSH for CLL1 and CLL2, respectively. After 24 hr co-culture, a 23% and 71.5% increase in GSH for CLL3 and CLL4, respectively was observed (**Fig 24A**). Again, these results highlight the heterogeneity in basal GSH levels between CLL patients as well as the degree to their response in up-regulating GSH levels upon stromal co-culture. The changes in GSH levels indicate a change in the redox status upon stromal co-culture and so our next question was whether this co-culture will alter ROS production induced by Auranofin treatment. CLL cells were seeded on NKTert cells and treated with 1μM Auranofin for one hour. As observed

earlier, CLL cells alone produced significant amounts of ROS upon Auranofin treatment (p=0.040, n=5). However, despite co-culture, ROS was still able to be significantly induced in all cases of patients (p=0.046, n=5) (**Fig 24B**).

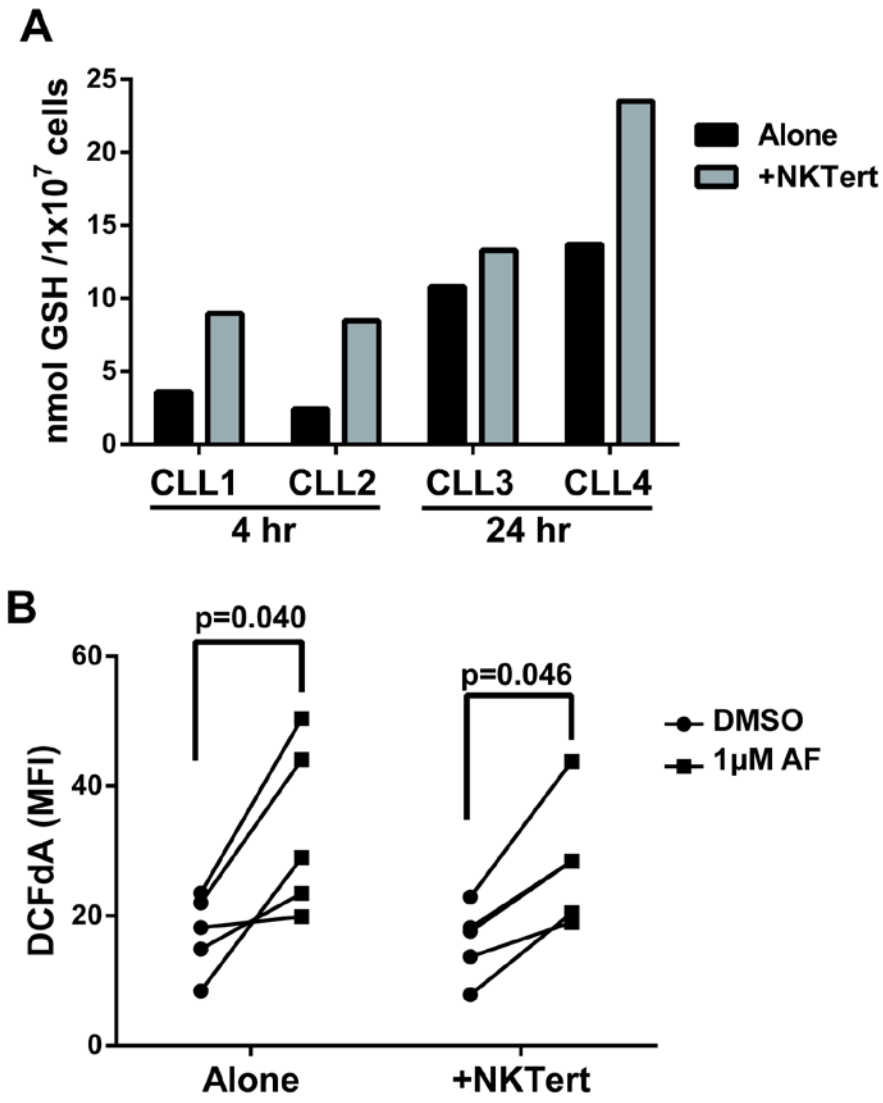


Figure 24. Stromal co-culture increases GSH in CLL cells. A, CLL cells were seeded on NKTert (CLL:NKTert, 20:1 ratio) for either 4 or 24hr and measured for total GSH concentrations. B, CLL cells were treated with 1μM Auranofin for 1hr either alone or on NKTert cells and assessed for ROS production by DCF-dA staining (n=5). Points represent mean fluorescence intensity. Student's t test was performed to determine p values.

As there was a change in the intracellular antioxidant pool, we next sought to determine if protein expression of Auranofin's target, TxnR was observed. CLL cells were seeded atop NKTert cells for 24 hr prior to collection. CLL cells appeared to have elevated expression of TxnR1 upon co-culture when comparing A (alone) versus +N (with NKTert) (**Fig 25A**). To ensure that the increase in TxnR1 was not due to artifact of NKTert cells, we collected NKTert supernatant (CM) from cells seeded at the same density and cultured CLL cells for 24 hr. Also, to determine if elevated TxnR1 can also occur in other stromal cells, CLL cells were seeded on HK cells at the same ratio (40:1, CLL:HK) for 24hr. In all cases, TxnR1 was elevated compared to the alone group (**Fig 25B**).

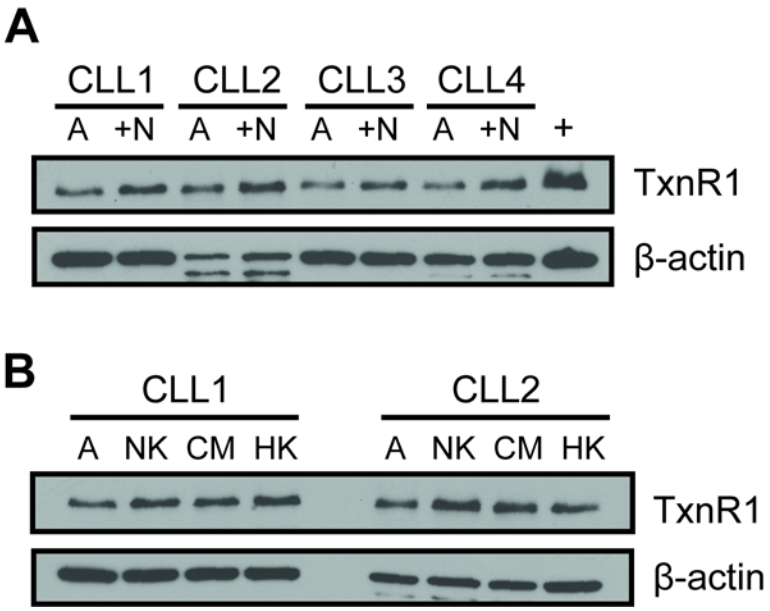


Figure 25. Stromal co-culture leads to increased expression of Thioredoxin Reductase 1 in CLL cells. A, Protein extracts from CLL cells co-cultured on NKTert stromal cells (+N) or alone (A) for 24hr were immunobloted for TxnR1 to measure expression levels (n=4). B, TxnR1 expression of CLL cells cultured alone (A), on NKTert (NK), with media from NKTert cells (CM) or on HK cells (HK). β-actin was used as an internal loading control for both blots.

It is unclear whether the direct interaction between the CLL and stromal cells is the reason for the increased sensitivity to Auranofin. To determine if this was the case, we used a transwell co-culture system where a 0.3μm porous membrane was used to separate the stromal cells and CLL cells. Despite being separated by a membrane, CLL cells were still more sensitized to Auranofin than when cultured alone; 21.4% and 32.1% viable cells remaining, respectively. However direct contact appears to still be more effective in killing the CLL cells (14.9% viable cells remaining) than alone or while separated by a membrane. (**Fig 26A**). Nonetheless, there consistently was significantly greater apoptosis to apoptosis even with transwell culture than alone when treated with 1μm Auranofin for 24hr (**Fig 26B**).

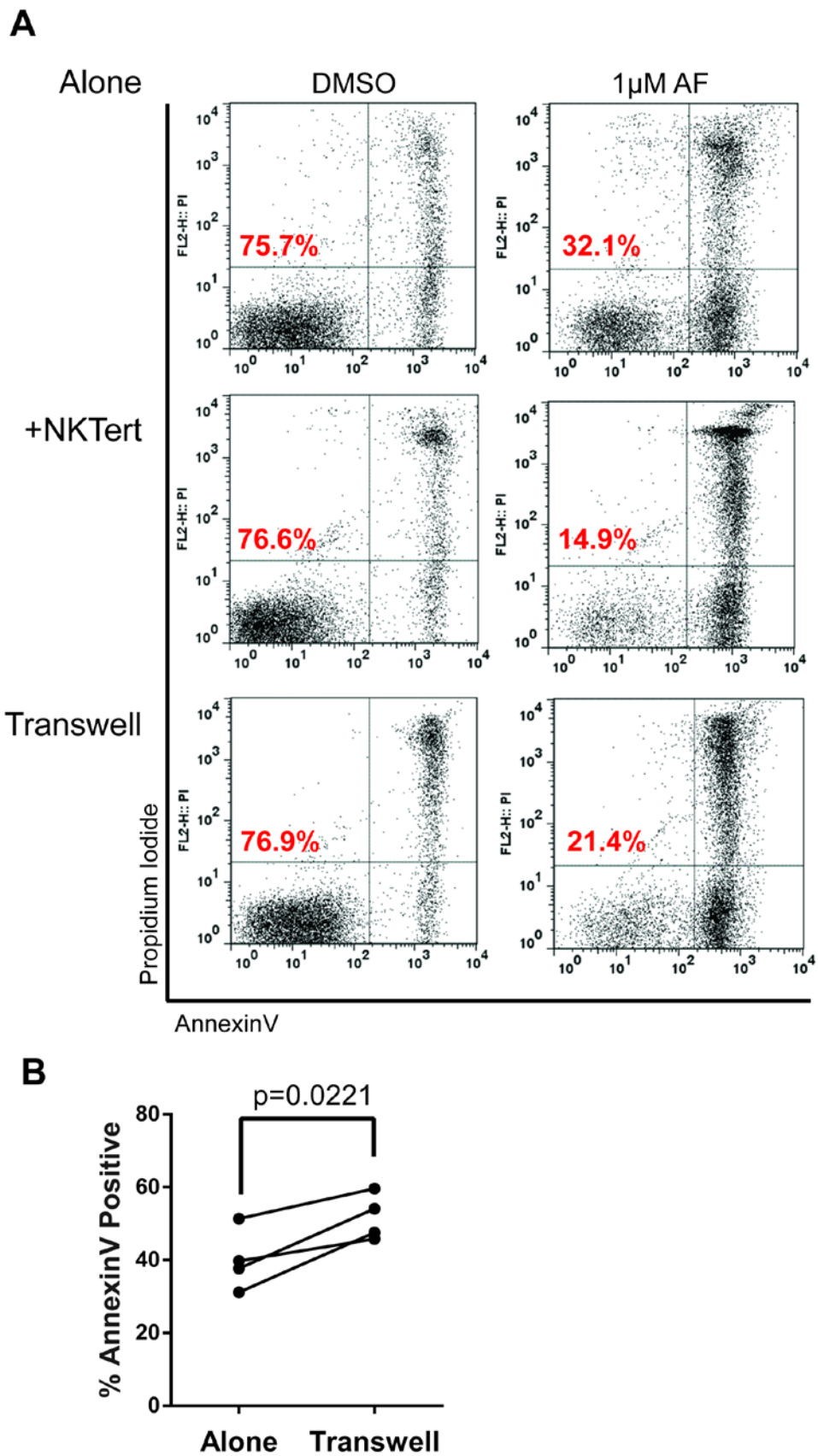

A
Alone
DMSO
1μM AF
+NKTert
Transwell
75.7%
32.1%
76.6%
14.9%
76.9%
21.4%
FL2-H:: PI
Propidium Iodide
AnnexinV
B
p=0.0221
% AnnexinV Positive
80
60
40
20
0
Alone
Transwell

Figure 26. Direct contact with stroma is not required for increased sensitivity to Auranofin for CLL. A, CLL cells were treated with 1μM Auranofin alone, on NKTert, or with NKTert but separated by 3μm membrane insert (transwell) for 24hr. Cells were assessed for apoptosis by AnnexinV/PI assay. B, AnnexinV positivity of CLL cells treated with1μM Auranofin alone or with NKTert transwell was determined after 24hr (p=0.0221, n=4). Student's t test was used to determine p value.

There are two major pathways for apoptosis: the intrinsic and extrinsic pathway. The intrinsic path utilizes the mitochondria to activate a cascade of events for cell death. The extrinsic pathway utilizes signals from receptors on the cell membrane to initiate cell death[119]. CLL cells were treated with 1μM Auranofin for three and five hours before collection. Probing for caspase-8, indicative of the extrinsic apoptosis pathway, was shown to be activated as there was an increased in the 43kDa caspase-8 product while decrease in the 57kDa band. Next, we probed for caspase-7, which is downstream and can be cleaved and activated, by caspase-8. Similar to caspase-8, there was a time-dependent decrease in the full length caspase-7 (FL Casp-7) at the 35kDa mark; whereas the cleaved product accumulated at the 20kDa region (**Fig 27**).

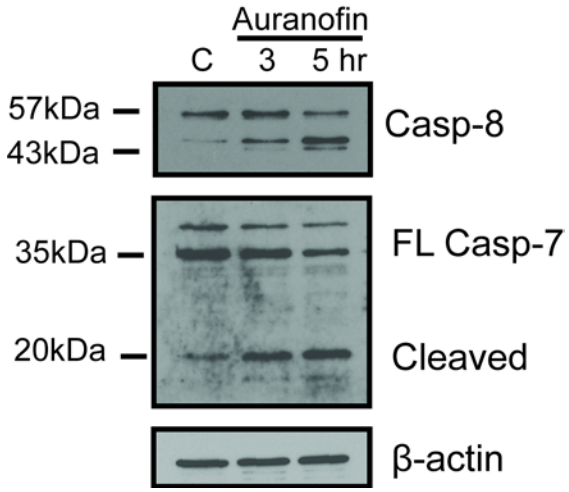
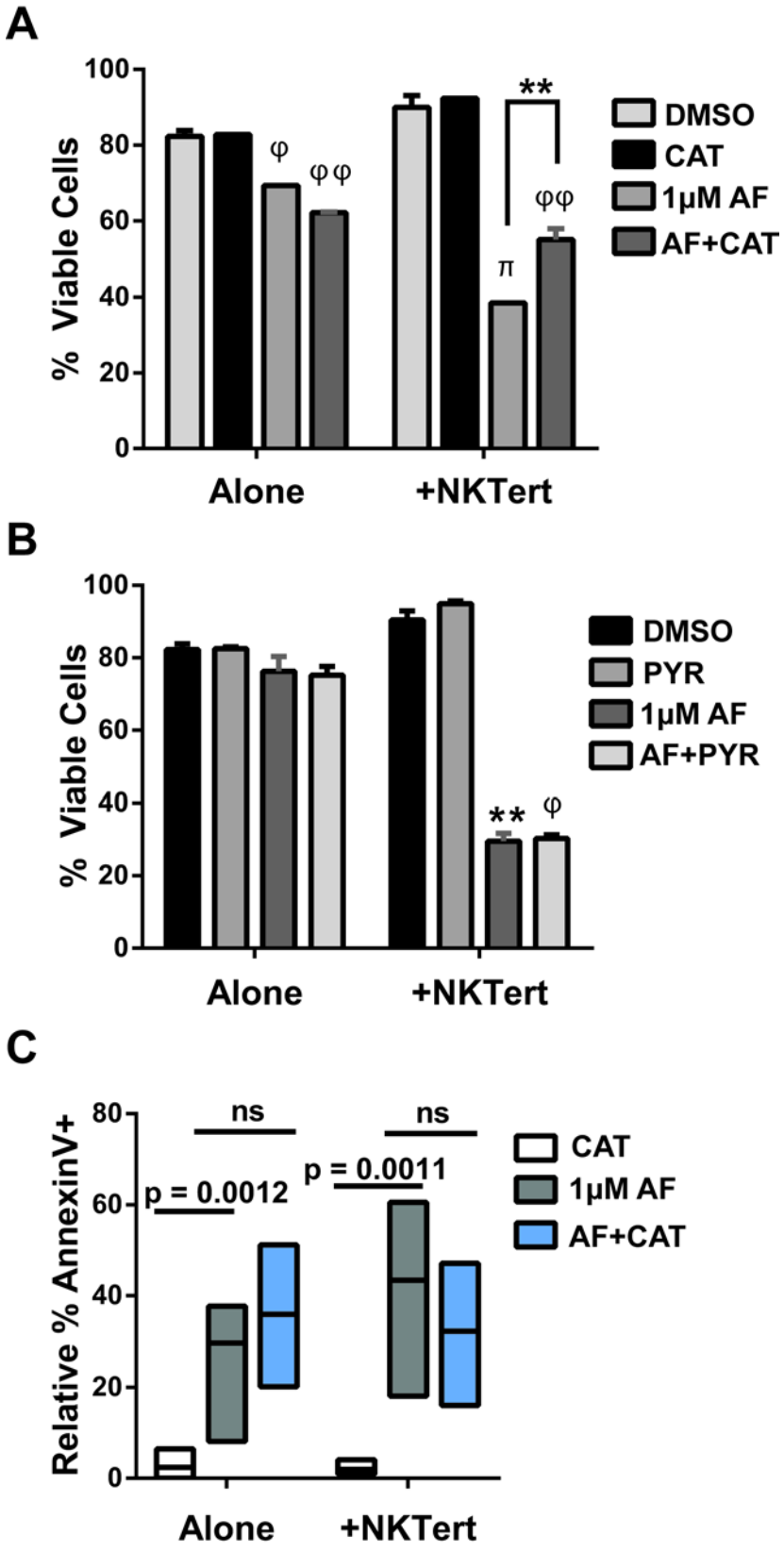


Figure 27. Auranofin activates caspases in CLL. Protein extracts of a CLL patient treated with 1μM Auranofin for 3 and 5hr were immunblotted for Caspase-8 and Caspase-7. β-actin was used as a loading control.

As Auranofin lead to significant ROS production in CLL cells and stromal co-culture resulted in increased cell death, we sought to determine if this was due to a ROS based mechanism. To test this, antioxidants: catalase (CAT) and sodium pyruvate (PYR) were used in combination with Auranofin on CLL cells. CAT provided minimal protection against Auranofin alone as significant AnnexinV positivity was measured with Auranofin alone (φ, $p<0.001$) and in combination with CAT (φφ, $P<0.0001$) (**Fig 28A**). Similarly, significant apoptosis was observed upon co-culture upon treatment with Auranofin alone (π, $p<0.005$) and in combination with CAT (φφ, $P<0.0001$). In contrast to the alone group, CAT did afford significant protection against Auranofin in NKTert co-cultured CLL cells (φφ, $P<0.0001$). In contrast to CAT, PYR afforded no protection to Auranofin for CLL cells. Despite minimal response to Auranofin for this patient in the 'alone' group, significant apoptosis was observed upon NKTert co-culture (**, $p<0.01$) and no protection when PYR was added (φ, $p<0.001$) (**Fig 28B**).

To see if this would consistently be the case, several more CLL patient samples were tested. In total, four of five CLL patients showed decreased apoptosis in the NKTert co-cultured group when 500U of CAT was used together with 1μM Auranofin (**Fig 28C**). However, all five CLL patients in the 'alone' group did not see a decrease in apoptosis despite the addition of CAT. Two CLL patients exhibited more cell death when combined with CAT. There was only one such case in the NKTert co-cultured group where CAT+ AF lead to greater apoptosis. For the other four patients, there was a trend in reduction in the degree of apoptosis when CAT was added to Auranofin (**Fig 28C**).To further determine if there was any selectivity in antioxidant protection, PYR and NAC was also tested in combination with Auranofin. 3mM PYR itself did not affect the viability of the CLL cells and had no significant protective effect when combined with 1μM Auranofin in either the alone

or NKTert co-cultured group. In both cases, CLL cell viability was significantly decreased with 1μM Auranofin alone ($p<0.01$) and when in combination with PYR ($p<0.001$) (**Fig 28B**)


A
100
80
60
40
20
0
% Viable Cells
φ
φφ
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φφ
π
DMSO
CAT
1μM AF
AF+CAT
Alone
+NKTert
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Relative % AnnexinV+
ns
ns
p = 0.0012
p = 0.0011
CAT
1μM AF
AF+CAT
Alone
+NKTert

Figure 28. Catalase protects Auranofin induced apoptosis on CLL cells co-cultured on stromal cells. A, CLL cells were treated with 500U catalase (CAT), 1μM Auranofin (AF), or in combination for 24hr either alone or on NKTert cells and assessed for Annexin positivity. Both treatment lead to significant apoptosis relative to CAT control: 1μM AF, φ, $p<0.001$, and AF+CAT, φφ, $p<0.0001$ in CLL cells alone and on NKTert: 1μM AF, π, $p<0.005$, and AF+CAT, φφ, $p<0.0001$. AF+CAT relative to AF alone when co-cultured on NKTert cells had significantly greater percentage of viable cells **, $p=0.0010$. B, CLL cells were treated with 1mM sodium pyruvate (PYR), 1μM Auranofin (AF), or in combination for 24hr either alone or on NKTert cells and assessed for apoptosis. AF lead to significant reduction in cell viability when co-cultured on NKTert, $p =0.0010$ while combination of AF + PYR did not afford any protection for CLL cells φ, $p<0.001$. C, CLL cells were treated with 500U CAT, 1μM Auranofin (AF) or in combination (AF + CAT) either alone or on NKTert for 24hr and assessed for Annexin positivity. AF lead to significantly increased percentage of AnnexinV+ cells relative to CAT, $p = 0.0012$ and $p = 0.0011$, either alone or with NKTert, respectivly. Combination with AF+CAT did not lead to significant changes relative to AF alone (n=5). Student's t test was used to determine p-values. (φ, $p<0.001$, φφ, $p<0.0001$).

When CLL cells were treated with 2mM NAC in combination with 1μM Auranofin and analyzed for apoptosis after 24h of treatment, the percentage of viable cells were significantly increased compared to no NAC ($p=0.0005$, n=4). Likewise, CLL cells co-cultured on NKTert cells were also protected when NAC was used ($p<0.0001$, n=4) (**Fig 29A**). The protection afforded by the addition of NAC may be either due to 1) NAC boosting the intracellular GSH pool in the CLL or 2) neutralizing the activity of Auranofin through binding of the sulfhydryl groups present in cysteine. In order to decipher which of these two could be the case, an Auranofin neutralization assay was done. CAT, PYR, NAC and GSH were each added to the medium followed by addition of Auranofin and incubated for one hour. Following the incubation, CLL cells were directly added to the respective wells and incubated for 24hr (**Fig 29B**). PYR and CAT had no neutralizing effect as CLL cells had significant cell death in response to Auranofin as the control 'alone' group. In contrast, NAC and GSH neutralized Auranofin by over 50% as there was an average 89 and 82% viability, respectively in comparison to the Auranofin alone group (40% viable).

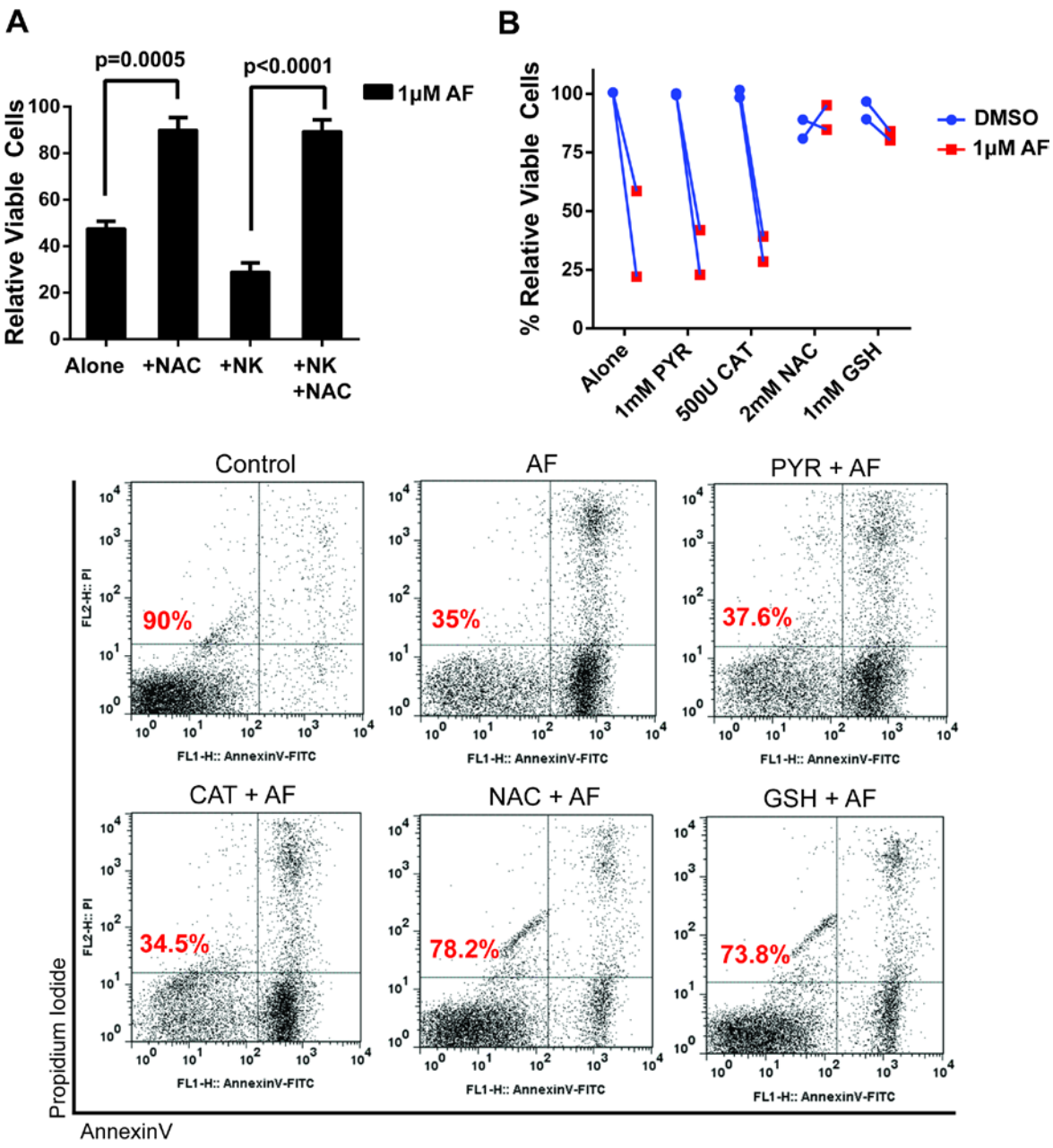

Figure 29. Neutralization of Auranofin by antioxidants. A, CLL cells were treated with 1μM Auranofin or in combination with 2mM NAC either alone or while seeded atop NKTert cells. Cells were treated for 24hr prior to assessing relative viability by AnnexinV/PI (n=4). B, Auranofin was incubated either alone, with 1mM PYR, 500U CAT, 2mM NAC or 1mM NAC for 1hr in culture medium prior to addition of CLL cells. Cells were incubated for 24hr prior to assessing for cell death (n=2). Representative dot graph of results are shown below. Student's t test was used to determine p values.

Auranofin falls into the category of gold-containing complexes and it is not known whether this increase in sensitivity of CLL cells upon co-culture is due to the presence of gold in the compound. Would the same response be seen if other metal containing compounds were used? To determine this, Auranofin (**Fig 30A**), Chloro[diphenyl(o-tolyl)phosphine]gold(I) (CDPPE) (**Fig 30B**) and trans-diamminedichloroplatinum(II) (TPLN) (**Fig 30C**) were compared. CDDPE also contains gold while TPLN, the trans form of the chemotherapeutic agent cisplatin, contains platinum. CLL cells were treated with either 1μM Auranofin, 1μM CDPPE or 10μM TPLN alone or on NKTert cells for 24hr and assessed for apoptosis induction. This patient showed minimal apoptosis induction with 1μM Auranofin alone but this was significantly increased to over 70% when co-cultured on NKTert ($p=0.003$). Oppositely CLL cells were quite sensitive to CDPPE alone but this was significantly diminished when cells were with NKTert ($p<0.0001$). TPLN showed minimal activity alone and this was even more so diminished when treated on stromal cells ($p=0.0211$) (**Fig 31**).

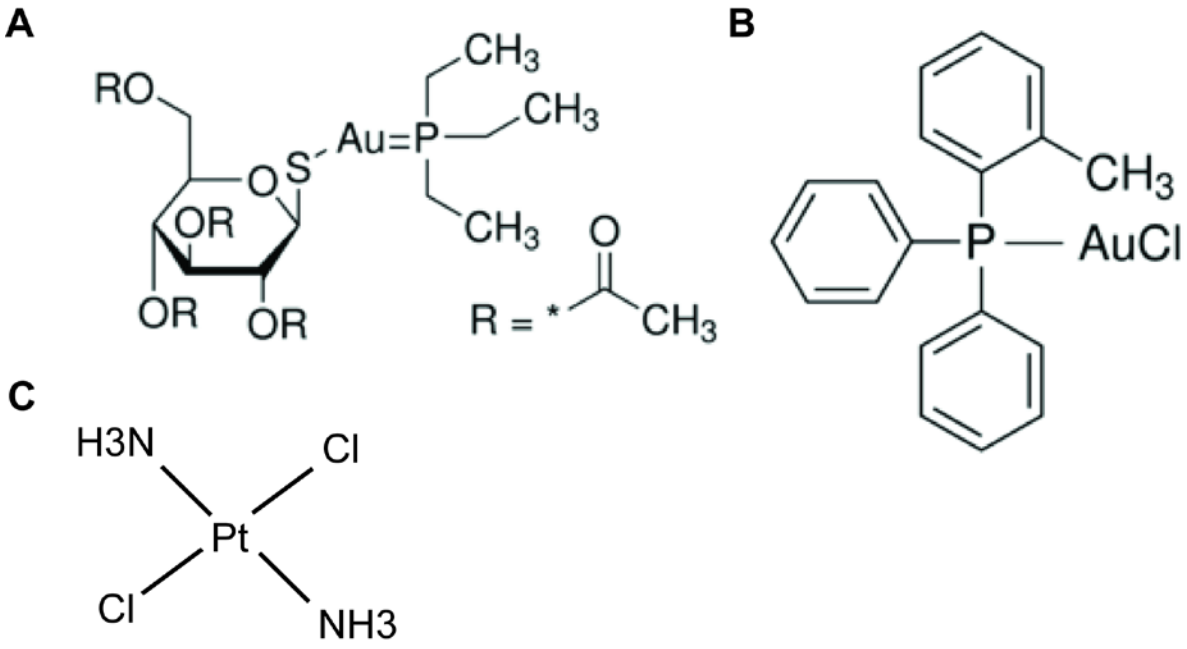

Figure 30. Structure of metal containing drugs. A, Auranofin. B, Chloro[diphenyl(o-tolyl)phosphine]gold(I) (CDPPE). C, trans-diamminedichloroplatinum(II) (TPLN).

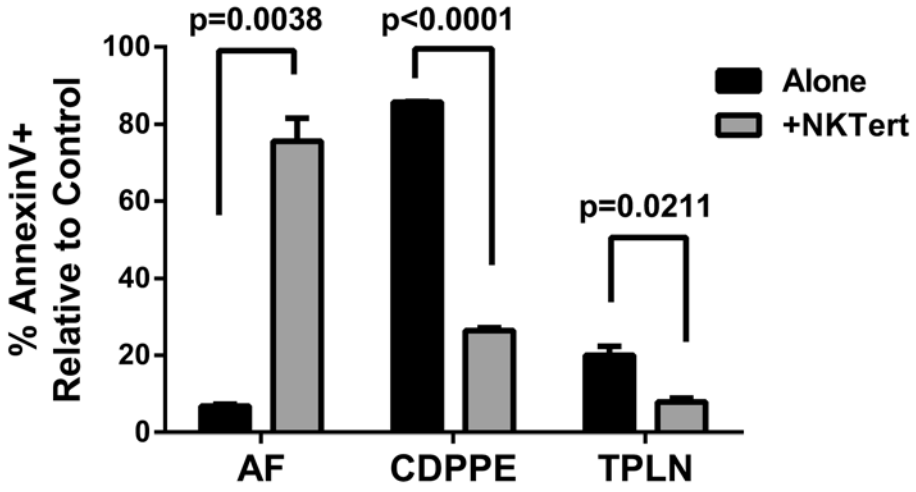

Figure 31. Apoptosis induction by metal containing drugs. CLL cells were treated with 1μM Auranofin, 1μM CDPPE or 10μM TPLN either alone or while co-cultured on NKTert cells. Cells were treated for 24hr prior to assessing for cell death by AnnexinV/PI assay. Student's t test was used to determine p values.

5.3 Discussion

The **aim of this study** was to determine whether altering the redox status in CLL cells can be a therapeutic strategy to overcome drug resistance. We utilized Auranofin, an FDA approved drug used to treat rheumatoid arthritis and known to inhibit TxnR1[88] . Previous studies from our and other labs have shown the therapeutic potential of redox altering drugs. Auranofin has been shown to be effective in inducing apoptosis in multiple types of tumor types[85, 89].

We determined that all CLL samples that underwent the MTT assay showed sensitivity to Auranofin although there was a range in IC50 values for each of the patients. It is possible that sensitivity towards Auranofin could be correlated to expression of TxnR1 as there also were varying levels of TxnR1 expression (Figure 18B, C). However, an increased sample pool would be required to determine whether there would be any positive correlation between TxnR1 protein expression levels and sensitivity towards Auranofin.

We also determined CLL cells underwent apoptosis upon treatment with Auranofin, but importantly, contrary to other studies where cells were more resistant to cytotoxic agents such as fludarabine and oxaliplatin upon co-culture[117], CLL cells were significantly more sensitized to Auranofin ($p<0.0001$, $n=23$). This is a significant finding as overcoming the protective effect of the microenvironment poses as a significant hurdle in not only CLL but other hematological and non-hematological malignancies. That this phenomenon was observed with not only one type of stromal cell line but also with HK lymph node stromal cells ($p=0.0139$, $n=4$) suggests that this response may also be possible in in vivo as well. In addition, no cell death was observed when stromal cells were assessed for cell death with Auranofin treatment. These results were encouraging as selective toxicity towards tumorigenic cells is always at the forefront of searching for a new therapeutic agent.

Intriguingly, we found that direct contact with the stromal cells is not required for the increased sensitivity towards Auranofin. We used a transwell insert to separate CLL cells from NKTert stromal cells. As a representative experiment depicted, direct contact leads to the most death (14.9% viable cells remaining) in response to Auranofin. However, there was still significant death in the transwell group compared to alone, 21.4% and 32.1% viable cells, respectively. We consistently observed this pattern upon testing further CLL patient samples ($p=0.0221$, $n=4$). These results hinted us towards two possibilities: 1) that Auranofin is leading to increased secretion of a product which is damaging to CLL cells or 2) Auranofin is inhibiting the release of cytoprotective molecules (i.e IL-6).

Auranofin falls into the category of gold(I) phosphine containing complexes and their analogs have been evaluated for anti-tumor activity[85, 87, 88, 93]. We wanted to determine if the increased activity or Auranofin towards CLL co-culture was due to the gold contained within the structure, or it if was the overall chemical itself. It was important to answer this question, as, if it was a metal-based mechanism for the increased activity, perhaps another metal - containing agent would be just as efficacious. To determine if this was the case, we obtained two other metal-containing agents: Chloro[diphenyl(o-tolyl)phosphine]gold(I) (CDPPE) and trans-diamminedichloroplatinum(II) (TPLN). CDPPE is analogous to Auranofin as it is a gold(I) containing phosphine complex while TPLN contains the metal platinum. The results from this study determined that it is because of Auranofin itself that there is an increase in sensitivity upon stromal co-culture. Despite CDPPE being significantly cytotoxic to CLL cells alone, there was diminished cell death upon co-culture. TPLN was even less cytotoxic to CLL cells both alone and in co-culture. We can conclude with these results that it is not the gold/metal itself that matters, but the overall structure of Auranofin which exerts the unique response that is observed.

In order to determine the mechanism by which CLL cells are more responsive to Auranofin, we sought to determine the redox alterations which occur upon treatment. We

observed a dose dependent increase in ROS production in CLL cells within an hour of treatment; but this elevation was diminished by 2hr of treatment. Despite this relatively short oxidative burst, we hypothesized that Auranofin may trigger extracellular release of ROS by the stromal cells. To test this, we treated cells with CAT, as this can readily quench H_2O_2 in the extracellular medium but is too large to enter into the cell. CAT significantly increased the viability of CLL cells that were co-cultured on stromal cells in 3 out of 4 cases but this was achieved for only one patient in the 'alone' group. Although this cannot completely explain the increased cell death, we can infer from the protective effect observed in the co-cultured group that extracellular ROS release by the stromal cells does have a part in the increased apoptosis.

Elevated antioxidants have been observed in cancer cells relative to their normal counterparts. Furthermore, genetic aberrations such as oncogenic mutations (KRAS) can lead to altered ROS production and thus result in an altered tolerance to oxidative stress[61]. We wanted to test if antioxidants could protect CLL cells against Auranofin. NAC was able to better protect both the 'alone' and co-cultured group in all cases; even more so than CAT. The least effective antioxidant was PYR with no protective effect in either the alone or co-cultured groups. Looking at the mechanisms of protection by each of the antioxidants, we can speculate as to how Auranofin can be more cytotoxic to CLL cells in the co-cultured condition. NAC could have prevented Auranofin-induced cell death by several mechanisms. First, NAC can function as an antioxidant itself and eliminate ROS. Second, NAC is a precursor to GSH and studies have shown supplementing cells with NAC can restore GSH levels. Thus, NAC could have prevented cell death by restoring the GSH that was depleted by Auranofin. Third, although not a mechanism by design, NAC itself may have neutralized Auranofin as it contains sulfyhdryls that are open to conjugation by Auranofin. Pharmacological studies of Auranofin showed it can bind to serum albumin as well as to the sulfhydryls within TxnR1[5].

In order to decipher which of these case(s) could be the protective effect by NAC, we performed an Auranofin neutralization assay. Pre-incubating Auranofin with CAT, PYR, NAC or GSH for an hour prior to addition of CLL cells showed whether it was Auranofin conjugating to the antioxidant or not. Auranofin was nearly completely neutralized with NAC and GSH; both cysteine and therefore, sulfhydryl containing antioxidants. CAT and PYR on the other hand, had no protective effect against Auranofin on CLL cells. These results are important as future combination studies with Auranofin need to be carefully planned. In addition, as Auranofin is administered orally, patients taking other oral supplements such as NAC and GSH may neutralize the effectiveness of the drug.

Previous studies in our lab have shown that stromal cells provide protection of CLL cells by modifying its redox environment[117]. Indeed, as previously shown, stromal co-culture was able to elevate total GSH levels with the CLL cells. It is also well known that GSH depletion can be deadly to cells[62, 63]. We sought to determine what redox alterations occur upon Auranofin treatment in CLL. We observed Auranofin significantly depleted GSH in after 3hr ($p=0.031, n=4$) and 5hr ($p=0.0078, n=4$). This suggests that GSH depletion, in addition to the extracellular ROS released by stromal cells can overwhelm the CLL cells; thus, leading to increased cell death. To compound matters, we observed that Auranofin altered the $NADPH/NADP^+$ ratios in CLL cells after one hour of treatment. This result was expected as TxnR1 utilizes NAPDH to obtain electrons to reduce oxidized TRX and thus, inhibition of TxnR1 will lead to accumulation of NADPH.

A novel finding of this study was that CLL cells were more sensitive to Auranofin whether in direct contact with the stromal cells or not. In addition, TxnR1 expression was elevated in CLL cells upon direct contact with NKTert or HK cells, or with the conditioned medium of NKTert cells. That TxnR1 expression levels increased despite lack of direct contact may imply that it may be through release of a cytokine which triggered increased expression of TxnR1. It is possible that the co-culturing, which lead to increased TxnR1, is

the cause for the greater cytotoxicity observed to Auranofin. As TxnR1 is targeted by Auranofin, increasing the target in CLL, whether it is initially triggered as a pro-survival response, may be the mechanism for this increased cell death.

5.4 Future Directions

To further determine the mechanism of increased sensitivity of CLL cells to Auranofin upon stromal co-culture, the following experiments are proposed:

As TxnR1 expression was elevated in CLL cells upon stromal co-culture and because TxnR1 is Auranofin's target, if one were to have TxnR1 knockout cells ($TxnR1^{-/-}$) one could compare the sensitivity to Auranofin relative to the wild-type ($TxnR1^{+/+}$). Cells with $TxnR1^{-/-}$ would expected to be less sensitive towards Auranofin than TxnR1+/+ cells. Further, all CLL cells that were immunoblotted for TxnR1 showed basal expression of the protein. Transient siRNA knockdown of TxnR1 may be performed on CLL cells and assessed for viability. It would be important to determine if the redox alterations observed with Auranofin would also be seen with specific knockdown of TxnR1. In a reverse experiment, full length cDNA ORFs of TxnR1 can be used for overexpression in CLL cells. For this case, one woud anticipate that rather than a decrease in sensitivity, increased cytotoxicity towards Auranofin would be observed. These experiments would help determine whether the response to Auranofin is due to the level of TxnR1 present.

Auranofin has been shown to reduce inflammation in patients with rheumatoid arthritis. Production of inflammatory cytokines: TNF-alpha, interleukin-beta, and interleukin-6 by macrophages were reduced when treated with Auranofin[120, 121]. As stromal cells are known to secrete cytokines which mediate survival signals, it is possible that Auranofin is inhibiting cytokine release and in turn secreting death-initiating signals. TxnR1 expression increased in CLL without direct stromal contact, as adding CLL cells to media collected

from NKTert cells was able to show. Further, the increased sensitivity towards Auranofin was replicated, although to a lesser extent, even when CLL and stromal cells were separated by a PVDF membrane insert. Auranofin may be inhibiting the release of pro-survival signals for CLL such as interleukin-6, while triggering aggregation of death domain-containing receptors. An ELISA of the following conditions: CLL alone, with and without Auranofin and CLL + stromal cells with and without Auranofin may give insight to changes in the microenvironment.

Lastly, further in vivo studies to understand how Auranofin reduced the tumor burden and extended the survival of TCL1/p53-/- is needed. The study by Fiskus et al. showed a two-week treatment of 5 days/week of Auranofin significantly reduced leukemia burden while increasing survival time[93]. However, the exact mechanism to the reduction of leukemic cell burden is yet unknown. It would be important to extend this study in not only TCL1/p53$^{-/-}$ mice, but also in TCL1/p53$^{+/+}$ and TCL1/p53$^{+/-}$ groups as survival times significantly differ with wildtype and heterozygous p53. Following Auranofin treatment, a complete examination of organs, particularly histology of spleens and lymph nodes should be performed. In addition, examination of other lymphoid cell types, to see whether any changes in cell number, or secretion of cytokines, are occurring in these cells could be important in understanding how leukemia cell burden is being reduced.

Chapter 6

Overall Discussion and Conclusions

Several new findings in CLL biology were made during this study. First, further characterization of the TCL1/p53$^{-/-}$ mice model was conducted. As there was only one published study of this mouse model during the time of this study, the biology of the mitochondria in these mice were yet unknown. Previous studies in p53 knockout mice have shown decrease in mitochondria biogenesis, while cell lines showed impaired mitochondria respiratory capacity upon loss of p53. Rather than seeing diminished mitochondria function and decrease in mitochondrial biogenesis, we observed a compensatory response in the mice. This is supported by the increased expression of TFAM and PGC1α. Furthermore, del17p CLL patients also had increased protein expression of both these transcriptional regulators. These results support the notion that upon loss of p53, leukemic cells induce a positive feedback signal to maintain, as much as possible, normal mitochondria function.

The second interesting finding of this study was the elevated expression of EZH2 in TCL1/p53$^{-/-}$ mice; although this pattern was not observed in del17p patients. The dichotomy of these observations may be due to biological differences between the CLL mouse and human models. It is also possible that other abnormalities found in CLL patients can trump the repressive activities of p53 on EZH2. Despite EZH2 expression differences, use of two EZH2 inhibitors, DZNep and GSK126 on CLL patients resulted in a response contrary to what has been reported in the literature. Rather than inhibition of H3K27(Me)$_3$, maintenance and elevation of trimethylation was observed. It would be worth looking at whether EZH1 is compensating for EZH2 upon treatment with GSK126. However, as the increased trimethylation was also observed with the less specific EZH2 inhibitor, DZNep, EZH1 compensation may not be the cause. This study was not able to identify the mechanisms to this increase in methyltransferase activity, but, future studies should look at

whether there are EZH2 mutations in CLL. Based on the findings of this study, EZH2 inhibitors may not be an ideal therapeutic strategy for CLL.

Lastly, we sought to determine if Auranofin would have any therapeutic efficacy in CLL. Auranofin indeed triggered apoptosis in CLL cells and importantly, upon co-culture with stromal cells, were consistently more sensitive to the drug. Numerous studies support the CLL microenvironment in maintaining CLL viability and protection against DNA damaging agents. That Auranofin can overcome this protective effect in CLL patients despite having prior therapy, makes it an intriguing agent. Further, TxnR1 expression was elevated in CLL cells upon stromal co-culture or incubation with stromal cell conditioned medium. As TxnR is a target of Auranofin, this increase in TxnR1 expression could be the reason for the elevated cytotoxicity. ROS production and GSH depletion were additional alterations that likely contributed to induction of apoptosis by Auranofin. However, the mechanism by which stromal co-culture increases the sensitivity of CLL cells towards Auranofin is yet unknown; and future experiments to decipher the mechanisms are proposed. The minimal cytotoxicity of Auranofin towards the stromal cells and that it is currently an FDA approved agent warrants further investigation as a therapeutic option in CLL.

Chapter 7

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Chapter 8

Vita

Marcia Azusa Ogasawara was born in Hilo, Hawaii on March 17, 1983, the daughter of Nui Ogasawara and Paul Shigeo Ogasawara. After completing her work at Hilo High School, Hilo, Hawaii in 2001, she entered New Mexico Institute of Mining and Technology in Socorro, New Mexico. She received the degree of Bachelor of Science with a major in biology from New Mexico Institute of Mining and Technology in May, 2006. In August of 2006, she entered The University of Texas Graduate School of Biomedical Sciences at Houston.