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Investigating the Mechanism of p53 Repression of Gfi1 and the Mechanism of Gfi1 Involvement in Lymphomagenesis

by

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Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Biology

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An abstract of

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GFI1 is a transcriptional repressor that plays a critical role in hematopoiesis and has also been implicated in lymphomagenesis. It is still poorly understood how GFI1 expression is regulated in the hematopoietic system. We show here that GFI1 transcription was repressed by the tumor suppressor p53 in hematopoietic cells. Knockdown of p53 resulted in increased GFI1 expression and abolished DNA damage-induced GFI1 downregulation. In contrast, GFI1 expression was reduced and its downregulation in response to DNA damage was rescued upon restoration of p53 function in p53-deficient cells. In luciferase reporter assays, wild type p53, but not a DNA binding-defective p53 mutant, repressed the GFI1 promoter. Chromatin immunoprecipitation (ChIP) assays demonstrated that p53 bound to the proximal region of the GFI1 promoter. Detailed mapping of the GFI1 promoter indicated that GFI1 core promoter region spanning from -33 to +6 bp is sufficient for p53-mediated repression. This core promoter region contains a putative p53 repressive response element, mutation of which abolished p53 binding to and repression of GFI1 promoter. Significantly, apoptosis induced by DNA damage was inhibited upon Gfi1 overexpression, but augmented following GFI1 knockdown. Furthermore, we show that Bcl-xL is upregulated by Gfi1. Overexpression of Gfi1
upregulates Bcl-xL protein and mRNA level. Knockdown or deficiency of Gfi1 is associated with decreased levels of Bcl-xL protein and mRNA expression. Significantly, our data also indicate that upregulation of Bcl-xL is responsible for Gfi1 inhibition of apoptosis induced by DNA damage. Overexpression of Bcl-xL reduces the sensitivity of Gfi1 knocked down cells to DNA damage-induced apoptosis. In contrast, knockdown of Bcl-xL partially abolishes Gfi1 inhibition of apoptosis induced by DNA damage, indicating that upregulation of Bcl-xL contributes to Gfi1 protection of DNA damage induced apoptosis. Our data establish for the first time that GFI1 is repressed by p53 and add to our understanding of the roles of GFI1 in normal hematopoiesis and lymphomagenesis.

Gfi1 has been shown to cooperate with c-Myc in regulating cell proliferation and lymphomagenesis. However, the underlying molecular mechanism remain poorly understood. Here we demonstrate that Gfi1 upregulate c-Myc expression. Overexpression of Gfi1 augment the expression of c-Myc. In contrast, decreased level of Gfi1 expression is accompanied by downregulation of c-Myc. In addition, our data indicate that Gfi1 can inhibit apoptosis induced by c-Myc overexpression. Based on these findings, we propose a potential mechanism by which Gfi1 cooperate with c-Myc in lymphomagenesis. Gfi1 promote c-Myc expression, elevating the risk of c-Myc induced transformation. Meanwhile, Gfi1 inhibits apoptosis caused by c-Myc overexpression. Thus, the presence of Gfi1 leads to more effective cell transformation by overexpression of c-Myc.
In the memory of my father, Shaoting Du. I wouldn’t be who I am now without his love and support.
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Table of Contents

Abstract.......................................................................................................................... iii

Acknowledgements ......................................................................................................... vi

Table of Contents ............................................................................................................. vii

List of Figures ................................................................................................................ xi

List of Abbreviations ....................................................................................................... xiii

1 Introduction..................................................................................................................... 1

1.1 Hematopoiesis ........................................................................................................... 1

1.2 Hematopoietic malignancies .................................................................................... 3

1.3 Hematopoietic cytokines ........................................................................................ 4

1.4 Hematopoietic transcription factors ....................................................................... 5

1.5 Growth factor independent 1 (Gfi1) ...................................................................... 6

1.6 c-Myc ....................................................................................................................... 9

1.7 The tumor suppressor p53 ..................................................................................... 11

1.8 Bcl-2 family members in apoptosis ..................................................................... 12
2 Material and Methods ........................................................................................................ 15

2.1 Cells and materials ........................................................................................................ 15
2.2 Construction of plasmids ............................................................................................ 16
2.3 Transfection and generation of stable cell lines .......................................................... 17
2.4 Cell proliferation assay (MTS assay) ............................................................................ 18
2.5 Chromatin immunoprecipitation assay (ChIP assay) .................................................... 18
2.6 Luciferase assay ........................................................................................................... 18
2.7 Semi-quantitative reverse transcriptase PCR and real-time PCR .............................. 19
2.8 RNA interference ......................................................................................................... 19
2.9 Apoptosis assay ........................................................................................................... 19
2.10 Statistics ..................................................................................................................... 20

3 Results ............................................................................................................................ 21

3.1 Gfi1 is repressed by p53 and inhibits apoptosis induced by DNA damage through
upregulating Bcl-xL expression. ......................................................................................... 21

3.1.1 p53 inhibits the expression of GFI1 protein and mRNA .......................................... 21
3.1.2 p53 represses GFI1 transcription through direct binding to its promoter ............... 25
3.1.3 The GFI1 core promoter contains a p53 response element (RE) ............................ 26
3.1.4 Repression of GFI1 by p53 is not dependent on class I, II histone deacetylases
(HDACs), p21Cip1 and E2F1 ....................................................................................... 29
3.1.5 Gfi1 inhibits DNA damage induced apoptosis in a p53-independent manner. 31
3.1.6 Gfi1 upregulates Bcl-xL, but does not affect Bax, Bak and Bcl-2 expression. ................................................................. 34

3.1.7 Bcl-xL is partially responsible for Gfi1 inhibition of DNA damage induced apoptosis. ................................................................................................................................. 37

3.2 Gfi1 upregulates c-Myc expression and inhibits c-Myc overexpression induced apoptosis .............................................................................................................................. 40

3.2.1 Overexpression of Gfi1 upregulates Myc protein and mRNA expression. ................................................................. 40

3.2.2 Gfi1 knockdown or deficiency leads to downregulation of Myc protein and mRNA expression. ................................................................................................................................. 42

3.2.3 Gfi1 overexpression inhibits Myc induced apoptosis. ................................................................................................. 43

3.2.4 Gfi1 abolishes Myc-mediated downregulation of Bcl-xL ................................................................................................. 45

4 Discussion .......................................................................................................................................................................... 46

4.1 Gfi1 is repressed by p53 through direct DNA binding ................................................................. 47

4.2 Biological significance of Gfi1 repression of p53 ......................................................................... 49

4.3 Gfi1 inhibits DNA damage induced apoptosis independent of p53 ................................................. 51

4.4 Gfi1 cooperates with c-Myc in lymphomagenesis by upregulating c-Myc expression and inhibiting c-Myc overexpression induced apoptosis. ......................................................... 52

4.5 Gfi1 upregulates Bcl-xL expression to inhibit apoptosis induced by DNA damage and Myc overexpression ........................................................................................................................................ 54

5 Future Directions ......................................................................................................................................................... 58

References ........................................................................................................................................................................ 60
List of Figures

1-1: Schematic representation of hematopoiesis and cytokines that regulate hematopoiesis. ................................................................. 2
1-2: Schematic representation of transcription factors in hematopoiesis. .............. 4
1-3: Schematic representation of Gfi1 protein structure........................................ 7
1-4: Schematic view of the role of Gfi1 in normal hematopoiesis. ....................... 9
1-5: Schematic representation of extrinsic and intrinsic apoptosis pathways........... 14
3-1: Gfi1 protein and mRNA levels are upregulated upon p53 knockdown. .......... 23
3-2: Restoration of p53 function results in GF11 downregulation in response to DNA damage. ................................................................. 24
3-3: p53 binds to and represses GFII. ................................................................. 26
3-4: Mapping of the GFII promoter region required for p53-mediated repression. ...... 28
3-5: Identification of the repressive p53 RE in GFII core promoter............................ 29
3-6: Repression of GFII by p53 is not dependent on class I, II HDACs, p21Cip1 and E2F1. ........................................................................................................ 31
3-7: Overexpression of Gfi1 inhibits DNA damage-induced cell death............... 33
3-8: Knockdown of GF11 increases cell death in response to DNA damage. ........... 34
3-9: Gfi1 upregulates Bcl-xL, but does not affect Bax, Bak and Bcl-2 expression....... 35
3-10: Gfi1 knockdown or deficiency is associated with decreased Bcl-xL expression.

3-11: Overexpression of Bcl-xL rescues Gfi1 knockdown U937 and HL-60 cells from DNA damage induced apoptosis.

3-12: Knockdown of Bcl-xL partially abolishes Gfi1 inhibition of DNA damage induced apoptosis.

3-13: Schematic representation of p53 repression of Gfi1 and Gfi1 inhibition of apoptosis through upregulating Bcl-xL.

3-14: Overexpression of Gfi1 upregulates Myc protein and mRNA levels.

3-15: GFI1 knockdown or deficiency leads to downregulation of Myc protein and mRNA expression.

3-16: Gfi1 overexpression inhibits Myc-induced apoptosis.

3-17: Gfi1 abolishes Myc-mediated downregulation of Bcl-xL expression.

4-1: Schematic representation of Gfi1 cooperating with c-Myc in lymphomagenesis.

4-2: Gfi1 does not upregulate Bcl-xL expression through Bcl-xL promoter or 3’UTR region.
List of Abbreviations

4-HT ......................... 4-Hydroxytamoxifen
7-AAD ...................... 7 amino-actinomycin
3'UTR ...................... three prime untranslated region

ABL ....................... Abelson tyrosine kinase
ALL ....................... Acute lymphoid leukemia
AML ....................... Acute myeloid leukemia
AP1 ....................... Activator protein 1

BAD ...................... Bcl-2 antagonist of cell death
Bak ....................... Bcl-2 antagonist killer 1
Bax ....................... Bcl-2 associated x protein
Bcl-2 ...................... B cell lymphoma-2
Bcl-xL .................... Bcl-2-related gene long isoform
BCR ....................... Breakpoint cluster region
Bid ....................... Bcl-2 interacting domain death agonist
Bim ....................... Bcl-2 interacting mediator of cell death
BMC ....................... Bone marrow cell
bp ......................... Base pair

C/EBP .................... CAAT/Enhancer binding protein
CD ....................... Cluster of differentiation
CDK ...................... Cyclin-dependent kinase
ChIP ..................Chromatin Immunoprecipitation
CIP1 ..................CDK-interacting protein 1
CLP ....................Common lymphoid progenitor
CML ..................Chronic myeloid leukemia
CMP ..................Common myeloid leukemia

EKLP ..................Erythroid Krüppel-like factor
EPO ..................Erythropoietin
ETO ..................Eight twenty one

FBS ..................Fatal bovine serum
FLT3 ..................Fms-like tyrosine kinase

G-CSF .................Granulocyte-colony stimulating factor
Gfi1 ..................Growth factor independent 1
GFP ..................Green fluorescent protein
GM-CSF ...............Granulocyte-macrophage-colony stimulating factor
GMP ..................Granulocyte/monocyte progenitor

HDAC ..................Histone deacetylase
HLH/LZ ...............Helix-loop-helix/leucine zipper
HSC ..................Hematopoietic stem cell

IL ..................Interleukin

KD ..................Knockdown

LSK ..................Lin-/Sca-1+/c-Kit+

MCL-1 ..................Myeloid cell leukemia sequence 1
M-CSF ..................Macrophage-colony stimulating factor
MOMP ..................Mitochondrial outer membrane permeabilization
NFκB.......................Nuclear factor kappa-light-chain-enhancer of activated B cells
NHL ......................Non-Hodgkin lymphoma

RE ......................Response element
RTK .....................Receptor tyrosine kinase

SCF ........................Stern cell factor
SD ..........................Standard deviation
shRNA .....................short hairpin RNA
SNAG ..................Snail/Slug
STAT ......................Signal transducer and activator of transcription
SV40 ........................Simian virus 40

TNF ........................Tumor necrosis factor
TRE ........................Tetracycline-response element
TSA .......................Trichostatin A

WB ........................Western Blot
WT ..........................Wild type

ZF ..........................Zinc finger
Chapter 1

Introduction

1.1 Hematopoiesis

Hematopoiesis is the process during which various lineages of mature blood cells develop from hematopoietic stem cells (HSC). HSCs are capable of self-renewal to maintain a pool of long-term stem cells. HSCs differentiate into multipotential progenitors (MPPs), which give rise to common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs) that are committed to myeloid lineages and lymphoid lineages, respectively. CMPs can further differentiate into mature myeloid cells including granulocytes, monocytes/macrophages, erythrocytes and megakaryocytes, whereas CLPs develop into mature lymphoid cells such as T cells and B cells. (Figure 1-1). Terminally differentiated hematopoietic cells are less capable of proliferation and eventually die of apoptosis [1, 2].

Hematopoiesis is generally regulated by three distinct mechanisms. The first mechanism involve interaction between hematopoietic cells and the bone marrow
microenvironment including stromal cells and extracellular matrix. Hematopoiesis is also regulated by a group of cytokines, which provide regulatory signals for proliferation, differentiation and apoptosis. The third mechanism is comprised of a network of hematopoietic transcription factors that function to direct hematopoietic cell development and lineage commitment. Deregulation of cytokine or transcription factor expression during hematopoiesis may lead to uncontrolled proliferation, a block of hematopoietic differentiation, and eventually hematopoietic malignancies such as leukemia and lymphoma [3].

Figure 1-1: Schematic representation of hematopoiesis and cytokines that regulate hematopoiesis. HSCs differentiate into common myeloid progenitor (CMPs) and common lymphoid progenitors (CLPs). CMPs and CLPs are further differentiated into mature myeloid cells including Neutrophils, monocytes, megakaryocytes/platelets and erythrocytes, and lymphoid cells including T-lymphocytes and B-lymphocytes. Some of the major hematopoietic cytokines for different stages of hematopoiesis are shown (also see text).
1.2 Hematopoietic malignancies

Leukemia is caused by uncoordinated proliferation and sustained survival of myeloid or lymphoid cells arrested at various stages of hematopoietic differentiation. According to the cellular origin and disease progression, leukemia can be classified into acute myeloid leukemia (AML), acute lymphoid leukemia (ALL), chronic myeloid leukemia (CML) and chronic lymphoid leukemia (CLL). Leukemia is believed to be the consequence of two classes of mutations. Class I mutations cause constitutive activation of receptor tyrosine kinase (RTK) or intracellular kinase, such as BCR/ABL and FLT3 mutants. Activation of these kinases usually confer cells the advantage for proliferation and survival, leading to uncontrolled expansion of hematopoietic cells. Class II mutation typically cause impaired hematopoietic differentiation. Mutations in transcription factors including RUNX1, C/EBPα and MLL usually belong to class II mutation.

Lymphoma is characterized by uncontrolled proliferation and tumor formation of transformed lymphocytes in lymphatic tissues such as lymph nodes and spleen. Malignant lymphocytes form mass of tumors and eventually invade other organs via the lymphatic system. Lymphoma was generally classified into Hodgkin lymphoma and non-Hodgkin lymphoma. Hodgkin lymphoma has a predictable characteristic pattern of migration. Non-Hodgkin lymphoma (NHL) tends to spread to other organs in comparison. Recent classification of lymphoma is based on the cell type from which malignancy was originated, including T, B and NK cells. For example, peripheral T-cell lymphoma is a subdivision of NHLs that originates from various types of peripheral T cells. As in other types of cancer, lymphomas develop as a consequence of mutations or chromosomal translocations that activate proto-oncogenes or inactivate tumor suppressors.
1.3 Hematopoietic cytokines

Hematopoietic cytokines are regulatory glycoproteins primarily produced by lymphocytes, monocytes, fibroblasts and stromal cells [1]. Hematopoietic cytokines function by activating cognate receptors which subsequently trigger activation of specific downstream signaling pathways. Cytokines exerts stimulatory or inhibitory regulation of cells at specific stages of hematopoiesis. Early acting cytokines such as IL-3 and IL-6 regulate early multipotential progenitors, whereas late acting cytokines such as macrophage-colony stimulating factor (M-CSF) and granulocyte-colony stimulating factor
(G-CSF) acts on more committed progenitor cells to regulate later stage of hematopoietic development. For example, IL-3 supports the growth of a wide variety of early progenitor cells. G-CSF is required for stimulating terminal differentiation of neutrophils [4]. Some hematopoietic cytokines act at both early and later stages of hematopoiesis. Granulocyte macrophage-colony stimulating factor (GM-CSF) regulates the development of early myeloid progenitors as well as terminal differentiation of granulocytes and monocytes [5].

1.4 Hematopoietic transcription factors

Hematopoietic transcription factors play a critical role in directing hematopoietic cell development and lineage commitment (Figure 1-2). The hematopoietic transcription factor network, similarly to the hematopoietic cytokine network, acts in a hierarchy manner. Some transcription factors, such as GATA-2 and AML1, affect the development of a wide spectrum of hematopoietic cells. Lack of GATA-2 causes global hematopoietic deficit of all cell lineages although the maturation of individual cells remains normal [6]. Lack of AML1 in mice leads to a variety of defects in embryonic fetal liver hematopoiesis [7]. On the contrary, some transcription factors such as GATA-1, EKLP, C/EBPα and C/EBPε, act on more lineage-specific cells. For example, GATA-1 and EKLP regulate terminal differentiation of erythrocytes. C/EBPα functions to promote development of granulocyte/monocyte progenitors (GMPs) from CMPs and favors transition of GMPs to granulocytes/neutrophils instead of monocytes/macrophages. C/EBPε is required for terminal neutrophil differentiation. In addition, some transcription factors function in both early and later stage of hematopoiesis. PU.1 regulates early development of myeloid and
lymphoid progenitor cells from HSCs, and also promote development towards monocytes/macrophages from GMPs. PU.1-deficient mice lack of disparate hematopoietic lineages [8].

Hematopoietic cytokines act in intimate functional collaboration with hematopoietic transcription factors to regulate hematopoiesis. Stimulation with cytokines may change the expression of transcription factors. Similarly, transcription factors are capable of altering the expression of cytokine signaling components. For example, STAT5 and Raf signaling pathways are activated in response to IL-7 stimulation in B cell development [9]. Lack of PU.1 leads to decreased expression of receptors for GM-CSF, G-CSF and M-CSF, which results in deficiency of mature granulocytes, macrophages and B cells [10].

1.5 Growth factor independent 1 (Gfi1)

The Gfi1 gene encodes a 423 amino acid nuclear transcription repressor. Gfi1 consists of an N-terminal SNAG domain and six C2H2-type zinc finger (ZF) domain at the C-Terminus (Figure 1-3). The N-terminal SNAG domain, which is conserved among the murine protein Snail and Slug family, contains the nuclear localization signal and is required for transcription repression activity. The 3rd, 4th and 5th zinc finger domains have been shown to be responsible for DNA binding activity [11-13]. The middle region between the SNAG and ZF domain is poorly characterized, but is believed to be responsible for protein-protein interaction.
Gfi1 functions as a transcription repressor through binding to specific DNA elements containing AAT/GC core sequences and mediates reversible transcriptional repression through recruiting co-factors such as ETO, HDAC and G9a histone lysine methyltransferase [14]. For example, Gfi1 represses expression of Id2 and Egr1/2 through direct binding to their promoters [15, 16]. Gfi1 can also indirectly repress gene expression. For example, Gfi1 cooperates with c-Myc to represses transcription of p21Cip1 (CDKN1A), p15INK4B (CDKN2B) by inhibiting Miz1 activation of CDKN1A and CDKN2B promoters [17, 18]. Moreover, Gfi1 forms complexes through protein-protein interaction to inhibit protein function. For example, Gfi1 interacts with PU.1 and antagonizes PU.1 mediated transcriptional regulation to promote neutrophil differentiation [19].

Gfi1 is a transcriptional repressor that plays a central role in normal hematopoietic development [20, 21]. Loss of Gfi1 leads to severely reduced thymus cellularity because of elevated apoptosis rate during T cell development and delayed cell cycle entry into S phase after TCR stimulation [22, 23]. Moreover, Gfi1 promote proliferation of mature T cells. Enforced expression of Gfi1 enhances expansion of Th2 cells [24]. In contrast, Gfi1 restricts proliferation of hematopoietic stem cells (HSCs), thus maintaining the functional integrity of HSCs. Gfi1−/− HSCs are hyperproliferative and impaired in long term proliferation [25]. Furthermore, Gfi1 promotes terminal development of granulocytes and
antagonizes the alternative development towards macrophages. Gfi1-/- mice are severely neutropenic with absence of mature granulocytes because of defective granulocyte differentiation [24, 26, 27] (Figure 1-4).

Besides the role in normal hematopoiesis, Gfi1 has also been implicated in hematopoietic malignancies. Gfi1 functions as a proto-oncoprotein in lymphoid system. Gfi1 was first identified as a proviral integration target in T lymphoma cells that selected for IL-2 independent growth. Overexpression of Gfi1 leads to growth factor independent growth in hematopoietic cells [13]. Gfi1 transgenic mice are predisposed to develop T cell lymphoma due to overexpression of Gfi1 in T cells [28]. Moreover, Gfi1 cooperates with Myc and Pim-1 to accelerate lymphomagenesis [29]. In contrast to its role in lymphomagenesis, Gfi1 may also function as a tumor suppressor in myeloid system. Loss of Gfi1 causes upregulation of HoxA9, Pbx1 and Meis1 in myeloid progenitors, which lead to accumulation of myeloid progenitors and leukemogenesis [30]. Gfi1-deficient mice are prone to myeloid leukemia [30]. In contrast to its role in lymphomagenesis, Gfi1 may also function as a tumor suppressor in myeloid system. Loss of Gfi1 causes upregulation of HoxA9, Pbx1 and Meis1 in myeloid progenitors, which leads to accumulation of myeloid progenitors and leukemogenesis [30]. Gfi1-deficient mice are prone to myeloid leukemia [30].
1.6 c-Myc

c-Myc is a member of Myc family which includes evolutionally related helix-loop-helix/leucine zipper (HLH/LZ) transcription factors that contribute to a wide variety of malignancies. c-Myc processes the most oncogenic potential comparing to other Myc family members such as N-Myc and L-Myc. Deregulated c-Myc expression has been associated with generation of leukemia and lymphoma in mice models, and is observed in a large number of hematopoietic malignancies.
The *c-Myc* gene is activated in various ways in human cancers, including gene viral insertion and chromosomal translocation. For example, retroviral insertion adjacent to *c-Myc* locus can result in c-Myc overexpression by elevating c-Myc mRNA [31]. In addition, the c-Myc gene can be juxtaposed by chromosomal translocations adjacent to genes that are highly expressed, leading to increased c-Myc expression level. For example, Burkitt’s lymphoma is characterized by the chromosomal translocation between the *c-Myc* gene and one of the immunoglobin heavy or κ and λ light chain loci such that the transcription of *c-Myc* is controlled by an immunoglobin locus, resulting in overexpression of c-Myc protein in B cells.

*c-Myc* is a vital transcription factor in regulation of cell proliferation as well as survival. c-Myc promotes cell proliferation partially by overcoming G1 phase and G1/S checkpoint cell cycle arrest in part through repressing the transcription of CDK inhibitors including p15\(^{\text{INK4B}}\) and p21\(^{\text{CIP1}}\). For example, c-Myc, together with Gfi1, binds to Miz1 to repress Miz1 activation of *CDKN2B (p15INK4B)* and *CDKN1A (p21Cip1)* expression, leading to downregulation of CDK inhibitor p15\(^{\text{INK4B}}\) and p21\(^{\text{CIP1}}\), respectively [17, 18].

In addition, c-Myc can also induce apoptosis, providing a fail-safe mechanism for eliminating unchecked cell proliferation caused by DNA damage, mutagenesis or radiation. c-Myc induced apoptosis can be triggered by either p53-dependent or independent mechanisms. Upon overexpression of c-Myc, ARF expression is upregulated leading to increased level of p53 and, eventually, apoptosis [32]. c-Myc has also been reported to induce DNA damage and genetic instability, which subsequently activates p53 signaling pathway that leads to apoptosis [33]. Additionally, c-Myc can also induce apoptosis independent of p53 through mitochondrial apoptotic pathway that involves Bcl-2 family
members, including Bcl-2, Bcl-xL, Bax and Bak. However, c-Myc overexpression in
tumor cells is often accompanied by additional mutations that disable the Myc-dependent
apoptosis.

1.7 The tumor suppressor p53

The tumor suppressor p53 regulates transcription of genes in various aspects of
hematopoiesis, including differentiation, proliferation, apoptosis and senescence. The $p53$
gene is less frequently inactivated in hematopoietic malignancies comparing to solid
tumors, but mutations of $p53$ do exist in leukemia and lymphoma [35, 36].

$p53$ regulates behavior of HSCs in normal hematopoietic development through
maintaining HSC quiescence and inhibiting HSC self-renewal [37]. For example, the
number of LSK cells (Lin$^{-}$ Sca-1$^{-}$ c-Kit$^{+}$) in p53-null mice is increased by two fold
comparing to wildtype mice. Bone marrow cells from p53-null mice are more competitive
than wildtype cells in competitive repopulation assays [38].

As a tumor suppressor, p53 functions to prevent errors during cell proliferation caused
by stress and inhibit oncogenic mutation by maintaining the fidelity of cell division. DNA
damage leads to a steady increase of p53 expression level. Expression of oncogenes such
as $c$-$Myc$ and $E1A$ can also activate p53 expression [39]. Upon activation by DNA damage
and oncogene expression, p53 functions mainly through transcription activation of its
target genes. p53 transactivates expression of the CDK inhibitor $p21^{CIP1}$, leading to G1
phase cell cycle arrest and inhibition of proliferation [40]. p53 can also transactivate pro-
apoptotic BCL-2 family members Bax, Puma and Noxa to induces apoptosis [41].
Moreover, p53 can be activated by oncogenic Ras and, by cooperating with Ras, induces
premature senescence [42, 43]. In addition to transactivation, p53 can function as a transcription repressor by repressing transcription of proto-oncogenes. p53 prevents tumor progression by repressing c-Myc expression [44]. However, comparing to the mechanism of p53 transcription activation, the mechanism of p53 transcription repression is less understood and needs to be further elucidated.

1.8 Bcl-2 family members in apoptosis

The Bcl-2 (B cell lymphoma-2) gene was first discovered in the t(14; 18) chromosome translocation in B-cell lymphoma, overexpression of which leads to oncogenesis due to inhibition of cell death. The Bcl-2 family members are a group of proteins, including Bcl-2 itself and others which share similar Bcl-2 homology (BH) domains of Bcl-2. The Bcl-2 family include anti-apoptotic and pro-apoptotic proteins. The anti-apoptotic proteins contain 4 Bcl-2-homology domains. Bcl-2, Bcl-xL (Bcl-2-related gene long isoform) and Mcl-1 (myeloid cell leukemia 1) are the major members of anti-apoptotic Bcl-2 family proteins. The pro-apoptotic Bcl-2 family member proteins includes the effector proteins and the BH3-only proteins. The effector proteins Bak (Bcl-2 antagonist killer 1) and Bax (Bcl-2-associated x protein) contain BH 1-4 motif. Upon activation, Bax and Bak form homodimers within the outer mitochondria membrane and initiate mitochondrial outer membrane permeabilization (MOMP) to allow soluble mitochondrial proteins like cytochrome c to diffuse into the cytosol. The BH-3 only proteins distinct scenarios of cellular stress. Bad (Bcl-2 antagonist of cell death) and Noxa are referred as “sensitizer” and/or “repressor” BH-3 proteins which only bind to anti-apoptotic proteins. Others such as Bid (Bcl-2 interacting domain death agonist) and Bim (Bcl-2 interacting mediator of cell death) interact with both anti-apoptotic proteins and effectors to activate MOMP. These
BH3-only proteins are referred as “direct activators” [45]. The interactions between anti-apoptotic, pro-apoptotic and BH3-only proteins determine the process of apoptosis.

Apoptosis is a controlled form of cell death which exhibits distinct characteristics in cell signaling pathways [46]. All pathways that lead to apoptosis eventually depends on the activation of caspases, which are cysteinyl aspartate proteases that coordinate programmed cell death. Apoptosis pathways can be distinguished as the extrinsic pathway and the intrinsic pathway, depending on the requirement of Bcl-2 family proteins (Figure 1-5). The extrinsic pathway, or death-receptor pathway, is depended on transmembrane receptor proteins such as tumor necrosis factor (TNF) family members. Apoptosis by extrinsic pathway is triggered by binding of death receptor such as Fas and tumor necrosis factor receptor-1 (TNFR-1) to the corresponding ligands, which in turn activates downstream caspases such as caspases-3, 6 or 7, without any involvement of Bcl-2 family members. On the other hand, the intrinsic pathway, also called the mitochondrial pathway, is activated by various development or cytotoxic factors such as DNA damage or viral infection, and is strictly controlled by members of Bcl-2 family proteins. The intrinsic pathway lead to initiation of mitochondrial outer membrane permeabilization (MOMP) which allows cytochrome c to diffuse from mitochondria to cytosol and engages Apaf-1 (apoptotic protease activating factor-1). Apaf-1 is subsequently oligomerized into apoptosome which binds and promotes activation of caspase-9 [47]. Eventually, executioner caspase-3 and 7 are activated to induce cell death.

Bcl-2 family members are regulated by Gfi1 during hematopoiesis in various ways. Bax and Bak have been shown to be repressed during Gfi1 inhibition of apoptosis in thymocytes and peripheral T-cells [48]. Gfi1-/− thymocytes have severely reduced
cellularity, possibly due to excessive apoptosis during T cell development in thymus [48, 49]. Bcl-2 have been implicated to repress Bax to mediate Gfi1 protection of HSCs against stress-induced apoptosis [50].

Figure 1-5: Schematic representation of extrinsic and intrinsic apoptosis pathways. Apoptosis can be induced through extrinsic pathway by activation of death receptors such as Fas and TNFR-1 (left), or intrinsic pathway by various cellular stress, UV or viral infections (right). Some of the major Bcl-2 family members and caspases are shown (also see text).
Chapter 2

Materials and Methods

2.1 Cells and materials

Murine Pro-B Ba/F3 cells were maintained in RPMI-1640 medium supplemented with 4% fetal bovine serum (FBS), 4% WEHI-3B cell-conditioned media as a crude source of murine interleukin-3, and 1% penicillin/streptomycin (P/S). Human Burkitt lymphoma cell line Ramos (American Type Culture Collection), human T lymphoblastic cell line Molt3, and human myeloid leukemia cell line U937 and HL-60 were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% P/S solution. Human umbilical cord blood CD34+ cells (American Type Culture Collection) were cultured in IMDM medium supplemented with 15% BIT9500, 50ng/µl Flt3 Ligand, 50ng/µl SCF, 10ng/ml TPO (thrombopoietin) and 1% P/S. Human megakaryocytic cell line MO7e was cultured in RPMI-1640 medium supplemented with 10% FBS and 5 ng/ml recombinant human GM-CSF. Human colon cancer cell line HCT116 and its p53−/− and p21−/− derivatives originally generated by Dr. Bert Vogelstein (Johns Hopkins University), were kindly provided by Dr.
William Taylor (University of Toledo) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS and 1% P/S solution. The cells were grown in a humidified incubator at 37°C with 5% CO₂.

Antibodies against Gfi1 (N-20) and human p53 (BP 53.12)0 were purchased from Santa Cruz Biotechnology. Antibodies against Bax and Bcl-2 were purchased from BD Transduction. Anti Bak and anti β-actin antibodies were from Calbiochem and Sigma, respectively. QuikChange™ Site-Directed Mutagenesis Kit was purchased from Stratagene.

2.2 Construction of plasmids

The human GFI1 promoter fragments spanning from -1933 bp to +468 bp and from -4840 to +184 bp, respectively, were generated by PCR and cloned into the pGL3-basic luciferase reporter plasmid. The -1933 bp/+468-bp fragment was used as a template for PCR to generate a series of truncated GFI1 promoter fragments. Single-stranded oligonucleotides corresponding to the different GFI1 core promoter sequences of both strands were annealed in vitro and cloned into the NheI and XhoI sites of the pGL3-control luciferase reporter plasmid. Mutations in the GFI1 promoter fragments were generated by site directed mutagenesis. HCT116 cells were transfected using TransIT-LT1 Transfection Reagent and harvested 36 hrs later. Luciferase activities were measured using a Molecular Devices Lmax luminometer (Sunnyvale, CA) and normalized on the basis of the co-transfected β-galactosidase activity.

For inducible expression of Gfi1, the Gfi1-RV retroviral vector was digested with BamHI and XbaI, and the resulting DNA fragment of approximate 2.8 kb, which contained
the rat Gfi1 cDNA, an internal ribosomal entry sequences (IRES) and humanized GFP cDNA, was used to replace the mSEAP cDNA of the lentiviral vector pTMPrtTA, a generous gift of Dr. Olivier Danos (Genethon-Centre National de la Recherche Scientifique, France).

The murine Bcl-xL promoter fragment spanning from -1.6 to +1.6 kb was generated by PCR from pGL2-Bcl-xL promoter construct originated from Dr. Nunez (Michigan University, Michigan) and cloned into the pRL-null luciferase reporter plasmid. A 2.0 kb fragment spanning from -3.6 to -1.6 kb was cloned from murine genomic DNA and inserted into pRL-Bcl-xL -1.6/+1.6 kb reporter construct upstream of Bcl-xL promoter to generate pRL-Bcl-xL -3.6/+1.6 kb reporter construct. The human Bcl-xL 3’UTR region was cloned from human genomic DNA and inserted to lentiviral reporter pFUGW-luc-GFP plasmid.

2.3 Transfection and generation of stable cell lines

The pBabePuro.p53ER\textsuperscript{tam} expression construct [51] was a generous gift from Dr. Douglas R. Green (La Jolla Institute for Allergy and Immunology). HL-60 and U937 cells were transfected with pBabePuro.p53ER\textsuperscript{tam} by electroporation and selected in 2 µg/ml puromycin 48 hrs later. Puromycin-resistant cells were subcloned by limiting dilution. Individual clones were examined for expression of p53ERtam by Western blot analysis.

The pMTrtTA-Gfi1-GFP plasmid was transfected into Ba/F3 and Ramos cells by electroporation and GFP\(^+\) cells were sorted. Ba/F3 cells were also transfected with retrovirus containing pBabe-puro-MycER and selected in 2 µg/ml puromycin for 48 hrs. Living cells were isolated and infected with lentivirus containing pMTrtTA-Gfi1-GFP expression construct. GFP\(^+\) cells were sorted.
2.4 Cell proliferation assay (MTS assay)

Viable cell numbers were quantitated by the CellTiter 96® AQueous Non-Radioactive Cell Proliferation (MTS) Assay (Promega, Madison, WI). After extensive washing, 2 x 10^4 cells were incubated in triplicate in 100 μl of RPMI 1640 medium in 96-well plates in the presence of doxycycline (1 μg/ml) for 24 hrs, followed by treatment with doxorubicin (100 ng/ml for Ba/F3 cells and 2 mg/ml for Ramos cells) for 18 hrs. CellTiter 96® AQueous One Solution Reagent was added to each well and the plates were read 2 hrs later at 490-nm wavelength in a microplate luminometer (Molecular Devices, Sunnyvale, CA).

2.5 Chromatin immunoprecipitation assay (ChIP assay)

ChIP assays were performed essentially as described [17, 18]. Briefly, cells were fixed with 1% formaldehyde and then lysed in hypotonic buffer [5 mM Tris-HCl (pH 7.5), 85 mM KCl and 0.5% Nonidet P-40]. After centrifugation at 6000 rpm for 5 min, nuclei were lysed in ChIP lysis buffer [1% SDS, 10 mM EDTA, and 50 mM Tris HCl (pH 7.5)] and sonicated to shear chromatin DNA to ~500-bp fragments. Nuclear lysates were precleared with protein A/G agarose beads and rabbit normal IgG for 1 h and subjected to immunoprecipitation using the anti p53 or a species-matched irrelevant antibody. Precipitated DNA was examined by semi-quantitative PCR.

2.6 Luciferase assay

HCT116 cells were transfected using TransIT-LT1 Transfection Reagent and harvested 36 hrs later. Luciferase activities were measured using a Molecular Devices Lmax luminometer (Sunnyvale, CA) and normalized on the basis of the co-transfected β-galactosidase activity.
2.7 Semi-quantitative reverse transcriptase PCR and real-time PCR

Total cellular RNA was extracted using TRIzol reagent (Invitrogen). 1µg of total RNA was reverse transcribed into cDNA by Oligo (dT)-15 primer (Promega) and GoScript™ reverse transcriptase (Promega). 1µl of reverse transcribed cDNA was subjected to PCR amplification with gene-specific primer pairs using GoTaq Green Mastermix (Promega) for semi-quantitative RT-PCR or SsoFast™ Eva Green Mastermix (Promega) for real-time PCR.

2.8 RNA interference

Lentiviral constructs encoding human GFI1 specific shRNAs have been described before [17, 18]. Lentiviral construct containing human p53 shRNA was a gift of Dr. Robert A. Weinberg (Addegene plasmid #19119). Lentiviral construct of mouse Bcl-xL shRNA was a gift of Dr. Mick Croft. 293T cells were transfected with the lentiviral constructs along with the packaging plasmids using the calcium phosphate coprecipitation procedure. The virus-containing supernatants were harvested and used to infect cells as described above. Cells were selected in 2 µg/ml puromycin 48 h later and shRNA-mediated knockdown of GFI1 and p53 was examined by Western blot analysis.

2.9 Apoptosis assay

Apoptosis was examined using the Annexin V-PE apoptosis detection kit (BD Biosciences). Briefly, 0.3 x 10^6 cells were collected and incubated with Annexin V-PE and 7 amino-actinomycin (7-AAD). Cells were analyzed by two-color flow cytometry using BD Cellquest Pro software (Becton Dickinson, San Jose, CA).
2.10 Statistics

GraphPad Prism software (GraphPad Software, La Jolla, CA, USA) was used for all statistical analysis. Data are shown as mean ± SD in all figures. A p value <0.05 was considered significant for all analyses and shown as *. ** denotes P<0.01, *** denotes P<0.001 and **** denotes p<0.0001. Significance of differences in cell viability was determined using Mann-Whitney U tests. Two-tailed paired T test was used to calculate differences of luciferase activities between samples in all luciferase assays, as well as differences of living cell number measured by trypan blue staining or MTS assays. Student’s t test was used to calculate sample differences in real-time PCRs between 2 samples. One way ANOVA was used in real-time PCRs with comparison of 3 samples, followed by Tukey’s multiple comparison test.
Chapter 3

Results

3.1 Gfi1 is repressed by p53 and inhibits apoptosis induced by DNA damage through upregulating Bcl-xL expression.

3.1.1 p53 inhibits the expression of GFI1 protein and mRNA

The tumor suppressor p53 induces apoptosis upon DNA damage, while Gfi1 promotes cell survival and contributes to lymphomagenesis [48]. We explored the role of p53 in the regulation of GFI1 expression in hematopoietic cells. MO7e and Molt3 cells are human megakaryocytic and T lymphoblastic leukemia cell lines, respectively, that express wild type (WT) p53 protein. The expression of p53 in MO7e and Molt3 cells was knocked down through lentiviral mediated delivery of a p53 shRNA. Significantly, p53 knockdown was associated with increased expression of GFI1 at both protein and mRNA levels (Figure 3-1A). Treatment with doxorubicin (Doxo), which induces topoisomerase II-mediated DNA double strand breaks, led to a steady increase in p53 protein level and this was accompanied by decreased GFI1 expression in both cell lines, which was most significant at 36 hrs of
treatment (Figure 3-1B and C). However, GFI1 expression was not downregulated in the p53 knocked-down (KD) cells in which the levels of p53 remained low, albeit upregulated, following Doxo treatment. We also examined whether p53 upregulation correlated with GFI1 downregulation in human umbilical cord blood CD34+ cells. As shown in Figure 3-1D, Doxo treatment upregulated p53 expression in the cells and this was associated with GFI1 downregulation at 36 hrs of treatment.

We further assessed the effect of restoration of p53 function on GFI1 expression in p53-deficient cells. Human myeloid leukemic U937 and HL-60 cells, which lacked functional p53, were transduced with the retroviral expression construct for the p53/estrogen receptor ligand binding domain fusion protein (p53ER<sup>TAM</sup>). It is worth noting that addition of 4-hydroxy tamoxifen (4-HT) does not overly activate p53ER<sup>TAM</sup>, but enables p53ER<sup>TAM</sup> to be activated by upstream signals [52]. Cells were subsequently treated with Doxo in the absence or presence of 4-HT. As shown in Figure 3-2, GFI1 protein and mRNA levels in the p53ER<sup>TAM</sup>-expressing cells declined significantly at 36 hrs of Doxo treatment in the presence of 4-HT, but did not or only slightly declined in its absence. GFI1 expression was not or barely downregulated by Doxo treatment in the control cells with or without added 4-HT. Together, these data demonstrated that p53 inhibited GFI1 expression, presumably by targeting its mRNA.
Figure 3-1: Gfi1 protein and mRNA levels are upregulated upon p53 knockdown. (A) MO7e and Molt3 cells were infected with the lentivirus containing a p53 shRNA. The expression of GFI1 and p53 proteins was examined by Western blot analysis. GFI1 mRNA levels were examined by RT-PCR. Subsequently, control (Ctr) and p53 knocked down (KD) MO7e (B) and Molt3 (D) cells were treated with Doxo (25 ng/ml) for times as indicated and examined for expression of p53 and GFI1. The results shown in B and D were quantitated using ImageJ and normalized based on the levels of b-actin protein and GAPDH mRNA for MO7e (C) and Molt3 (E) cells. (F) Human umbilical cord blood CD34+ cells were treated with Doxo prior to evaluation of the expression of p53 and GFI1.
Figure 3-2: Restoration of p53 function results in GFI1 downregulation in response to DNA damage. U937 (A) and HL-60 (B) cells untransfected or transfected with p53ERTAM were either left untreated or treated with Doxo (50 ng/ml) for times as indicated in the presence or absence of 4-HT. The expression of GFI1 and p53ER was examined by Western blotting and RT-PCR (upper panels), and the data were quantitated using ImageJ (lower panels). The moderate increase in GFI1 mRNA level following Doxo treatment in the control HL-60 cells without Doxo treatment was not reproducible in two other independent experiments.
3.1.2 p53 represses GFII transcription through direct binding to its promoter

As p53 inhibits Gfi1 expression through downregulation of Gfi1 mRNA, we explored whether p53 downregulates GFII mRNA by repressing GFII promoter activity. We examined whether p53 repressed the activity of a human GFII promoter fragment spanning from -1933 bp to +468 bp (relative to human GFII mRNA sequence [NM_005263]) in the p53\textsuperscript{−/−} HCT116 cells. As shown in Figure 3-3A, luciferase activity driven by the GFII promoter was markedly inhibited by the wild type (WT) p53, but not by the W248 mutant which is defective in DNA binding, indicating that DNA binding activity was required for p53 repression of GFII promoter. Consistent with the role of p53 in repressing GFII, Doxo treatment inhibited the activity of the GFII promoter fragment in the p53\textsuperscript{+/+} HCT116 cells, but not in the p53\textsuperscript{−/−} cells (Figure 3-3B).

We further performed chromatin immunoprecipitation (ChIP) assays to investigate whether p53 bound to the GFII promoter. The p53\textsuperscript{−/−} HCT116 cells were transiently transfected with a human GFII promoter fragment spanning from -4840 bp to +184 bp either alone or together with WT p53 expression construct. Protein-DNA complexes were prepared and immunoprecipitated with an anti-human p53 antibody or a species-matched control antibody. As shown in Figure 3-3C, p53 specifically bound to the proximal, but not the distal, promoter region of GFII. ChIP assays were also carried out on Molt3 cells to address whether p53 bound to GFII in the endogenous setting. As in the p53\textsuperscript{−/−} HCT116 cells, endogenous p53 bound to the GFII core promoter region (Figure 3-3D). Together, these data indicate that p53 repressed GFII transcription by directly binding to its promoter.
3.1.3 The *GFI1* core promoter contains a p53 response element (RE)

To identify the p53 RE in the *GFI1* promoter, we assessed the effects of p53 on the activities of a series of progressively truncated *GFI1* promoter fragments (Figure 3-4A). Notably, the promoter fragment spanning from -63 bp to +6 bp was still repressed by p53, but further truncation resulted in loss of promoter activity (Figure 3-4C). To precisely
define the *GFI1* core promoter region required for p53-mediated repression, we determined which *GFI1* core promoter region was sufficient to confer p53 response to a non-p53 responsive promoter. A number of oligonucleotides corresponding to the different regions of *GFI1* core promoter were synthesized and inserted upstream of the *SV40* promoter of the pGL3 control plasmid (Figure 3-4B). The *SV40* promoter was not repressed by p53 (Figure 3-4D). Insertion of any of the three oligonucleotides containing the 39-bp core promoter sequence (-33 bp/+6 bp) resulted in the repression of the *SV40* promoter by p53. In contrast, the two oligonucleotides lacking this sequence had no effect on p53 response. These data demonstrated that the p53 RE is located within the *GFI1* core promoter region between -33 bp and +6 bp.

A close examination of *GFI1* core promoter sequence revealed that the promoter region spanning from -34 bp to -9 bp appeared to match the loosely defined repressive p53 RE [53]. We therefore addressed whether mutation of the two half-sites in the potential repressive p53 RE of *GFI1* promoter blocked p53-mediated repression. Interestingly, mutation of the CGAG sequence in the downstream half-site, but not the CCCG sequence in the upstream half-site, abolished p53 repression of the *GFI1* promoter (Figure 3-5B). Consistent with loss of repression by p53, ChIP assays demonstrated that p53 was unable to bind to the *GFI1* promoter when the downstream half-site was mutated (Figure 3-5C). Together, these data indicated that the *GFI1* core promoter contains a p53 RE that is required for p53-mediated repression.
Figure 3-4: Mapping of the GFI1 promoter region required for p53-mediated repression. (A and B) Schematic diagrams of GFI1 promoter fragments cloned into pGL3-basic vector (A) or inserted upstream of the SV40 promoter of the pGL3-promoter vector. (C and D) p53^-/- HCT116 cells were transfected with the indicated GFI1 promoter luciferase reporter constructs without or with p53. Luciferase activities were measured 36 hrs after transfection.
3.1.4 Repression of \( GFI1 \) by p53 is not dependent on class I, II histone deacetylases (HDACs), \( p21^{Cip1} \) and E2F1

Recent studies have indicated that p53 may repress certain target genes through the recruitment of HDACs via its interaction with mSIN3a [54, 55]. We therefore addressed whether trichostatin A (TSA), a potent inhibitor of class I and II HDACs, would affect p53-
mediated repression of GFI1. TSA treatment significantly augmented the activity of the GFI1 promoter, but did not abolish p53-mediated repression (Figure 3-6A), suggesting that the function of the class I and II HDACs is involved in the negative regulation of GFI1 transcription, but is not required for p53-mediated repression. Consistent with this, forced expression of p53 in p53−/− HCT116 cells did not enhance mSIN3A binding to the GFI1 promoter (Figure 3-6B).

p53 has also been shown to repress its target genes indirectly through transcriptional activation of p21Cip1, which inhibits the phosphorylation of retinoblastoma protein, leading to decreased activation of E2F-regulated genes [54, 55]. To exclude this possibility, we examined whether Doxo was able to inhibit GFI1 promoter activity in p21Cip1−/− HCT116 cells. As shown in Figure 3-6C, the activity of the GFI1 promoter was suppressed upon Doxo treatment in both p21Cip1+/+ and p21Cip1−/− HCT116 cells, indicating that repression of GFI1 by p53 is independent of p21Cip1. In support of this, luciferase reporter assays indicated that E2F1 was unable to activate the GFI1 promoter (Figure 3-6D).
3.1.5 Gfi1 inhibits DNA damage induced apoptosis in a p53-independent manner

Gfi1 has been shown to promote survival of hematopoietic cells [28, 48-50]. We addressed the role of Gfi1 in DNA damage-induced apoptosis. Murine pro-B Ba/F3 cells
were transduced with a lentiviral construct that expressed Gfi1 from the tetracycline-response element (TRE) in the presence of doxycycline (Doxy). The expression of endogenous Gfi1 was barely detectable in Ba/F3 cells by Western blot analysis, but was strongly induced upon addition of Doxy (Figure 3-7A). Cells were then treated with Doxo and evaluated for apoptotic cell death by trypan blue staining and annexin V assays. Treatment of Ba/F3 cells with Doxo led to markedly reduced cell viability and increased number of cells undergoing apoptosis (Figure 3-7B and C). Notably, Doxo-induced apoptosis was significantly reduced following addition of Doxy.

To address whether the protective effect of Gfi1 on cell death was dependent on functional p53, we introduced the Doxy-responsive Gfi1 lentiviral construct into Ramos cells (Figure 3-7D), a p53-deficient Burkitt’s lymphoma cell line. Ramos cells were highly resistant to DNA damage, but treatment with Doxo at 2μg/ml resulted in a significant decrease in cell viability and the number of living cells as determined by exclusion of trypan blue staining and MTS assay (Figure 3-7E and F). MTS assay instead of annexin V staining was used because Doxo at 2μg/ml interfered with flow cytometric analysis of annexin V stained cells. As in Ba/F3 cells, Doxo-induced cell death was significantly inhibited upon Gfi1 induction in Ramos cells, indicating that Gfi1 inhibition of Doxo-induced cell death was independent of p53.

We further assessed the effect of GFI1 knockdown on Doxo-induced cell death in myeloid leukemia cell lines U937 and HL-60, both of which expressed high levels of endogenous GFI1 [17, 18]. GFI1 expression was knocked down in cells transduced with the lentiviral vectors containing two different GFI1 shRNAs, but not with the empty vector
Figure 3-8: Notably, GFI1 knockdown was associated with a significant increase in the sensitivity of cells to Doxo-induced cell death. Because U937 and HL-60 cells lacked functional p53, these data further indicate that the protective effect of GFI1 on DNA damage-induced apoptosis was independent of p53 in hematopoietic cells.
3.1.6 Gfi1 upregulates Bcl-xL, but does not affect Bax, Bak and Bcl-2 expression.

Gfi1 has been shown to repress the pro-apoptotic Bcl-2 family members Bax and probably also Bak [11, 45], but appears to upregulate the expression of the anti-apoptotic Bcl-2 in hematopoietic cells [23, 50]. To investigate the mechanism whereby Gfi1 inhibited DNA damage-induced apoptosis, we examined the expression of Bcl-2 family members including Bcl-xL, Bax, Bak and Bcl-2 in Ba/F3 and Ramos cells transduced with the Doxy-
responsive Gfi1 expression construct. As shown in Figure 3-9, the expression of Bcl-xL protein and mRNA was upregulated by Gfi1 induction. However, other Bcl-2 family members were not affected by Gfi1 induction. Treatment of Ba/F3 and Ramos cells with Doxo following Gfi1 induction downregulates Bcl-xL expression, but also had no apparent effects on the expression of these Bcl-2 family members.

Figure 3-9: Gfi1 upregulates Bcl-xL, but does not affect Bax, Bak and Bcl-2 expression. (A) Ba/F3 and Ramos cells transduced with the inducible Gfi1-expressing lentiviral construct were preincubated with Doxy (1 mg/ml) for 24 hrs and then treated with Doxo (100 ng/ml) for times as indicated. The expression of Gfi1 and the Bcl-2 family members as indicated was examined by Western blot analysis. (B) The levels of the Bcl-2 family members in control and GF11 knocked down U937 and HL-60 cells were examined by Western blot analysis. (C) Ba/F3 PMTrtTA-Gfi1-GFP (Left) and Ramos (Right) PMT-rtTA-Gfi1-GFP cells were treated with doxycycline (1ug/ml) for 24 h, mRNA level of Bcl-xL was examined by real-time PCR.
We further addressed the effect of Gfi1-deficiency on Bcl-xL expression. As shown in Figure 3-10, expression of Bcl-xL protein and mRNA was downregulated in both U937 and HL-60 cells with GFI1 knockdown. Additionally, Bcl-xL protein and mRNA expression was also downregulated as Gfi1 expression decreases in primary lineage depleted bone marrow cells from Gfi1 +/+, +/- or -/- mice. Thus, instead of Bax and Bak,

![Image of Western blot and Real-Time PCR graphs showing downregulation of Bcl-xL expression with Gfi1 knockdown or deficiency.](image-url)

Figure 3-10: Gfi1 knockdown or deficiency is associated with decreased Bcl-xL expression. (A and B) HL-60 and U937 were infected with lentivirus containing pLKO empty vector, Gfi1 shRNA #65 or #68. (A) Expression of Bcl-xl, Bax and Gfi1 was examined by Western blot. (B) Expression of Bcl-xL was examined by Real-Time PCR. (C and D) Bone marrow mononuclear cells were extracted from femur and tibia of 8-12 weeks old C57BL/6J mice, followed by depletion of lineage positive cells. Expression of Bcl-xL in Gfi1 +/-, +/- and -/- mice was examined by real-time PCR (C) and Western blot (D).
Bcl-xL is likely to be the major player in Gfi1-mediated inhibition of DNA damage-induced apoptosis in the cells used in this study.

3.1.7 Bcl-xL is partially responsible for Gfi1 inhibition of DNA damage induced apoptosis.

To address the role of Bcl-xL in Gfi1 inhibition of DNA damage induced apoptosis, we examined whether Bcl-xL rescues cells with knockdown of Gfi1 from increased sensitivity to DNA damage induced cell death. U937 and HL-60 cells were transfected with control vector or Bcl-xL expression construct, followed by GFI1 knockdown. Subsequently, cells were treated with 200ng/ml of Doxo for 16 hours, living cell numbers were determined by trypan blue staining and MTS assays. As shown in Figure 3-11, living cell number was significantly lower in cells with only GFI1 knockdown, comparing to control cells. However, overexpression of Bcl-xL in U937 and HL-60 cells with GFI1 knockdown reduced the difference in cell number comparing to cells without GFI1 knockdown, indicating Bcl-xL can rescue GFI1 knockdown U937 and HL-60 cells from DNA damage induced apoptosis. Notably, the rescue effect of Bcl-xL on GFI1 knockdown cells were not complete, suggesting other factors in play to mediate Gfi1 inhibition of DNA damage induced apoptosis.

We further assessed the effect of Bcl-xL knockdown on Gfi1 inhibition of DNA damage-induced apoptosis. Ba/F3 cells with inducible Gfi1 were infected with lentivirus containing Bcl-xL shRNA or control vector. Gfi1 expression was induced by Doxy treatment for 24 hours, followed by Doxo treatment. Cell viability was determined by trypan blue exclusion and Annexin V staining. As shown in Figure 3-12, the protective effect of Gfi1 on DNA damage induced apoptosis in cells with Bcl-xL knockdown was present, but not as significant as that in control cells, suggesting Bcl-xL is one of the factors
that mediates Gfi1 inhibition of DNA damage induced apoptosis. Together, these data indicates that Bcl-xL is partially responsible for Gfi1 inhibition of DNA damage induced apoptosis.

Figure 3-11: Overexpression of Bcl-xl rescues Gfi1 knockdown U937 and HL-60 cells from DNA damage induced apoptosis. U937 and HL-60 cells were infected with retrovirus containing MSCV-GFP vector or MSCV-GFP-BCL-xl expression construct. U937 and HL-60 Bcl-xl cells were obtained by sorting of GFP positive cells. U937 and HL-60 Ctr or Bcl-xl expressing cells were subsequently infected with lentivirus containing pLKO empty vector, Gfi1 shRNA #65 or #68. U937 WT or Gfi1 KD cells were treated with Doxorubicin (200ng/ml) for 16 h. Two-tailed Student’s t test used, p<0.05. (A) Living cell number of U937 cells were counted with trypan blue staining. (B) MTS assay of U937 cell were performed. (C) Living cell number of HL-60 cells were counted with trypan blue staining. (D) MTS assay of HL-60 cell were performed. (E) Expression of Gfi1 and Bcl-xl were examined by Western Blot.
Based on these data, we propose a signaling pathway which mediates apoptosis induced by overexpression of p53. p53 represses expression of Gfi1 which can inhibit apoptosis by upregulating Bcl-xL expression (Figure 3-13). Upon activation, p53 may induce apoptosis by repressing Gfi1 expression in hematopoietic cells. Our findings have provided new insights into the understanding of p53 transcriptional repression and the role of p53 in hematopoietic system.

Figure 3-12: Knockdown of Bcl-xL partially abolishes Gfi1 inhibition of DNA damage induced apoptosis. BaF3 rTA-GFP-Gfi1 cells were transduced with lentivirus containing Bcl-xL shRNA or scramble control. Cells were then exposed to Doxo (100 ng/ml) for 18 hrs with or without preincubation with Doxy for 24 hrs. (A) Percentages of apoptotic (annexin V-positive) cells were assessed by flow cytometry after staining with annexin V and 7-AAD. (B) Cell viabilities were determined by exclusion of trypan blue staining. (C) Knockdown of Bcl-xL in BaF3 rTA-GFP Gfi1 cells were examined by Western Blot.
3.2 Gfi1 upregulates c-Myc expression and inhibits c-Myc overexpression induced apoptosis

3.2.1 Overexpression of Gfi1 upregulates Myc protein and mRNA expression.

High level of Gfi1 accelerates T-cell proliferation and inhibits activation induced T cell death [28]. Likewise, c-Myc promotes cell proliferation by overcoming G1 phase and G1/S checkpoint cell cycle arrest by repressing transcription of CDK inhibitors including p15INK4B and p21CIP1. Gfi1 also cooperates with c-Myc to induce T-cell lymphoma [29]. To further understand the relationship between Gfi1 and Myc, we examined the role of Gfi1 in c-Myc expression. Gfi1 was induced with Doxy in Ba/F3 cells and Ramos cells, followed by Doxo treatment for 2, 4, 6 or 8 hours. Expression of c-Myc in Ba/F3 and Ramos cells was upregulated upon Gfi1 induction, while downregulated by Doxo treatment over time. Notably, c-Myc expression is prolonged with Gfi1 induction, suggesting Gfi1 upregulates c-Myc protein expression (Figure 3-14A and B).

To address whether Gfi1 upregulates c-Myc mRNA expression, real-time PCR was performed. As shown in Figure 3-14C and D, c-Myc mRNA expression is upregulated.
upon Gfi1 induction in Ba/F3 and Ramos, suggesting overexpression of Gfi1 upregulates c-Myc expression at mRNA level. Together, these data indicate that overexpression of Gfi1 upregulates Myc protein and mRNA expression.
3.2.2 Gfi1 knockdown or deficiency leads to downregulation of Myc protein and mRNA expression.

To further address the role of Gfi1 in c-Myc expression, we examined c-Myc expression in cells with GFI1 knockdown. U937 and HL-60 cells were infected with lentivirus containing GFI1 shRNA or control vector. Protein and mRNA expression of c-Myc was examined. As shown in Figure 3-15A and B, knockdown of Gfi1 leads to

Figure 3-15: GFI1 knockdown or deficiency leads to downregulation of Myc protein and mRNA expression. U937 and HL-60 cells were infected with lentivirus containing pLKO empty vector, Gfi1 shRNA #65 or #68. (A) Expression of Myc and Gfi1 was examined by Western Blot. (B) mRNA level of Myc was examined by Real-Time PCR. (C) Bone marrow mononuclear cells were extracted from femur and tibia of 8-12 weeks old C57BL/6J mice, followed by depletion of lineage positive cells. Expression of c-Myc in Gfi1 +/-, +/- and -/- mice was examined by real-time PCR.
downregulation of c-Myc protein and mRNA in both U937 and HL-60 cells. Additionally, c-Myc mRNA was examined in lineage depleted bone marrow cells from 8-12 weeks old C57BL/6J mice. Notably, Gfi1 deficiency in cells from Gfi1+/− or −/− mice is associated with decrease of c-Myc mRNA expression as compared to that in WT cells (Figure 3-15C). Together, these data indicate that knockdown or deficiency of Gfi1 downregulates expression of c-Myc protein and mRNA.

3.2.3 Gfi1 overexpression inhibits Myc induced apoptosis.

We addressed the role of Gfi1 in apoptosis induced by overexpression of c-Myc. Ba/F3 cells were transfected with Myc/estrogen receptor fusion protein MycER, which induces c-Myc overexpression upon 4-HT induction. Subsequently, Ba/F3 cells were transfected with lentiviral mediated Doxy inducible Gfi1 expression construct. Upon 4-HT induction, MycER is activated and transported from cytoplasm to nucleus, which induces apoptosis by overexpression. Cells were treated with Doxy for 24 hours, followed by 4-HT treatment. Cell viability was determined by trypan blue exclusion and Annexin V staining. Activation of MycER leads to gradual decline of cell viability due to increase of apoptosis in Ba/F3 cells, while induction of Gfi1 markedly protected cells from apoptosis (Figure 3-16A and B). Together, these data indicate that Gfi1 can inhibit apoptosis induced by overexpression of c-Myc.
Figure 3-16: Gfi1 overexpression inhibits Myc-induced apoptosis. Ba/F3 cells transfected with MycER and PMTrtTA-GFI1-GFP were treated with Doxycycline for 24 hours, followed by 4-HT treatment for 24 hours. (A) Cell Viability was examined by cell counting with trypan blue staining at 24 and 48 hours. (B) Cell Viability was analyzed by flow cytometry with Annexin V-PE staining by 24 hours. (C) Expression of MycER and Gfi1 was examined by Western blot.
3.2.4 Gfi1 abolishes Myc-mediated downregulation of Bcl-xL.

Expression of Bcl-xL protein and mRNA is inhibited by Myc activation in primary myeloid and lymphoid progenitors, as well as in precancerous B cells from Eμ-Myc transgenic mice. Additionally, the suppression of Bcl-xL by Myc is bypassed during lymphomagenesis [56]. We examined whether Gfi1 plays a role in Myc downregulation of Bcl-xL. Ba/F3 cells were transfected with MycER and inducible Gfi1. Cells were induced with Doxy for 24 hours, followed by 4-HT treatment to activate MycER. Interestingly, we show that Gfi1, which upregulates Bcl-xL, can abolish Myc-mediated downregulation of Bcl-xL (Figure 3-17A) in Ba/F3 cells. However, expression of Bax and Bcl-2 was not affected by Gfi1 induction or MycER activation.

Figure 3-17: Gfi1 abolishes Myc-mediated downregulation of Bcl-xL expression. Ba/F3 cells transfected with MycER and PMTrtTA-Gfi1-GFP were treated with doxycycline (1ug/ml) for 24 h, followed by 4-HT for 24 hours. (A) Expression of MycER, Gfi1 and Bcl2 family members was examined by western blot.
Chapter 4

Discussion

Gfi1 is a ZF transcriptional repressor that plays a central role in normal hematopoiesis [11, 19, 57]. Gfi1 regulates HSC self-renewal and restricts the proliferation of HSCs to preserve their function integrity [25, 58]. Gfi1 also promotes the differentiation of granulocytes and lymphocytes [30]. In addition to its role in normal hematopoiesis, Gfi1 plays an important role in hematopoietic malignancies. Gfi1 is a proto-oncogene that promotes cell proliferation by abolishing G1 cell cycle arrest and accelerating entry of S phase [11]. Gfi1 promotes cell survival in various cell lines and mouse models. Gfi1−/− mice have severely reduced thymocyte cellularity due to increased apoptosis during T cell development [23]. However, there is still a gap in the understanding of the role of Gfi1 in apoptosis and lymphomagenesis. In spite of its critical role in hematopoiesis and hematopoietic malignancies, very little is known about how Gfi1 expression is regulated in hematopoietic system.

Here we show that GFI1 transcription is repressed by p53 in hematopoietic cells. p53 inhibits expression of GFI1 protein and mRNA through binding GFI1 core promoter,
which contains a p53 response element essential for mediating p53 transcription repression identifying \textit{GFI1} as a target gene for p53 mediated transcriptional repression. Our data also demonstrate that Gfi1 upregulates expression of c-Myc and inhibits apoptosis induced by c-Myc overexpression and DNA damage. Interestingly, Gfi1 upregulates the expression of the anti-apoptotic Bcl-2 family member, Bcl-xL, and abolishes c-Myc downregulation of Bcl-xL expression. By abolishing c-Myc downregulation of Bcl-xL, Gfi1 contributes to bypassing this apoptotic pathway and lymphomagenesis [56]. These data have important implication for understanding the role of Gfi1 in normal hematopoiesis and lymphomagenesis.

\textbf{4.1 Gfi1 is repressed by p53 through direct DNA binding}

The tumor suppressor p53 is a transcription factor that is activated by cellular stresses such as DNA damage and oncogene stimulation. Upon activation, p53 may induce cell cycle arrest, DNA repair, cellular senescence or apoptosis. Thus, p53 acts to maintain genome stability and eliminate damaged or abnormally proliferating cells. Loss of p53 function is the most common event during tumorigenesis. The biological activities of p53 are mediated mainly through transcriptional regulation of its target genes. p53 has been shown to activate a wide range of target genes including those that exert a negative effect on cell proliferation and survival. In addition to transcriptional activation, p53 has been shown to repress a number of genes and recent studies indicate that transcriptional repression by p53 is required for its tumor suppressor function [53-55, 59]. However, much less is known about p53 transcription repression, comparing to transcription activation of p53.
An important mechanism by which p53 regulates gene expression is through direct binding to the REs in the promoters of its target genes. The canonical p53 RE involved in p53-mediated transcriptional activation has been defined as two 10-bp half-sites 5’-RRRCWWGYYY-3’ separated by a spacer of 0-13 bp (R is purine, Y is pyrimidine and W is A or T) with certain level of tolerance to mismatches from the consensus sequence [53-55]. Significantly less is known about the p53 REs involved in p53-mediated repression and a number of models of suppressive p53 REs have been proposed. Our data indicate that the 39-bp GFI1 core promoter is sufficient for p53 binding to and repression of GFI1. Interestingly, this core promoter region appears to contain the repressive p53 RE loosely defined as RRXCXXGXYX-XRXCXXGXYY (X can be A, C, G or T). However, mutation of the CGAG sequence in the downstream half-site, but not the CCCG sequence in the upstream half-site, abolishes p53 binding to and repression of GFI1. Thus, the precise sequence or motif of the repressive p53 RE in the GFI1 core promoter remains to be determined. Nonetheless, our data indicate that the 39-bp core promoter of GFI1 contains a p53 RE that is required for p53-mediated repression.

It is still unclear how p53 represses GFI1 transcription through the repressive p53 RE. Several models of p53 transcriptional repression have been proposed, some of which require direct binding of p53 to DNA of its target genes [59]. For example, p53 could bind to the promoter region of its target gene and displace a transcriptional activator which binds to adjacent region of p53 binding site, resulting in downregulation of target gene expression. Interestingly, after scrutinizing the promoter sequence of GFI1, we have discovered a potential binding site of E2F1 which overlaps with the p53 RE responsible for transcriptional repression. However, luciferase assay showed that mutation of E2F1
binding site does not affect $GFI1$ promoter activity (data not shown). Transcriptional repression by p53 may also occur through recruiting of HDACs, leading to subsequent modification of core histones and promoter closing. For example, p53 has been shown to recruit HDACs to its target genes such as $c$-$Myc$, $survivin$, $MAP4$, $Nanog$ and $Arf$ [53-55, 59, 60]. Therefore, treatment of TSA should abolish p53 repression of $GFI1$ promoter activity since TSA blocks transcription factors to access DNA inside chromatin by interfering the removal of acetyl group from histones. However, TSA only inhibits class I and class II of HDAC families, but not class III HDACs [61]. As shown in Figure 3-6A, Gfi1 promoter activity is still repressed by p53 overexpression, although upregulated by TSA treatment, suggesting the functions of class I and II HDAC family members are not required for p53-mediated repression of $GFI1$. The roles of class III HDAC family members in p53 repression of Gfi1 remain to be elucidated. Further studies are needed to elucidate the mechanism of p53 repression of $GFI1$ transcription.

4.2 Biological significance of Gfi1 repression of p53

p53 plays an important role in normal hematopoiesis. In the hematopoietic system, p53 is primarily expressed in hematopoietic stem cells, regulating quiescence and self-renewal of HSCs. p53 deficient mice have HSCs with elevated proliferation rate, leading to a 2-3 fold larger pool of HSCs. Total bone marrow cells from p53-null mice can easily outgrow their counterparts from wildtype mice in competitive bone marrow transplantation assay [62-65]. However, unregulated proliferation is detrimental to the long-term fitness of HSCs. Recipient mice of p53-null HSCs typically die much earlier of hematopoietic malignancies due to accelerated proliferation and accumulation of mutation [66]. On the contrary,
constitutive activation of p53 in mice leads to a drastic depletion of HSC population by impairing the stemness of HSCs [65, 67, 68].

Gfi1 has been shown to restrict proliferation and preserve the functional integrity of HSCs. Gfi1 can also inhibit apoptosis of HSCs [25, 50]. Our data show that expression of Gfi1 is repressed by p53 in myeloid, lymphoid cells and Lin- murine primary bone marrow cells. Therefore, in p53-deficient HSCs, Gfi1 expression is elevated, thus inhibiting apoptosis caused by accumulated mutations, contributing to the unregulated proliferation and, eventually, tumorigenesis. Although Gfi1 has restricting effect on proliferation of HSCs, more potent proliferation inhibitor like p21Cip1 has been downregulated in p53-deficient HSCs, which overcomes the effect caused by Gfi1 and leads to cell proliferation. On the other hand, Gfi1 expression is downregulated in HSC with constitutively activated p53. Therefore, the preservation of HSC functional integrity by Gfi1 is attenuated, contributing to the impaired stemness of HSCs. Downregulation of Gfi1 can also lead to increased apoptosis of HSCs, contributing to the depletion of HSCs. Together, our data have provided new insight into the understanding of p53 regulation of HSCs.

A previous report demonstrated p53 maintains HSC quiescence by upregulating Gfi1 [62]. However, total bone marrow from Gfi1-/- mice have severely impaired competitive long-term reconstituting abilities after transplantation [58]. As stated previously, bone marrow cells from p53-null mice have proliferative advantage compared to that from wildtype mice, indicating that Gfi1 and p53 has conflicting effect in HSCs. Thus, Gfi1 is more likely to be repressed instead of being activated by p53.

In addition to normal hematopoiesis, p53 also suppresses the development of hematopoietic malignancies. As a tumor suppressor, p53 is activated by oncogene
activation. For example, p53 is activated in response to activation of oncogene E1A and Ras [39]. Activated p53 functions as a tumor suppressor by inhibiting the expression of oncogenes. For example, p53 has been shown to downregulate c-Myc expression through binding to c-Myc promoter or induction of tumor suppressor miR-145 [44, 69]. Additionally, c-Myc has been shown to cooperate with Gfi1 in lymphomagenesis. Our findings have provided a new possibility that p53 suppresses lymphomagenesis through repression of Gfi1, thus inhibiting Gfi1 cooperation with c-Myc in lymphomagenesis.

4.3 Gfi1 inhibits DNA damage induced apoptosis independent of p53

Our data demonstrated that GFI1 acts to inhibit cell death induced by DNA damage, consistent with recent reports that Gfi1−/− HSCs and lymphoblastic leukemia cells exhibited increased rates of apoptosis in response to DNA damage [50, 70]. Consistent with this, it has been shown that Gfi1 restricts p53-dependent DNA damage response, which may be responsible for its anti-apoptotic activity [71]. However, our data suggest that the protective effect of Gfi1 is not dependent on p53 as Gfi1 also protects cells lacking functional p53 from DNA damage-induced cell death. Thus, it is possible that upon activation by DNA damage, p53 may induce apoptosis in part through repression of GFI1. Additionally, we also demonstrated that overexpression of Gfi1 has no significant effect on the expression of Bcl-2 family members which had been implicated as Gfi1 target genes including Bax and Bak.

51
4.4 Gfi1 cooperates with c-Myc in lymphomagenesis by upregulating c-Myc expression and inhibiting c-Myc overexpression induced apoptosis.

Gfi1 has been shown to cooperate with c-Myc in regulating cell proliferation and lymphomagenesis. However, the underlying molecular mechanism remains poorly understood. Gfi1, together with c-Myc and Miz1, can form a ternary complex which binds to the promoter region of CDKN1A and CDKN2B through Miz1 to inhibit Miz1 activation of the expression of the two genes [17, 18]. Subsequently, this promotes cell proliferation by overriding the cell cycle arrests caused by CDK inhibitors p21\(^{Cip1}\) and p15\(^{INK4B}\).

Myc is frequently activated by overexpression in cancers. For example, overexpression of c-Myc is the primary cause of lymphoma. Here we demonstrate that overexpression of Gfi1 augments the expression of c-Myc. In contrast, decreased level of Gfi1 expression is accompanied by downregulation of c-Myc. In addition, expression of c-Myc mRNA is decreased in Lin- murine primary bone marrow cells from Gfi1\(^{-/-}\) mice, comparing that from WT mice. Together, these findings reveal an important role of Gfi1 in the regulation of c-Myc expression.

In addition to proliferation and tumorigenesis, overexpression of c-Myc can also lead to apoptosis, providing a fail-safe mechanism for c-Myc induced tumorigenesis. c-Myc has been reported to induced apoptosis through extrinsic and intrinsic pathways. The extrinsic pathway involves c-Myc sensitization of cells to apoptosis induced by ligand binding to death receptors like CD95/Fas [72]. In the intrinsic pathways, c-Myc can induce apoptosis by upregulating ARF and, subsequently, p53 expression to induced apoptosis [32].
Alternatively, c-Myc can induce apoptosis through regulating the expression of Bcl-2 family members in mitochondria to induce apoptosis [34, 56]. However, c-Myc induced apoptosis is often inhibited during lymphomagenesis. Our data indicated that Gfi1 can inhibit apoptosis induced by c-Myc overexpression.

As a transcriptional repressor, Gfi1 mainly functions through repressing the expression of its target genes. Gfi1 may indirectly upregulate c-Myc by downregulating a gene which represses c-Myc expression. Additionally, Gfi1 have been shown to regulate miR-21 and miR-196b to control myelopoiesis [73]. Therefore, Gfi1 may also upregulate microRNAs that affects c-Myc 3’UTR. To elucidate the mechanism, we inserted c-Myc promoter or 3’UTR region into luciferase constructs. However, with overexpression of Gfi1, we were unable to observe any change in the luciferase activity from c-Myc promoter or 3’UTR region. Further experiments are needed to elucidate the mechanism by which Gfi1 upregulates c-Myc expression.

Based on these findings, we propose a potential mechanism by which Gfi1 cooperate with c-Myc in lymphomagenesis. Gfi1 promote c-Myc expression, elevating the risk of c-Myc induced transformation. Meanwhile, Gfi1 inhibits apoptosis caused by c-Myc overexpression. Thus, the presence of Gfi1 leads to more effective cell transformation by overexpression of c-Myc (Figure 4-1).
4.5 Gfi1 upregulates Bcl-xL expression to inhibit apoptosis induced by DNA damage and Myc overexpression.

The Bcl-2 family members are vital regulators of apoptosis, including pro-apoptotic members such as Bax, Bak and Bad, as well as anti-apoptotic members like Bcl-2. Gfi1 has been shown to repress the pro-apoptotic Bcl-2 family members Bax and probably also Bak [48, 49]. In line with these studies, Bax expression was significantly augmented following irradiation in Gfi1−/− HSCs and thymocytes [50, 70]. In contrast, the expression of the anti-apoptotic Bcl-2 appeared to be downregulated in Gfi1−/− T cells and HSCs [23, 50].
Here we demonstrated that Bcl-xL is upregulated by Gfi1. Overexpression of Gfi1 upregulates Bcl-xL protein and mRNA level. Knockdown of Gfi1 leads to downregulation of Bcl-xL protein and mRNA expression. Furthermore, expression of Bcl-xL is downregulated in Lin- bone marrow cells from Gfi1+/- and -/- mice, as compared to that in WT mice. Together, these findings provide solid evidence that Gfi1 can upregulate Bcl-xL expression.

Our data also indicate that upregulation of Bcl-xL is responsible for Gfi1 inhibition of apoptosis induced by DNA damage and c-Myc overexpression. Overexpression of Bcl-xL reduces the sensitivity of Gfi1 knocked down cells to DNA damage-induced apoptosis. In contrast, knockdown of Bcl-xL partially abolishes Gfi1 inhibition of apoptosis induced by DNA damage, indicating that upregulation of Bcl-xL contributes to Gfi1 protection of DNA damage induced apoptosis. In cells transfected with inducible Gfi1 and MycER expression construct, induction of Gfi1 leads to upregulation of Bcl-xL and increased viability of 4-HT treated cells. We have also shown that Bcl-xL expression is downregulated in Lin- bone marrow cells from Gfi1-/- mice, suggesting that Bcl-xL may play a role in Gfi1 protection of HSCs against apoptosis. Together, these data suggest Bcl-xL the key player in mediating Gfi1 inhibition of apoptosis of hematopoietic cells. Our findings provide a possible explanation for the roles of Gfi1 in hematopoiesis. However, our data indicate that Bcl-xL is partially responsible for mediating Gfi1 inhibition of DNA damage induced apoptosis. Further studies are needed to examine whether other factor are also in play.

Bcl-xL expression has been reported to correlate with p53 status and sensitivity to apoptosis in ALL cells. Loss or mutation of p53 is associated with higher level of Bcl-xL
in multiple ALL cell lines, suggesting Bcl-xL plays a role in p53 induced apoptosis [74]. However, Bcl-xL is not a known p53 target gene. Our data show that GFI1 is repressed by p53, while Bcl-xL is upregulated by Gfi1 and mediates Gfi1 inhibition of apoptosis in hematopoietic cells. Together, our data suggest a potential mechanism by which p53 inhibits Bcl-xL expression through repressing the expression of Gfi1.

C-Myc has been shown to downregulate the expression of Bcl-xL and induce apoptosis [56]. Interestingly, our data show that overexpression of Gfi1 abolishes C-Myc downregulation of Bcl-xL expression and inhibits C-Myc induced apoptosis. It is possible that Gfi1 collaborates with C-Myc in lymphomagenesis in part through upregulation of Bcl-xL expression.

The expression of Bcl-xL mRNA is upregulated by overexpression of Gfi1, suggesting Gfi1 regulates Bcl-xL expression at RNA level. However, the mechanism by which Gfi1 upregulates Bcl-xL is not clear. Previous reports have shown that Bcl-xL is regulated by multiple transcription factors including ETS, NFκB, STAT and AP1 through Bcl-x promoter [75]. As a transcriptional repressor, Gfi1 may indirectly upregulate Bcl-xL by inhibiting the expression of a negative regulator of Bcl-xL. We have cloned a 3.6kb Bcl-xL promoter fragment into reporter construct. However, luciferase assay have shown that Bcl-x promoter activity does not change upon Gfi1 overexpression in hematopoietic cells (Figure 4-2A). Additionally, Gfi1 have been shown to regulate miR-21 and miR-196b to control myelopoiesis [73]. We have cloned the 3’UTR region of Bcl-x into luciferase reporter construct and observed no change in reporter activity, indicating that Gfi1 does not upregulating Bcl-xL expression through the 3’UTR region of Bcl-x (Figure 4-2B). Further studies are needed to understand how Gfi1 upregulate Bcl-xL.
Figure 4-2: Gfi1 does not upregulate Bcl-xL expression through Bcl-xL promoter or 3’UTR region. (A) Ba/F3 PMTrtTA-Gfi1-GFP were transfected with Bcl-xL promoter (-3.6/1.6 kb) luciferase construct, followed by doxycycline (1ug/ml) treatment after 2 hrs. Luciferase activity was measured after 24 hrs and normalized for protein concentration. (B) Ramos PMTrtTA-Gfi1-GFP cells were transduced with lentivirus reporter containing Bcl-xL 3’UTR region, followed by doxycycline (1ug/ml) treatment after 24 hrs. Luciferase activity was measured after 24 hrs and normalized for protein concentration.
Chapter 5

Future Directions

5.1 Explore the mechanism of Gfi1 upregulation of Bcl-xL and c-Myc.

We will continue examine for potential Gfi1 binding site on Bcl-xL and Myc promoter region and loci. We will also examine possible microRNAs which mediates Gfi1 repression of 3’UTR. In addition, we will explore the effect of DNA methylation and acetylation on repressing Bcl-xL or c-Myc expression.

5.2 Examine the effect of Gfi1 on c-Myc induced apoptosis in primary cells.

We will extract bone marrow mononuclear cells from femur and tibia of 8-12 weeks of Gfi1 +/+, +/- or -/- C57BL/6J mice and isolate hematopoietic stems cells or early lymphoid progenitor cells. The HSCs or pro-B cells will be infected with retrovirus containing MycER expression construct. Subsequently, infected cells will be sorted for RFP+ and treated with 4-HT to activated MycER and cell viability will be examined by
Annexin V staining. The effect of Gfi1 deficiency on Myc induced apoptosis will be evaluated.

5.3 Examine whether overexpression of Bcl-xL rescues the phenotype in Gfi1 knockout mice.

We have demonstrated Gfi1 inhibit apoptosis partially through upregulation of Bcl-xL. Gfi1−/− mice have distinct phenotypes including reduced size of thymus due to apoptosis [50]. We will examine whether overexpression of Bcl-xL in thymus of Gfi1−/− mice could abolish this phenotype.

5.4 Examine whether overexpression of Bcl-xL inhibits c-Myc induced apoptosis in primary cells.

We will extract bone marrow mononuclear cells from femur and tibia of 8-12 weeks of Gfi1 +/+, +/- or −/− C57BL/6J mice and isolate hematopoietic stems cells or early lymphoid progenitor cells. The HSCs or pro-B cells will be infected with retrovirus containing MycER and Bcl-xL expression construct. Subsequently, cells will be sorted for RFP+ (MycER) and GFP+ (Bcl-xL). Sorted cells will be treated with 4-HT to induced MycER. Cell viability will be examine by Annexin V staining. We will evaluated the effect of Bcl-xL overexpression on Myc induced apoptosis in Gfi1-deficient cells.
References


## Appendix A

### List of Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>MO7e</td>
<td>Human Megakaryoblastic Leukemia cell line</td>
</tr>
<tr>
<td>Molt3</td>
<td>Human T leukemia cell line</td>
</tr>
<tr>
<td>U937</td>
<td>Human Leukemic Monocytic Lymphoma cell line</td>
</tr>
<tr>
<td>HL-60</td>
<td>Human Promyelocytic Leukemia cell line</td>
</tr>
<tr>
<td>HCT116</td>
<td>Human Colorectal Cancer cell line</td>
</tr>
<tr>
<td>Ba/F3</td>
<td>Immortalized Murine Pro-B cell line</td>
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<tr>
<td>Ramos</td>
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