TARGETING THE EPIGENETIC MECHANISM IN ACUTE MYELOID LEUKAEMIA

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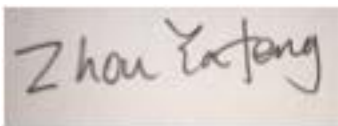
To them all, I dedicate this thesis.

Declaration

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



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Summary

Target therapy has always been the focus of studies of therapeutic approaches in cancer, especially in treatment of AML. Although chemotherapy and stem cell transplantation are current first line therapy in treating AML, new therapies with more accurate targeting and fewer side effects are required to improve the prognosis of AML patients. Therefore, the new small molecule drug, JQ1, showing anti-tumor effect through inhibiting the epigenetic reader BRD4, was considered a promising drug in multiple types of cancers including AML. JQ1 has been shown to induce cell cycle arrest in different cancers through inhibiting the well-known oncogene, MYC. However, other mechanisms of JQ1 have not been well studied in AML.

In this study, I found that JQ1 is able to induce cell death in AML cells through activating the apoptotic pathway. The connection between JQ1 and apoptosis is a tumor suppressor TXNIP, which has been known to regulate glucose metabolism and redox homeostasis. After JQ1 treatment, TXNIP was elevated, inducing apoptosis through activating the ASK1-MAPK pathway in mitochondria. Furthermore, I found the up-regulation of TXNIP level under JQ1 was due to the key regulator MYC. Further studies confirmed that MYC could repress the expression of TXNIP through miR-17-92 cluster. Depleted expression of MYC after JQ1 treatment also led to decreased miRNAs from miR-17-92 cluster, which released the expression of TXNIP and finally led to apoptosis.

These findings demonstrated that JQ1 is able to induce cell death in AML. The connection between MYC and TXNIP enhanced our understanding of the MYC regulatory network, while reactivation of TXNIP was demonstrated to be effective in treating AML. All together, these results provide valuable data in our fight against AML.

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List of Abbreviations

AML	acute myeloid leukemia
ALL	acute lymphoid leukemia
APL	acute promyelocytic leukemia
ASK1	apoptosis signal-regulating kinase 1
BRD4	bromodomain containing protein 4
Caspase	cysteine-aspartic proteases
CDK	cyclin-dependent kinases
CDKN2A	cyclin-dependent kinase inhibitor 2a
ChIP	chromatin immunoprecipitation
CLP	common lymphoid progenitor
CML	chronic myeloid leukemia
CMP	common myeloid progenitor
CTD	C-terminal domain
C/EBPA	CCAAT/enhancer binding protein alpha
DMSO	dimethyl sulfoxide
DNMT	DNA methyltransferase
DZNep	3-deazaneplanocin A
ERK	extracellular signal-regulated kinases
EZH2	enhancer of zeste 2
FISH	fluorescence in situ hybridization
FDA	Food and Drug Administration
FBS	fetal bovine serum
HSC	hematopoietic stem cell
HAT	histone acetyltransferase
HDAC	histone deacetylase
HEXIM1	hexamethylene bis-acetamide inducible 1
IL	interleukin
JAK	janus kinase

JNK	c-Jun N-terminal kinase
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinases
MAX	MYC associated factor X
MEK	mitogen-activated protein kinase kinase
MEMα	minimum essential media alpha
MIZ1	MYC-interacting zinc finger protein 1
MLL	mixed-lineage leukemia
MPN	myeloproliferative neoplasms
MYC	v-myc avian myelocytomatosis viral oncogene homolog
NPM	nucleophosmin
NLRP3	NACHT, LRR and PYD domains-containing protein 3
NMC	NUT midline carcinoma
PCR	polymerase chain reaction
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Tween-20
PI3K	phosphoinositide 3-kinase
PRC2	polycomb repressive complex 2
PRMT	protein arginine methyltransferase
P-TEFb	positive transcription elongation factor b
Pol II	RNA polymerase II
RAS	GTPase KRas
RAF	RAF proto-oncogene serine/threonine-protein kinase
RBC	red blood cell
RPMI	Roswell Park Memorial Institute medium
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription ploymerase chain reaction
RUNX1	Runt-related transcription factor 1
SCF	stem cell factor

shRNA	short hairpin RNA
siRNA	small interfering RNA
TRX	thioredoxin
TXNIP	thioredoxin-interacting protein
TRED	transcriptional regulatory element database
WHO	World Health Organization

Chapter 1: Introduction

1.1 Acute Myeloid Leukemia

1.1.1 Hematopoiesis

Hematopoiesis is the formation of cellular components in blood. During hematopoiesis, mature blood cells carrying various functions were differentiated from a very small population of pluripotent hematopoietic stem cells (HSCs)(Forsberg, Bhattacharya, & Weissman, 2006). As mature blood cells only have a very short life span, they must be replaced continuously. In a healthy adult person, approximately 10^{11}-10^{12} new blood cells are produced every day so as to maintain steady peripheral circulation(Medvinsky, Rybtsov, & Taoudi, 2011).

Hematopoiesis starts from the expansion and differentiation of pluripotent hematopoietic stem cells, which are characterized by their ability to self-renew and differentiate into all other kinds of blood cells(Morrison & Kimble, 2006). The HSCs are derived from ventral mesoderm during embryonic development and enriched in yolk sac, fetal liver and other sites to begin the production of blood stem cells in infants(Fernández & de Alarcón, 2013). However, in adults, the primary and most important hematopoiesis site is the bone marrow(Orkin & Zon, 2008).

In the bone marrow, HSCs go through sequential differential processes and finally form mature blood cells(Doulatov, Notta, Laurenti, & Dick, 2012). Firstly, the multipotential hematopoietic stem cells differentiate into common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs), which later develop into myeloid line and lymphoid line blood cells,

respectively. The CLP cells develop into pro-B cells and pro-T cells and eventually mature B-lymphocytes and T-lymphocytes, while the CMP cells differentiate into megakaryocyte-erythroid progenitors (MEPs) and granulocyte-macrophage progenitors (GMPs). These two progenitors then develop into red blood cells (RBCs), platelets, macrophages, etc. and finally enter the peripheral circulation.

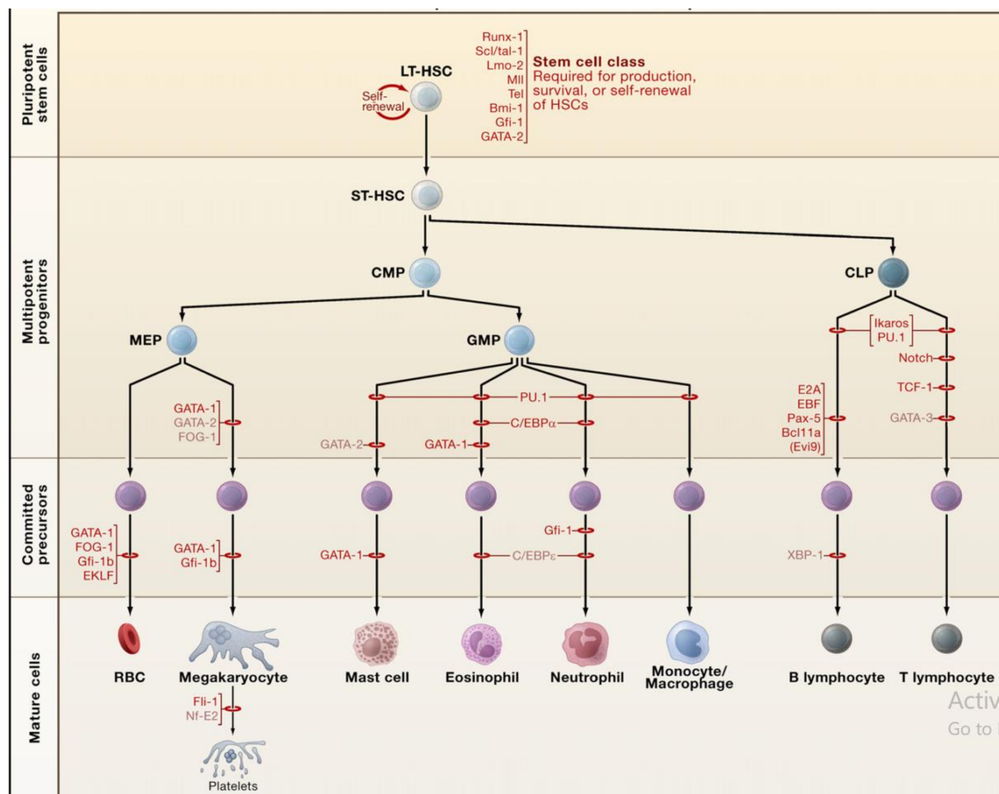


Figure 1.0.1 Hematopoiesis from HSC to mature cells. modified from Orikin *et al*. Cell. 2008.

The production of blood cells is tightly controlled during hematopoiesis and requires the participation of many factors(Broudy, 1997). The proliferation and self-renewal of HSCs are regulated by growth factors like stem cell factor (SCF), absence of which is lethal. During differentiation, interleukins like IL-2, IL-3, IL-6, IL-7 and colony-stimulating factors like GM-CSF, G-CSF and M-CSF are all important regulators that either regulate proliferation or stimulate

differentiation. These growth factors could initiate signal transduction pathways that lead to activation of multiple transcription factors.

The activated transcription factors then regulate the hematopoiesis through activating or silencing certain downstream target genes(Jiang Zhu & Emerson, 2002). One important transcription factor is CCAAT-enhancer binding protein alpha (C/EBPA), activation of which stimulates the HSCs to differentiate into multipotent progenitors and be prepared for further differentiation. Next, the levels of two main transcription factors, Pu.1 and GATA-binding factor 1 (GATA-1), determine whether these multipotent progenitors give rise to myeloerythroid or myelolymphoid lineages. Other transcription factors, such as Runt-related transcription factor 1 (RUNX1), Growth factor independent 1 transcription repressor (Gfi-1), Ikaros family zinc finger protein 1 (Ikaros), also play important roles in the development of multiple blood cell lineages.

1.1.2 Epidemiology and diagnosis of AML

Acute Myeloid Leukemia (AML) is the malignancy of myeloid line of blood cells, characterized by the uncontrolled expansion of immature myeloid lineage of blood cells in the bone marrow and blood(Löwenberg, Downing, & Burnett, 1988). The accumulation of immature blood cells causes the reduction of normal blood cells like red blood cells and platelets and finally leads to mortality. If left untreated, AML can cause rapid death within several months.

The American Cancer Society estimates that about 54, 270 new cases of leukemia will occur in the United States for 2015 and about 20,830 new cases will be AML and mostly in adults. Despite being a relative rare cancer type, this disease takes a large number of lives every year. The estimated death from AML in 2015 is 10,460. What is worse, patients with AML have a poor five-year survival rate as low as 25%, making it a malignant disease(American Cancer Society, 2015).

Age is one of the most important prognostic factors in AML(van Putten & Löwenberg, 1997). The median age of AML patients at diagnosis is 67 years in the United States(Siegel, Miller, & Jemal, 2015). For patients who are under 60, the five-year survival rate is about 30% -35% while for patients older than 60, the five-year survival rate is even lower than 10%(Howlader et al., n.d.; Montalban-Bravo & Garcia-Manero, 2014). Recurrent chromosome rearrangement is another important prognostic factor(van Putten & Löwenberg, 1997; Zeisig, Kulasekararaj, Mufti, & So, 2012). Genetic changes like t (8; 21), t (15; 17), and inv (16) usually associate with relatively favorable prognosis. Patients with these chromosome rearrangements respond much better to chemotherapy, with a complete remission rate around 90 % and a five-year survival rate higher than 60 %(Calgb et al., 2002; Grimwade et al., 1998). However, the presence of poor-risk genetic arrangements, such as complex karyotype, -5, del (5q), or abnormalities of 3q, usually associates with an adverse prognosis and the long-term survival rate may be less than 10%. Patients carrying other chromosome changes are classified as intermediate risk group(Breems et al., 2005; Slovak et al., 2009). Other

factors, such as mutations in FLT3, C/EBPA, NPM or TET2 also affect the prognosis of AML patients(Barjesteh van Waalwijk van Doorn-Khosrovani et al., 2003; Cazzaniga et al., 2005; Chou et al., 2011; Fröhling et al., 2004; Gaidzik et al., 2012; Metzeler et al., 2011; Schnittger et al., 2002; C Thiede et al., 2002; Christian Thiede et al., 2006; Verhaak et al., 2005; Weissmann et al., 2012). The incidence rates of developing AML is higher in men than in women, with a ratio about 1.4: 1(Siegel et al., 2015).

Table 1 Cytogenetic risk groups and molecular abnormalities

Favorable	Intermediate	Adverse
t(8;21) t(15;17) inv(16) Normal cytogenetics with NPM1 mutation or CEBPA mutation in absence of FLT3-ITD mutation	Normal +8 +21 +22 del(7q) del(9q) Abnormal 11q23 and all other structural/numerical abnormalities t(8;21), inv(16), or t(16;16) with c-KIT mutation	-5 -7 del(5q) Abnormal 3q Complex Normal cytogenetics with FLT-ITD mutation

Clinically, AML patients often show diverse signs and symptoms, usually as a result of leukemic infiltration of the bone marrow. Common symptoms in AML patients include fatigue, anemia, easy bleeding and increased risk of infection, whereas leukemic infiltration of the liver, spleen, lymph nodes or bone could produce a variety of other symptoms(Hoffman, 2005).

For those suspected to have AML, a bone marrow aspirate is usually the first step in diagnosis, allowing for morphological examination to identify blast cells and differentiation arrest. Often the bone marrow sample is also sent for immunophenotyping using flow cytometry. Flow cytometry allows the identification of the expression of different surface and cytoplasmic antigens on the leukemia cells as well as abnormal differentiation patterns of the blood cells. Together with morphological assessment, this will allow the confirmation of the lineage and type of leukemia. Rarely, the leukemia cells may express antigens of different lineages, representing a rare subtype of leukemia called the mixed phenotype acute leukemia (MPAL). As chromosome abnormalities are commonly detected in adult AML, cytogenetic analysis is mandatory for patients suspected with acute leukemia(Döhner, Estey, Amadori, Appelbaum, Büchner, Burnett, Dombret, Fenaux, Grimwade, Larson, et al., 2010; Estey, 2012). According to the World Health Organization (WHO) classification, seven recurrent translocations and inversions were listed in “AML with recurrent genetic abnormalities”(Vardiman, Harris, & Brunning, 2002; Yin, Medeiros, & Bueso-Ramos, 2010).

Table 2 FAB classification of AML

FAB subtype	Description	Comments
M0	Undifferentiated	Myeloperoxidase negative; myeloid markers positive
M1	Myeloblastic without maturation	Some evidence of granulocytic differentiation

M2	Myeloblastic with maturation	Maturation at or beyond the promyelocytic stage of differentiation; can be divided into those with t(8;21) AML1-ETO fusion and those without
M3	Promyelocytic	APL; most cases have t(15;17) PML-RARα or another translocation involving RARα
M4	Myelomonocytic	
$M4_{Eo}$	Myelomonocytic with bone-marrow eosinophilia	Characterized by inversion of chromosome 16 involving CBFβ, which normally forms a heterodimer with AML1
M5	Monocytic	
M6	Erythroleukemia	
M7	Megakaryoblastic	GATA1 mutations in those associated with Down's syndrome

Table 3 WHO classification of AML

AML with certain genetic abnormalities
AML with a translocation between chromosomes 8 and 21
AML with a translocation or inversion in chromosome 16
AML with a translocation between chromosomes 9 and 11
APL (M3) with a translocation between chromosomes 15 and 17
AML with a translocation between chromosomes 6 and 9
AML with a translocation or inversion in chromosome 3
AML (megakaryoblastic) with a translocation between chromosomes 1 and 22
AML with myelodysplasia-related changes
AML related to previous chemotherapy or radiation
AML not otherwise specified
AML with minimal differentiation (M0)

AML without maturation (M1)
AML with maturation (M2)
Acute myelomonocytic leukemia (M4)
Acute monocytic leukemia (M5)
Acute erythroid leukemia (M6)
Acute megakaryoblastic leukemia (M7)
Acute basophilic leukemia
Acute panmyelosis with fibrosis

Besides conventional cytogenetic analysis, fluorescence *in situ* hybridization (FISH) is another option when the former one failed, or to identify MLL fusion partners in 11q23 translocations(Coleman, Theil, Tubbs, & Cook, 2011; Gulley, Shea, & Fedoriw, 2010). The advantage of FISH is that it could be performed using fixed cell pellets instead of fresh blood. For cells carrying mutations on FLT-3, or IDH, or NPM1, or C/EBPA, reverse transcription polymerase chain reaction (RT-PCR) is the most sensitive assay to detect these mutations(Ahn, Seo, Weinberg, Boyd, & Arber, 2009; Bianchini et al., 2003; Elsayed, Nassar, Zaher, Elnoshokaty, & Moneer, 2014; Hollink et al., 2011; Kassem et al., 2011; Mark Levis, 2013; Quan et al., 2015). Besides these methods, DNA sequencing is a powerful tool to demonstrate subtle mutations and provide evidence for more personalized therapy(Braggio, Egan, Fonseca, & Stewart, 2013; Ilyas et al., 2015; Kohlmann et al., 2014; Luthra et al., 2014).

1.1.3 Conventional therapy

The current therapy of AML usually consists of two phases, the induction phase and the consolidation phase(Roboz, 2012). In the induction phase, the goal is to kill the leukemic cells in the blood and bone marrow, which puts the leukemia into remission. After induction treatment, up to 70% patients could achieve a complete remission(Roboz, 2011). However, the remission does not mean the disease has been cured; it only indicates that no leukemic cells could be detected in blood and bone marrow with current diagnostic methods. Usually a small number of leukemic cells persist after the induction therapy and almost all patients will relapse within several months without further treatment. Therefore, consolidation treatment after remission is necessary. The goal of consolidation treatment is to eliminate any remaining leukemia cells at inactive status and therefore prevents the relapse of disease. This phase is crucial in decreasing the risk of relapse. The specific consolidation therapy is usually based on the risk groups. For patients in the low risk group, usually chemotherapy alone is enough, while for patients in the normal and high risk groups, stem cell transplantation is often required(Döhner, Estey, Amadori, Appelbaum, Büchner, Burnett, Dombret, Fenaux, Grimwade, & Larson, 2010).

1.1.4 Chemotherapy

Chemotherapy is the current standard treatment for most subtypes of AML. In chemotherapy, cytotoxic drugs are used to stop the continuous growth of

cancer cells, either through inducing cell death or through interrupting the cell division. The most common regimen in the induction therapy is the combination of cytarabine and anthracycline or anthracenedione, known as "7+3". About 60-65% patients aged 60 years or younger could achieve remission after no more than two courses of therapy(Roboz, 2012; Zeisig et al., 2012).

However, the specific treatment for each patient may be different as younger patients and older patients respond differently to chemotherapy. For example, studies showed that patients younger than 60 could benefit from high dose of daunorubicin while high-dose of cytarabine showed no better outcome(J. H. Lee et al., 2011). In consolidation therapy, high-dose of cytarabine is often used for several cycles to achieve complete remission in younger patients. For older people who may not be able to tolerate intensive chemotherapy, 1-2 cycles of lower dose cytarabine are given(Bradstock, 2006; Döhner, Estey, Amadori, Appelbaum, Büchner, Burnett, Dombret, Fenaux, Grimwade, & Larson, 2010).

Besides cytotoxic drugs, other small molecular drugs are also used in the treatment of AML. For example, Decitabine is a hypomehylating agent approved by the US Food and Drug Administration (FDA) for the treatment of myelodysplastic syndromes (MDS) and AML(Ritchie & Feldman, 2013). It could hypomethylate DNA by inhibiting DNA methyltransferase. Decitabine treatment in patients older than 60 years with either *de novo* AML or AML arising out of MDS results in a remission rate at 78%, including complete

remission and partial remission(Malik & Cashen, 2014). However, a similar study showed no significant advantage against traditional chemotherapy(H. Kantarjian, Wilson, Ravandi, & Estey, 2013).

Clofarabine is a drug approved by the US FDA for the treatment of relapsed or refractory pediatric acute lymphoblastic leukemia (ALL)(Barba et al., 2012; McGregor, Brown, Osswald, & Savona, 2009). This drug must be used after at least two other types of treatment have failed and not many evidence have shown much benefits from using clofarabine alone. However, studies in patients older than 50 years with newly diagnosed AML showed that combination of Clofarabine and Cytarabine produced a good complete response rate at 52%(Burnett et al., 2013; Faderl, 2006; Tiley & Claxton, 2012).

1.1.5 Stem cell transplantation

Stem cell transplantation, also called bone marrow transplantation, is often considered in consolidation therapy for younger patients(Döhner, Estey, Amadori, Appelbaum, Büchner, Burnett, Dombret, Fenaux, Grimwade, & Larson, 2010; Zeisig et al., 2012). During stem cell transplantation, the patient's bone marrow is replaced by healthy hematopoietic stem cells (HSCs), which allows the re-establishment of healthy hematopoietic system. An intensive chemotherapy is given before transplantation to completely destroy the bone marrow generating leukemia cells. Then HSCs are infused into the patient through a central line and migrate to bone marrow and begin

the hematopoiesis. The HSCs used could be from a compatible donor or from patients' own stem cells, which may be stored when they reached remission previously.

The stem cell transplant therapy is usually considered for younger patients, as elderly patients, especially those who are older than 40 years, bear higher risk of death from such surgery, which may due to the intensive chemotherapy required prior to transplantation being intolerable for them(Niewerth, Creutzig, Bierings, & Kaspers, 2010). Considering that the median age of patients with AML is 67 years, only a minority of AML patients is suitable for such therapy. This therapy is also largely limited by the availability of suitable donors(Sierra & Djulbegovic, 2011).

1.2 Target therapy

Although widely used, the toxicity of chemotherapy is a major problem, especially for those patients with older age or poor health condition. Most chemotherapy drugs attack all cells undergoing rapid division, thus also damaging dividing cells in normal tissues such as hair, lining of the mouth, intestines. As a result, side effects of chemotherapy include hair loss, mouth sores, nausea, and vomiting. Increased bruising, bleeding, and fatigue are also common side effects as normal bone marrow cells are also rapidly dividing and thereby, destroyed during chemotherapy.

Compared to young patients, the elder patients usually suffer more from chemotherapy and they are less able to tolerate stem cell transplantation. The complete remission rate is only 47% for patients older than 60 years after standard chemotherapy. About 30% of patients die of aplastic anemia, and only 9% of patients are alive after 4 years. Even worse, many patients never received chemotherapy due to their serious medical conditions and also the poor prognosis of chemotherapy in elderly patients(Eleni, Nicholas, & Alexandros, 2010; Krug, Büchner, Berdel, & Müller-Tidow, 2011). In Menzin *et al* ‘s study, approximately 90% of patients older than 60 years stayed in the hospital for about one third of their remaining days while only 30% of them received chemotherapy(Menzin, Lang, Earle, Kerney, & Mallick, 2002). Thus, novel treatments need to be developed especially for this patient population.

Targeted therapy is a type of treatment using drugs or other substances to identify and attack specific cancer cells while doing minimal damage to normal cells(Aggarwal, 2010). As these drugs could identify specific parts of cancer cells at the molecular level, they often have less severe side effects and thus are promising in the treatment of AML. Two most commonly studied classes of targeted therapies are monoclonal antibodies (mAb) and small molecule drugs(M. Huang, Shen, Ding, & Geng, 2014; Society, 2013).

Monoclonal antibodies are produced in monoclonal B lymphocytes using cancer cell specific antigens, allowing them to recognize either cancer cells or signal proteins helping cancer cells to grow. These antibodies then attach to their antigens and either induce immune responses against the target cancer

cells or block the signal transduction pathway that stimulates cancer growth. It is also possible to modify these mAbs to deliver a toxin, radioisotope, cytokine or other active conjugate that directly kills the cancer cells(Chames, Van Regenmortel, Weiss, & Baty, 2009; Waldmann, 2003).

The first targeted cancer drug approved in FDA history is a monoclonal antibody called rituximab. It binds to the surface marker protein CD20 and activates the immune response executed by natural killer cells, which results in elimination of these target cells. As CD20 is mainly found on the cellular membrane of B cells, rituximab is used in the treatment of cancers of the white blood system like non-Hodgkin's lymphoma(Dotan, Aggarwal, & Smith, 2010; Plosker & Figgitt, 2003).

Small molecule drugs are usually synthesized using chemical methods. They interact with proteins that are critical for cancer cells and block their functions, eventually leading to cell death through apoptosis or necrosis(M. Huang et al., 2014).

The most well-known target drug in cancer treatment is imatinib, which targets the Philadelphia chromosome in chronic myeloid leukemia (CML)(Capdeville, Buchdunger, Zimmermann, & Matter, 2002; Deininger, Buchdunger, & Druker, 2012). Philadelphia chromosome means a t (9; 22) translocation, resulted in a fusion protein called BCR-ABL, which is the key oncogenic factor in CML(Koretzky, 2007; Kurzrock, Kantarjian, Druker, & Talpaz, 2003). Imatinib is a small molecule aiming to bind to the BCR-ABL protein and inhibit its tyrosine kinase activity. It is not only the first small

molecular target drug in cancer treatment, but also demonstrates the possibility of targeting tyrosine kinase using chemically synthesized small molecules. The US FDA approved imatinib for the treatment of CML just after three months of review, which is the fastest approval in FDA history. Imatinib soon became the standard treatment for CML patients harboring t (9; 22) translocation(An et al., 2010; Deininger et al., 2012). Later, imatinib was also found to be effective in other types of cancer like gastrointestinal stromal tumor with KIT mutation, acute lymphoblastic leukemia with Philadelphia chromosome, etc.(Growney et al., 2005; Rosenberg, 2005)

As AML is a highly heterogeneous malignancy, there are numerous reported potential targets in AML, either mutated genes or aberrant pathways. For the M3 subtype AML, also named acute promyelocytic leukemia (APL), the most common treatment is combination of chemotherapy and all-transretinoic acid (ATRA), a carboxylic acid form of vitamin A(Döhner, Estey, Amadori, Appelbaum, Büchner, Burnett, Dombret, Fenaux, Grimwade, & Larson, 2010; Zeisig et al., 2012). After the combine treatment of daunorubicin and ATRA, most APL patients could achieve complete remission. Another drug having good therapeutic effect in APL is arsenic trioxide (formula: As_2O_3). It was discovered from traditional Chinese medicine and first used as an anti-leukemia drug by Chinese researcher Zhang TingDong(Rao, Li, & Zhang, 2013). Now arsenic trioxide has been approved by the US FDA for treatment of APL patients who are unresponsive to ATRA(Antman, 2001; Bian et al., 2012; Soignet et al., 2001). The combination of these two drugs was also approved

for treatment of certain types of leukemia(Jun Zhu, Chen, Lallemand-Breitenbach, & de Thé, 2002).

However, most of them are only present in a relatively small percentage of all AML patients and only a small number of these targets could be employed in further drug development process(Mardis et al., 2009; Runde & Aul, 1992; The Cancer Genome Atlas Research Network, 2013). These potential drugs could be classified based on their targets.

1.2.1 Genetic targets

1.2.1.1 FMS like tyrosine kinase 3

FMS like tyrosine kinase 3 (FLT3) is a transmembrane protein and belongs to the class 3 family of receptor tyrosine kinases (RTKs). It plays an important role in normal hematopoiesis and cellular growth of HSCs(Boyer, Schroeder, Smith-Berdan, & Forsberg, 2011; Kikushige et al., 2008; Weisel, Yildirim, Schweikle, Kanz, & Möhle, 2007). After binding of FLT3 ligand, FLT3 is activated through dimerization and autophosphorylation, followed by the activation of its downstream targets like STAT5, RAS, phosphatidylinositol 3-kinase (PI3K), and phosphatase of regenerating liver-3. Through activating these genes, FLT3 could stimulate the survival and proliferation of leukemia cells(Gilliland & Griffin, 2002). About 30% of AML patients carry FLT3 activating mutations, mainly consisting of internal tandem duplications

(ITDs)(Mark Levis, 2013). Patients with FLT3 mutations have worse responses to the traditional chemotherapy, compared to those with wild type FLT3(M Levis & Small, 2003). Thus, it is of great interest to develop FLT3 inhibitors.

The first-generation of FLT3 inhibitors, instead of specifically targeting FLT3, were developed against a range of RTKs in malignancies other than AML. Most of them competitively bind to the active site of RTKs and inhibit ATP from accessing, therefore preventing the activation of RTKs and its downstream targets(Fathi & Chen, 2011).

Sorafenib is a small molecule drug targeting multiple kinases including several tyrosine protein kinases like FLT3, vascular endothelial growth factor receptor (VEGFR), and platelet derived growth factor receptor (PDGFR)(Adnane, Trail, Taylor, & Wilhelm, 2005; Wilhelm et al., 2008; W. Zhang et al., 2008). Sorafenib also targets other types of kinases like Raf kinases (RAS)(Adnane et al., 2005). It has been approved for use in renal cell carcinoma and hepatocellular carcinoma(Eisen, 2006; Escudier et al., 2009; Llovet et al., 2007; Palmer, 2008). In two phase I studies, the safety and clinically effectiveness of sorafenib were demonstrated in relapsed or refractory AML patients(Borthakur et al., 2011; Metzelder et al., 2009). A phase II study showed that sorafenib is safe to use in combination with some conventional cytotoxic drugs such as azacytidine(Ravandi et al., 2010). However, the combination failed to show any significant advantage in clinical benefits. In a placebo-controlled, Phase III SORAML study, the combination of sorafenib and standard chemotherapy produced a significantly longer median event-

free survival compared to the results from placebo plus standard chemotherapy, 21 months versus 9 months, respectively (Christoph Röllig et al., 2014). However, the improved outcome with sorafenib was not only observed in patients with FLT3 expression but also in patients without FLT3, which indicated that other targets besides FLT3 might be involved. Meanwhile, there is no statistically significant advantage on the overall survival rate. Other phase III trials testing the efficiency of sorafenib either combined with bortezomib or in the context of allogeneic stem cell transplantation are still ongoing or completed(Inaba et al., 2011; Metzelder et al., 2009).

Other non-specific FLT3 inhibitors, such as sunitinib, midostaurin and lestaurtinib, have similar drawbacks and therefore the second-generation FLT3 inhibitors with significantly greater specificity were developed, including quizartinib, crenolanib, and PLX3397. Quizartinib showed much lower IC50 for FLT3 inhibition compared to the first-generation inhibitors of tyrosine kinases(Chao et al., 2009). Quizartinib also showed significant tumor inhibition effect including rapid and complete tumor regression in mice models, while sunitinib only partially reduced tumor size and weight, and failed to prevent re-growth of tumor immediately after the treatment(Zarrinkar et al., 2010). In phase I/II trails, quizartinib used alone demonstrated an appreciable response rate, including complete and partial remission, in AML patients with FLT3 mutation and much less toxic side effects(Cortes et al., 2013; Tallman et al., 2013). Additional trials are ongoing aiming to explore the monotherapy of quizartinib in FLT3-ITD positive

patients, the application of combination of quizartinib and standard chemotherapy, and of quizartinib and stem cell transplantation(Cortes, 2014; Foran et al., 2013; Malvar et al., 2013). These trials would provide valuable data about the tolerability, safety and efficacy of quizartinib in the treatment of AML. Other second-generation FLT3 inhibitors, such as crenolanib and PLX3397, also demonstrate effects against FLT3 positive AML and are in phase I/II clinical trials(Galanis et al., 2013; Lin et al., 2013; Zimmerman et al., 2013).

1.2.1.2 RAS-RAF-MEK-ERK

The RAS-RAF-MEK-ERK pathway is critical for the proliferation of many cancer cells(Dhillon, Hagan, Rath, & Kolch, 2007; Kim & Choi, 2010; McCubrey et al., 2007). In AML, about 30% patients carry mutated oncogenic forms of RAS, which makes the RAS-RAF-MEK-ERK pathway a potential therapeutic target(Neubauer et al., 2008). Due to its central role in RAS pathway, MEK inhibitors were developed as potential target drugs(Nishioka, Ikezoe, Yang, & Yokoyama, 2010; Ricciardi et al., 2012). In solid tumors, three MEK inhibitors, CI-1040, PD-0325901, and AZD6244, had been tested in clinical trials(Catalanotti et al., 2013; Haura et al., 2010; Rinehart et al., 2004). However, the results were not very promising with only 13 remissions among about 500 patients all together, which may be due to the severe side effects, including rash, diarrhea, nausea, vomiting, peripheral edema, fatigue, and

optical disturbances. Other MEK inhibitors, including trametinib (GSK1120212) and pimasertib, have demonstrated their antitumor activities in a variety of cancers(Burgess et al., 2014; Infante et al., 2015; Martinelli et al., 2013; Wright & McCormack, 2013). In a phase I/II trail, trametinib showed promising clinical responses almost exclusively in patients with refractory myeloid malignancies characterized by somatic RAS mutations(Borthakur et al., 2012), whereas, a phase II trial showed that pimasertib failed to demonstrate clinical benefits in patients with poor prognosis AML and other hematological malignancies (ClinicalTrials.gov Identifier: NCT00957580).

1.2.1.3 JAK

The Janus kinase (JAK) protein family consists of four cytoplasmic tyrosine kinases, Janus kinase 1 (JAK1), Janus kinase 2 (JAK2), Janus kinase 3 (JAK3), and tyrosine kinase 2(TYK2), which all play a role in hematopoiesis(Staerk & Constantinescu, 2012; Ward, Touw, & Yoshimura, 2000). Although the mutations of JAK2 are rarely found in AML, the activation of its downstream targets STAT3 and STAT5 are reported in a majority of AML patients, which may be the result of mutations on other stimulators, like FLT3(Birkenkamp, Geugien, Lemmink, Kruijer, & Vellenga, 2001; H. J. Lee, Daver, Kantarjian, Verstovsek, & Ravandi, 2013; Spiekermann, Bagrintseva, Schwab, & Schmieja, 2003; Treaba et al., 2012; Vicente et al., 2007). Therefore, JAK inhibitors may be useful in treating AML.

Ruxolitinib is a JAK2 inhibitor that has been approved by the US FDA for the treatment of intermediate or high-risk myelofibrosis, a rare type of myeloproliferative neoplasms (MPNs) affecting the bone marrow, which often coexist with AML(Cervantes et al., 2013; Ganetsky, 2013; Verstovsek et al., 2012). A phase II study in patients with refractory leukemia showed that ruxolitinib was very well tolerated but only showed anti-leukemia activity in patients with post-MPN AML(Eghtedar et al., 2012). Trials to investigate the effectiveness of the combination of ruxolitinib and dacitabine in post-MPN AML are currently being conducted (ClinicalTrials.gov Identifier: NCT02076191, NCT02257138).

Due to their common targets, stat3 and stat5, increased JAK2 signaling is a common resistance mechanism against FLT3 inhibitors(Chu & Small, 2009; Weisberg et al., 2009). A novel JAK2-FLT3 inhibitor, fedratinib, could target JAK2 and FLT3 simultaneously, which allows it to confer the resistance to other FLT3 inhibitors(Kesarwani, Huber, & Azam, 2013). Researchers have shown the effectiveness of fedratinib in AML cell lines and AML patient samples(Ciceri et al., 2014). Further clinical studies will be required to investigate fedratinib as a therapeutic option of AML patients. Other JAK2 inhibitors, like AZ960, SB1518, are also under investigation(Hart et al., 2011; J. Yang, Ikezoe, Nishioka, Furihata, & Yokoyama, 2010).

1.2.1.4 Messenger RNA processing

The expression of eukaryotic translation initiation factor 4E (eIF4E) is elevated in about 30% of human malignancies, which enhances mRNA export from nucleus and the translation of a subset of transcripts coding for proteins involved in survival and proliferation of cancer cells(Furic et al., 2010; Mamane et al., 2004; Proud, 2015; Rhoads, 2009; Strudwick & Borden, 2002). In AML, the M4 and M5 subsets harbor high level of eIF4E, which allows target therapy to be considered in these subsets of AML(Hariri et al., 2013).

Ribavirin is a well-characterized nucleoside analogue known to block eIF4E activity and has been used as a broad-spectrum antiviral drug for nearly 40 years(A Kentsis et al., 2005; Alex Kentsis, Topisirovic, Culjkovic, Shao, & Borden, 2004; Y. Yan, Svitkin, Lee, Bisaillon, & Pelletier, 2005). In 2009, Assouline *et al* showed that the proliferations of M4/M5 AML cells were inhibited by ribavirin treatment(Assouline et al., 2009)(Kraljacic, Arguello, Amri, Cormack, & Borden, 2011). These studies also showed that the concentrations required to inhibit normal or M1/M2 AML specimens were much higher. This provided important clues for the anti-tumor potential of ribavirin in eIF4E positive AML. A followed study demonstrated the clinical benefits of ribavirin in eIF4E positive M4/M5 AML patients in a small clinic trial(Assouline et al., 2009). The clinical response was associated with reduced eIF4 level and relocalization of eIF4E from the nucleus to the cytoplasm. A phase I trial was conducted to determine the recommended phase II dose of ribavirin when combined with low dose cytarabine(Assouline et al., 2013). The results provided evidence for the potential usage of ribavirin in treatment of M4/M5 subtypes of AML.

1.2.2 Epigenetic targets

Not only genes with mutations or translocations could be targeted in cancer therapy, but also epigenetic changes either due to epigenetic modulators or the epigenetic marker readers. Epigenetic changes, including DNA methylation and histone acetylation/ deacetylation, occur frequently in acute leukemia, which provide targets for therapeutic approach(Greenblatt & Nimer, 2014; O'Brien, Prideaux, & Chevassut, 2014; Oki & Issa, 2010).

1.2.2.1 Epigenetic "Writers & Erasers"

1.2.2.1.1 DNA methylation

Dysregulation of DNA methylation has now been recognized as a hallmark of cancer(Feinberg & Tycko, 2004). The aberrant hypermethylation at the promoter region of certain genes could be found in most cases of AML. The increased level of DNA methylation leads to shutting down of multiple tumor suppressor genes like p53, etc. DNA methylation is executed by DNA methyltransferases (DNA MTase), including DNMT1, DNMT3A, and DNMT3B. These DNA MTases catalyze the transfer of a methyl group from S-adenosyl methionine (SAM) to DNA. DNMT3A is a common recurrent mutation site in AML patients. About 22% of AML patients harbor DNMT3A mutations and

their prognosis are much worse than patients with wild type DNMT3A(Ley et al., 2010). Therefore, inhibitors targeting this gene were developed.

Available inhibitors of DNA methylation include azacitidine and 5-aza-2'-deoxycytidine (decitabine), both of which are able to bind to the DNA MTases, inhibiting their functions and leading to the re-expression of tumor suppressor genes(Čihák, 1974). The US FDA has approved azacitidine and decitabine for treatment of myelodysplastic syndromes (MDS). However, the US FDA declined the decitabine therapy in AML after a large scale randomized international phase III trial in AML patients who are 65 years or older failed to show statistically significant improvement in overall survival, despite the significantly improved complete remission rates(H. M. Kantarjian et al., 2012). The good news is, in the same year, the European Commission approved decitabine treatment in AML patients who are 65 years or older. Although not getting approval from US FDA, decitabine could still be used off-label in the US.

1.2.2.1.2 Histone methylation

Histone methylation also plays an important role in epigenetic regulation(Greer & Shi, 2012). Methylation usually happens on the lysine or arginine residues, while trimethylation only occurs on lysine residues. The methyl marks are written by histone methyltransferase including SET domain containing methyltransferases and protein arginine methyltransferases

(PRMTs) and erased by demethylases(Yi Zhang & Reinberg, 2001). These various methyl marks are recognized by proteins with the ability to compact nucleosomes or directly regulate gene expressions. Unlike DNA methylation, methylated histone marks can be associated with either transcriptional activation or repression, depending on which epigenetic reader they are able to interact with. For example, the trimethylated lysine 4 of histone H3 (H3K4me3) represents a status open for transcription while the trimethylated lysine 27 of histone H3 (H3K27me3) and the trimethylated lysine 9 of histone H3 (H3K9me3) represent silencing of genes(Cheung & Lau, 2005; J. C. Rice et al., 2015).

One of the histone methyltranferases, enhancer of zeste homolog 2 (EZH2), is able to catalyze the trimethylation of histone H3 on lysine 27 and consists of the enzymatic component of polycomb repressive complex 2 (PRC2), which is responsible for maintaining the cellular epigenetic status during development and differentiation(H Chen, Rossier, & Antonarakis, 1996; Margueron & Reinberg, 2011; Morey & Helin, 2015; Vire et al., 2006). EZH2 is found commonly up-regulated in a wide range of cancers including breast, prostate, bladder, and renal cancers, as well as lymphoma and leukemia(C.-J. Chang & Hung, 2012; Hock, 2012; Lund, Adams, & Copland, 2014; Mishra, Kanchan, & Bhargava, 2012). The up-regulation of EZH2 usually results in the silencing of multiple tumor suppressor genes and thus promotes tumor progression(J. A. Simon & Lange, 2008). Inhibition of EZH2 impaired tumor growth and induce apoptosis in either cell lines or mice models(C. Simon et al., 2012). Therefore, EZH2 could be a druggable target in cancer therapy.

Several drugs targeting EZH2 have been developed. 3-Deazaneplanocin (DZNep) is the first drug proposed to inhibit EZH2(Glazer, Knode, Tseng, Haines, & Marquez, 1986). It soon showed promising anti-tumor activity in multiple cancers including breast cancer, colon cancer, non-small cell lung cancer, and leukemia, through inhibition of EZH2 level and inducing apoptosis(Fiskus et al., 2009; Fujiwara et al., 2014; Kikuchi et al., 2012; Jing Tan et al., 2007). Our lab also reported the effect of DZNep in AML and multiple myeloma(Z. Xie et al., 2011; J. Zhou et al., 2011). DZNep could decrease the level of EZH2 in AML cell lines and patient samples, thereby inducing apoptosis through up-regulation of a tumor suppressor thioredoxin interacting protein (TXNIP). However, other researchers had shown that DZNep is not an EZH2 specific inhibitor and other DNA methyltransferases could also be inhibited by DZNep, which increased the concern for off-target effect(L. L. Cheng et al., 2012; Miranda et al., 2009). Other drugs were also developed targeting the conserved SET domain of EZH2, including EPZ005687, EI1, and GSK126(Knutson et al., 2012; McCabe et al., 2012; W. Qi et al., 2012). GSK126 showed amazing selectivity against EZH2, which is more than 1000 fold higher than 20 other human methyltransferases. It not only inhibits the proliferation of lymphoma cell lines, but also showed remarkable antitumor effect in mice models. Inhibition of EZH2 also showed great effect in eliminating leukemia stem cells, which addded more value to this drug as traditional chemotherapy had failed to kill stem cells and therefore, unable to prevent relapse of disease(McCabe et al., 2012).

Inactivating mutations of EZH2 were also found in myelodysplastic syndrome, which indicates EZH2 also has tumor suppressing activity(Ernst et al., 2010; Nikoloski et al., 2010). Therefore, the inhibition of EZH2 must be carefully evaluated to distinguish patients who would benefit from EZH2 inhibition from those who would not.

1.2.2.1.3 Histone acetylation

Histone acetylation and deacetylation are other mechanisms to control gene expression at epigenetic level(Kurdistani & Grunstein, 2003; Vogelauer, Wu, Suka, & Grunstein, 2000). Two families of enzymes, histone acetyltransferases (HATs) and histone deacetyltransferases (HDACs) control these dynamic processes in opposite ways. HATs family includes GNAT family, MYST family, p300/CBP family, and other HATs(K. K. Lee & Workman, 2007). They are able to transfer acetyl groups to the lysine residues on the histone tails, neutralizing the positive charge on the lysine residues and facilitating the histone tails detaching from the nucleosomes(Ogryzko, 2001). As a result, the chromatin is transformed from heterochromatin to euchromatin, which is associated with a greater level of transcription(Sterner & Berger, 2000; Verdone, Caserta, & Mauro, 2005). HDACs act in the opposite way by reversing the acetylation and turning the chromatin back to silencing status(Leipe & Landsman, 1997). The HDACs superfamily includes 18 different HDACs classified into four different classes in human(de Ruijter, van Gennip,

Caron, Kemp, & van Kuilenburg, 2003). There are a variety of acetylation sites on tails of either histone H2A, H2B, H3 or H4. Most of these sites are lysine residues and one of them, the lysine 16 on histone 4 (H4K16), is the most crucial one in regulating chromatin status(Shahbazian & Grunstein, 2007).

HDACs have been associated with tumorigenesis and metastasis(Dokmanovic, Clarke, & Marks, 2007; Fraga et al., 2005; Paul A Marks et al., 2001; Yasui et al., 2003). Aberrantly expressed HDACs have been found in a variety of cancer types, including gastric cancer, prostate cancer, breast cancer, colon cancer, and cervical cancer(Choi et al., 2001; Halkidou et al., 2004; B. H. Huang et al., 2005; A. J. Wilson et al., 2006; Z. Zhang et al., 2004, 2005). In hematopoietic malignancies, HDACs might be involved in the leukemogenesis caused by fusion proteins produced by recurrent translocations. HDACs could be recruited to the specific target genes by oncogenic transcription factors, such as PML-RARα in acute promyelocytic leukemia (APL), LAZ3/BCL6 in non-Hodgkin's lymphoma, and AML1/ETO in M2 AML harboring t (8; 21)(Dhordain et al., 1998; P A Marks & Xu, 2009; J. Wang, Hoshino, Redner, Kajigaya, & Liu, 1998; J. Wang, Saunthararajah, Redner, & Liu, 1999). The aberrant recruitment of HDACs changes the genomic chromatin status and represses the expression of tumor suppressors, providing a strong rationale to target these HDACs in patients. Numerous inhibitors of HDACs (HDACi) have been developed and have shown single-agent clinical activity against various hematologic malignancies, including T-cell lymphomas and AML(P A Marks & Xu, 2009). The US FDA has approved several HDACi, such as vorinostat and romidepsin for treatment of cutaneous T-cell lymphoma, panobinostat for

treatment of multiple myeloma, etc.(Andreu-Vieyra & Berenson, 2014; Piekarz et al., 2011; Richardson et al., 2013; C. Zhang, Richon, Ni, Talpur, & Duvic, 2005). Several studies have proposed the therapeutic potential of HDACi in t (8; 21) AML cell lines and mouse models, although the detailed mechanism remains unclear(Bots et al., 2014; Fredly, Gjertsen, & Bruserud, 2013). The combination of HDACi and other target drugs such as the JAK2 inhibitor pacritinib also showed promising effectiveness in AML cell lines and mouse models carrying AML with either FLT3-ITD or JAK2 mutation(Novotny-Diermayr et al., 2012). Clinical trials in AML patients, including use of combination of entinostat and sorafenib, entinostat and Azacitidie, entinostat and sargramostim, AR-42 and decitabine, are also ongoing or already completed(Fiskus et al., 2009; Garcia-Manero et al., 2006; Gore et al., 2006; Ngamphaiboon et al., 2015; Novotny-Diermayr et al., 2012)(ClinicalTrials.gov Identifier: NCT01159301; NCT01798901).

1.2.2.2 Epigenetic "readers"

Besides regulating chromatins, histone acetylation also promotes transcription through providing a platform for the binding of transcriptional regulators, which is similar to the situation of the histone methylation(Verdone et al., 2005). The recruited transcriptional regulators could "read" acetylated lysine residues on the histone tails and initiate transcription of target genes.

1.2.2.2.1 BRD4 family

The most intensively “reader” protein is the bromodomain-containing family(Zeng & Zhou, 2015). A bromodomain consists of 110 amino acids and is able to recognize the acetyl group on specific lysine residues within histone tails, which allows proteins containing bromodomains to “read” the post-translational modification through protein-protein interaction(Dhalluin et al., 1999; Moriniere et al., 2009; Owen et al., 2000). Although bromodomains play an important role in the epigenetic regulation of gene expression and have the potential to become a therapeutic target, it is not easy to develop inhibitors against bromodomains as their interaction structure with acetylated histone tails is very small(H. Huang et al., 2007; Sanchez et al., 2000; Schultz, Copley, Doerks, Ponting, & Bork, 2000). Another difficulty is a total number of 61 bromodomains have been found within 46 different human proteins, so there is a high requirement on specificity of bromodomain inhibitors(Filippakopoulos et al., 2015).

1.2.2.2.2 BRD4 function

In 2011, Bradner’ group announced that they had synthesized a small molecule drug targeting the Bromodomain named JQ1(Filippakopoulos et al., 2010). JQ1 targets only a specific family of bromodomain-containing proteins

known as the bromodomain and extra C-Terminal (BET) family. This family consists of four members, BRD2, BRD3, BRD4 and BRDT, characterized by two conserved amino-terminal bromodomains and a divergent carboxy-terminal recruitment domain. Of all the four members, BRD4 is the main target of JQ1(Filippakopoulos et al., 2010).

BRD4 regulates gene expression through forming a complex with positive transcription elongation factor b (P-TEFb)(Jang et al., 2015; Z. Yang et al., 2005). P-TEFb is a complex mainly consisting of a kinase subunit, cyclin-dependent kinase 9 (Cdk9) and one of its regulatory subunits, Cyclin T1, T2, or K(Fu, Peng, Lee, Price, & Flores, 1999; Junmin Peng, Zhu, Milton, & Price, 1998). P-TEFb is found to stimulate the elongation of mRNA through activating the RNA polymerase II (Pol II) which pauses at the proximal promoters after beginning of transcription(Price, 2000; Rahl et al., 2015). Previous work showed that P-TEFb is able to directly phosphorylate the Ser2 site on the C-terminal domain (CTD) of the subunit of Pol II(Marshall, Peng, Xie, & Price, 1996; Ramanathan et al., 2001). However, the phosphorylated CTD is more likely to play a role in RNA processing through recruiting multiple RNA processing factors like the mRNA capping enzymes (CE), 3' end processing complex, and the mRNA splicing enzymes(Sims, Belotserkovskaya, & Reinberg, 2004).

Besides phosphorylating CTD of Pol II, P-TEFb is also able to interact with two negative regulators of transcription, the DRB sensitivity inducing factor (DSIF)

and the negative elongation factor (NELF)(Hui Chen et al., 2009; Yamada et al., 2015). These two factors together could sensitize the ATP analog 5, 6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) and promote the stalling of Pol II at the proximate promoter regions(Wada et al., 1998; Yamaguchi et al., 2015). P-TEFb could phosphorylate the large subunit of DSIF and thus promote mRNA elongation(Rahl et al., 2015). Phosphorylation of NELF by P-TEFb has also been demonstrated to associate with its detachment from Pol II during the transition into mRNA elongation(Fujinaga et al., 2004). These evidence provide clues for the detailed mechanism of P-TEFb-regulated mRNA elongation.

As the proximally paused Pol IIs are prevalent across genomes, tight regulation of P-TEFb activity is required for the proper control of global gene expression. Common mechanisms like expression of isoform, transcriptional, translational, and post-translational control have been found to be involved in the regulation of P-TEFb(Cho, Schroeder, & Ott, 2010; C. H. Herrmann, Carroll, Wei, Jones, & Rice, 1998; Kiernan et al., 2001; Shore, Byers, Dent, & Price, 2005). However, one small nuclear ribonucleic protein, 7SRNP, is the most important regulator of P-TEFb activity(Nguyen, Kiss, Michels, & Bensaude, 2001; Z. Yang, Zhu, Luo, & Zhou, 2001).

In cellular components, P-TEFb could form a complex with 7SK snRNP and a RNA binding protein hexamethylene bis-acetamide inducible 1 (HEXIM1), which could inactivate the enzymatic component Cdk9 using an inhibitory domain(Barboric et al., 2005; Q. Li et al., 2005; Michels et al., 2004; Peterlin,

Brogie, & Price, 2012; Yik et al., 2003). The inactive form of P-TEFb is sequestered and no longer binds to the proximally paused Pol II(Q. Li et al., 2005). In active cells undergoing rapid growth, the sequestered P-TEFb consists of 90% whole P-TEFb, therefore, preventing the inappropriate activation of genes across the genome(Z. Yang et al., 2001). Further experiments showed that 7SK snRNP exists at a diffusion status in the nucleus and only a few free P-TEFb exist and are tightly associated with genes undergoing transcript elongation(Biglione et al., 2007). This indicates that the specific extraction of P-TEFb from 7SK snRNP is necessary to achieve selective P-TEFb function on target genes(J. Peng, Liu, Marion, Zhu, & Price, 1998).

BRD4 was found to be involved in the regulation of P-TEFb(Bisgrove, Mahmoudi, Henklein, & Verdin, 2007; S.-Y. Wu & Chiang, 2007; Z. Yang et al., 2005). It releases P-TEFb from the 7SK snRNP complex through directly binding to P-TEFb at two specific regions, the C-terminal domain (CTD) interacting with Cyclin T1 and Cdk9, and the second bromodomain recognizing an acetylated region of Cyclin T1(Jang et al., 2015; Krueger, Varzavand, Cooper, & Price, 2010; Schröder et al., 2012). Therefore, BRD4 is able to carry P-TEFb to specific target genes that are associated with acetylated histone tails and initiate the Cdk9 mediated activation of Pol II and the subsequential mRNA elongation(Q. Zhou & Yik, 2006). The interaction between BRD4 and P-TEFb is particularly important for fast transcription

induction like in response to signal transduced activation of transcription factors(Hargreaves, Horng, & Medzhitov, 2009).

BRD4 executes its transcription activator function not only through recognizing acetylated histone tails, but also through direct interaction with other transcription factors. A recent study using purified factors evaluated the interaction between BRD4 and various factors, including pre-initiation complex components, chromatin regulators, and sequence-specific DNA binding factors(S. Y. Wu, Lee, Lai, Zhang, & Chiang, 2013). Besides the known binding with P-TEFb, they also found a group of BRD4-interacting transcriptional factors including p53, YY, c-Jun, AP2, C/EBPA, and the MYC/MAX heterodimer. For example, the C-terminal regulatory domain of p53 interacts with two distinct regions of BRD4 and facilitates the transcriptional activation of p53 downstream genes.

1.2.2.2.3 BRD4 inhibition

Besides JQ1, a lot of small molecular inhibitors of BRD4 were developed afterwards, including iBET-151, iBET-762, RVX-208, OTX-015, CPI-0610(Boi et al., 2015; Dawson et al., 2011; Mirguet et al., 2013; Picaud et al., 2013). These inhibitors showed strong binding to BRD4 and were able to inhibit the normal function of BRD4. NUT midline carcinoma (NMC) is a rare squamous carcinoma characterized by a recurrent chromatin translocation that often

results in a fusion protein between NUT and BRD4(C A French et al., 2007; Christopher A French et al., 2001, 2003). Most BRD4 inhibitors were tested in a NUT midline carcinoma model and inhibited proliferation in either cell lines or mouse models. For example, treatment of JQ1 induced tumor regression and a significant survival advantage in a mouse NMC model(Filippakopoulos et al., 2010). The JQ1 analog TEN-010 is currently in phase I clinical trial for treating advanced solid tumor including NMC (ClinicalTrials.gov Identifier: NCT01987362). Other drugs also showed significant therapeutic potency in preclinical studies or undergoing clinical trials for NMC, Type 2 diabetes, Leukemia, and other hematological malignancies (ClinicalTrials.gov Identifier: NCT01058018, NCT01728467, NCT01713582, NCT01949883).

1.2.2.2.4 JQ1 in cancer

JQ1 showed significant antitumor effect not only in NMC, but also in many hematopoietic malignancies. JQ1 could inhibit proliferation and induce cell cycle arrest in Burkitt lymphoma, multiple myeloma, ALL, and AML(Delmore et al., 2011; Mertz et al., 2011; Ott et al., 2012; Zuber et al., 2011).

Analysis of gene expression profile data revealed the mechanism of JQ1's anti-tumor effect. One of the most important driver oncogenes in hematopoietic cancer, c-MYC, is the major target of JQ1. In Burkitt lymphoma and multiple myeloma, the study of microarray data revealed that MYC was the most down-regulated gene after JQ1 treatment and the depletion of MYC

was dose-dependent(Mertz et al., 2011). Knockdown of BRD4 using shRNA in multiple myeloma showed similar changes of MYC level and cell cycle arrest compared to JQ1 treatment(Delmore et al., 2011). The expression level of MYC target gene p21 increased significantly after JQ1-induced inhibition of MYC expression, which may explain the cell cycle arrest caused after JQ1 treatment(Mertz et al., 2011). Ectopically expressed MYC rescued the cell cycle arrest caused by JQ1 treatment in multiple myeloma(Delmore et al., 2011). In AML, the inhibition of cell proliferation and cell cycle arrest were also observed either under JQ1 treatment or knockdown of BRD4 using shRNA(Zuber et al., 2011). Besides, inhibition of BRD4 also led to myeloid differentiation and depletion of leukemia stem cells, which could also be rescued by ectopically expressed MYC(H. Herrmann et al., 2012). Chromatin Immunoprecipitation assay demonstrated that BRD4 and Cdk9 bind to the promoter region of MYC and this binding is depleted after JQ1 treatment in multiple myeloma and AML cells, which confirmed the direct control of MYC by BRD4(Mertz et al., 2011; Zuber et al., 2011).

JQ1 and super enhancer

Through direct targeting BRD4, JQ1 inhibits the acetyl-lysine binding ability of BRD4, preventing it from interacting with acetylated histone and thus, controlling the expression of BRD4 target genes. Using genomic ChIP-seq analysis, Young and collleges found that BRD4 and its partners co-occupies a small set of exceptionally large specific sequences which are associated with certain genes involved in cancer biology, including MYC oncogene. These

specific sequences are named super enhancers due to their sizes and abilities to control gene expression. Treatment with JQ1 was also found to preferentially affect genes with super enhancers, which explained why targeting a ubiquitous regulator BRD4 caused gene specific effect.

1.3 MYC

MYC has been identified as a human homolog of the retroviral oncogene v-MYC for more than thirty years(Jansen et al., 1984; M. A. Lane, Neray, & Cooper, 1982). It is one of the most intensively studied human genes, yet new findings about functions and regulations of MYC have kept emerging, which have increased not only our understanding of MYC biology, but also of numerous biological processes including molecular tumorigenesis in general.

1.3.1 MYC regulates gene expression

MYC functions mainly as a master regulator of gene transcription across the genome. MYC is able to control multiple cellular processes including proliferation, cell cycle, differentiation, through activation or depression of its various downstream target genes(Luscher & Eisenman, 1990; Marcu, Bossone, & Patel, 1992). The helix-loop-helix/leucine zipper (HLH/LZ) domain is first identified in MYC and this domain is the primary contributor of its MYC

the DNA binding ability(Murre, McCaw, & Baltimore, 1989). In 1991, a partner protein of MYC, MAX, was identified(Blackwood & Eisenman, 1991). MAX could form heterodimer with MYC and recognize a CACGTG E-box sequence on DNA(Blackwood, Luscher, & Eisenman, 1992). This finding provided strong evidence for the regulatory role of MYC. There are several different ways of MYC-mediated transcription activation. For example, MYC recruits histone acetyltransferases (HATs) through an adaptor protein Transformation/transcription domain-associated protein (TRRAP)(S.-W. G. Cheng et al., 1999; S. B. McMahon, Van Buskirk, Dugan, Copeland, & Cole, 1998; Steven B McMahon, Wood, & Cole, 2000). The recruitment of HATs leads to acetylation of histone tails and trigger activation of transcription by epigenetic regulators like TIP60 and p300(Faiola et al., 2005; Frank et al., 2003). MYC is also able to activate transcription elongation by directly recruiting the P-TEFb complex and the transcription factor II human (TFIIH), which is critical for the formation of RNA polymerase II pre-initiation complex(Bentley, 2005; Cowling & Cole, 2007). Direct interaction between MYC and RNA polymerase II has also been reported(Eberhardy & Farnham, 2002). MYC can thus activate transcription through various mechanisms and is a critical positive regulator of gene expression.

As a master regulator of gene expression, MYC does not only act as a transcription activator, but also plays an important role in gene repression(Gartel & Shchors, 2003; Wanzel, Herold, & Eilers, 2003). Besides recognizing the E-box and activating gene expression, the MYC-MAX heterodimer can also bind to other transcription factors, replacing their co-

activating partners, or recruiting co-repressing partners, and thus inhibit the gene expression(Mao et al., 2003). For example, MIZ1 is a zing-finger protein which acts as a transcription factor, either by activating or repressing target genes through binding to different partners(Adhikary et al., 2005; Mao et al., 2004). When MIZ1 forms a complex with co-activator p300 histone acetyltransferase, it stimulates the expression of target genes(Staller et al., 2001). However, the MYC-MAX heterodimer is also able to bind to MIZ1 and inhibit transcription activation through displacing p300 and recruiting the DNA methyltransferase DNMT3A, repressing multiple target genes such as p21 and p15(Seoane et al., 2001; Seoane, Le, & Massague, 2002; S. Wu et al., 2003). The MYC-MAX heterodimer also executes similar functions by binding with other partners such as Nuclear Factor Y and SP1(Kyo et al., 2000; Taira et al., 1999).

Besides direct regulation, MYC also regulates gene expression indirectly through microRNAs(Jackstadt & Hermeking, 2014). MicroRNAs are small non-coding RNAs binding to the 3’ UTR of target mRNAs and mediating translational repression or mRNA degradation(Ambros, 2004; Bartel, 2004). MYC is now known to regulate multiple miRNAs through several different mechanisms. The first is directly activating or repressing miRNA transcription through binding to their promoters as mentioned above. Other than that, MYC also activates genes encoding the miRNA binding proteins such as Lin28 and Lin28b, which are able to interact directly with let-7 pre-miRNA and block

the maturation process(T.-C. Chang et al., 2009; Heo et al., 2008; Rybak et al., 2008). In addition, direct induction of the expression of DROSHA, which is critical for pri-miRNA transfer, is also an important mechanism in MYC regulated miRNAs expression(X. Wang, Zhao, Gao, & Wu, 2013).

The first identified miRNA cluster regulated by MYC is the miR-17-92 cluster, which encodes miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a-1(He et al., 2005; O'Donnell, Wentzel, Zeller, Dang, & Mendell, 2005). The miR-17-92 cluster was found to be frequently amplified in hematopoietic malignancies and solid tumors such as B-cell lymphoma, non-small cell lung cancer, colon cancer(Ota et al., 2004; Tanzer & Stadler, 2004). Ectopic expression of miR-17-92 is able to induce tumorigenesis in either hematopoietic malignancies or solid tumors(Olive, Jiang, & He, 2010). MYC could directly activate expression of miR-17-92 and two of its paralogs, miR-106b-25 and miR-106a-363, through binding to the E-box in their promoters(Dews et al., 2006; O'Donnell et al., 2005), affecting a lot of downstream targets involved in cell cycle, angiogenesis, metabolism, apoptosis(Dews et al., 2006; O'Donnell et al., 2005; Xiang & Wu, 2010).

miR-9 is another microRNA directly activated by MYC, which directly targets E-cadherin and induces expression of VEGF, affecting both epithelial-mesenchymal transition (EMT) and angiogenesis, and thus, promoting metastasis in glioblastoma and breast cancer(Ma et al., 2010; Selcuklu et al., 2012). However, the role of miR-9 varies among different types of cancer(Nass et al., 2009; Schraivogel et al., 2011). For example, miR-9 is

silenced by hypermethylation in gastric cancer and overexpressed miR-9 suppresses proliferation in either ovarian cancer and chronic lymphoblastic leukemia through inhibition of the NF-κB pathway(Guo et al., 2009; Tsai et al., 2011; L. Q. Wang et al., 2013). miR-9-3p also act as a tumor suppressor in hepatocellular carcinoma through targeting WW Domain Containing Transcription Regulator 1(WWTR1)(Higashi et al., 2015).

The inhibitory role of MYC in regulating microRNAs is also well recognized(T.-C. Chang et al., 2008). Let-7 was found to be suppressed in multiple types of cancer and ectopically expressed let-7 was able to inhibit tumor growth in vivo(Calin et al., 2004; Esquela-kerscher et al., 2008; Kumar et al., 2008; Takamizawa et al., 2004; Yanaihara et al., 2006). MYC represses let-7 expression through direct binding to its promoter, while let-7 also targets the 3'UTR of MYC, forming a negative regulatory feedback loop(Boyerinas, Park, Hau, Murmann, & Peter, 2010). Besides direct regulation, LIN28, a MYC-induced RNA binding protein, also contributes to the inhibition of let-7 by disrupting the maturation process of let-7. This demonstrated the complexity of miRNA regulation(T.-C. Chang et al., 2009).

MYC also forms a negative feedback regulatory loop with miR-34a and inhibits its anti-tumor function of miR-34a in hematopoietic malignancies and solid tumors(Craig et al., 2011; Misso et al., 2014; Yamamura et al., 2012). Other miRNAs, such as miR-15a/16-1, miR-22, are also repressed either by MYC itself or by collaboration with other transcription factors including HDAC and SP1(Kong et al., 2014; Xue et al., 2015; X. Zhang et al., 2012). These MYC-

regulated miRNAs have a broad function in cell cycle control, proliferation, epithelial-mesenchymal transition (EMT), and apoptosis(T.-C. Chang et al., 2008; Jackstadt & Hermeking, 2014).

1.3.2 MYC and cellular activity

1.3.2.1 MYC and cell cycle

The association between MYC and cell cycle has been well recognized ever since the discovery of MYC(Amati, Alevizopoulow, & Vlach, 1998). Forced expression of MYC helps cells overcome the cell cycle arrest at G0/G1 induced by different growth inhibition factors(Freytag, 1988). Cell cycle progression is under control of cyclin-dependent kinases (CDKs) and their regulatory partner cyclins(Nigg, 1995). MYC is able to promote the cell cycle progression through activating the expression of these growth-promoting proteins, including CDK2, CDK4, cyclin D2, and cyclin E(Amati et al., 1998; Meyer & Penn, 2008). Besides transcriptional activation, MYC could also increase activities of CDKs through regulating one of the CDK activating phosphatases, cdc25(Galaktionov, Chen, & Beach, 1996; Hernández et al., 1998). Inhibiting the expression of inhibitors, including p15/INK4B, p21/Cip1, and p27/Kip1, which target CDK and cyclin complex, is another important mechanism in regulating cell cycle by MYC(Claassen & Hann, 2000; Seoane et al., 2001; S. Wu et al., 2003; W. Yang et al., 2001). Besides these, MYC also directly activates expression of E2F1, E2F2, E2F3 that are responsible for

regulating gene expression for the progression of cell cycle(M. R. Adams, Sears, Nuckolls, Leone, & Nevins, 2000; Fernandez et al., 2003; Sears, Ohtani, & Nevins, 1997; Stevens & La Thangue, 2003). Furthermore, MYC could abrogate the transcription of cell cycle checkpoint genes such as GADD45 and GADD153(Amundson, Zhan, Penn, & Fornace, 1998; Marhin, Chen, Facchini, Fornace, & Penn, 1997). Regulation of these genes allows cell to pass the G1/S checkpoint point and enter cell cycle(Bush et al., 1998; Salvador, Brown-Clay, & Fornace Jr., 2013).

1.3.2.2 MYC and cell death

The role of MYC in regulating cell death was found much later compared to its role in cell cycle control. Ectopically expressed MYC induces cell death through apoptosis and MYC-null cells are resistant to diverse apoptotic stimuli(Evan et al., 1992; Shi et al., 1992). MYC has been demonstrated to regulate apoptosis through interacting with CDKN2A(Y. Qi et al., 2004; Zindy et al., 1998). CDKN2A could inhibit the p53 inhibitor MDM2, therefore stabilize p53 protein(Yanping Zhang, Xiong, & Yarbrough, 1998). Activation of MYC increases expression of CDKN2A, eventually leading to increased p53 protein and apoptosis(Christine M Eischen, Weber, Roussel, Sherr, & Cleveland, 1999; Finch et al., 2006; Jacobs et al., 1999; Schmitt, McCurrach, de Stanchina, Wallace-Brodeur, & Lowe, 1999). MYC also induces apoptosis in a p53-independent way through indirectly suppressing the anti-apoptotic

proteins BCL-2 and BCL-XL or inducing the pro-apoptotic proteins BAK and BAX(Dansen, Whitfield, Rostker, Brown-Swigart, & Evan, 2006; C M Eischen et al., 2001; Christine M Eischen, Woo, Roussel, & Cleveland, 2001; Juin et al., 2002). However, concurrent expression of MYC and BCL-2 may facilitate tumorigenesis in B-cell lymphoma, indicating the complexity of MYC functions(Johnson et al., 2012; Strasser, Harris, Bath, & Cory, 1990).

1.3.2.3 MYC and differentiation

MYC is also important in determining whether a cell proceeds to proliferation or to differentiation. Although MYC blocks differentiation in many types of cells, the pro-differentiation role of MYC has also been reported(Coppola & Cole, 1986; Dmitrovsky et al., 1986; Gandarillas & Watt, 1997; Langdon, Harris, Cory, & Adams, 1986). The situation is even more complicated in hematopoietic cells(Delgado & León, 2010). For example, overexpression of c-MYC in mice leads to increased proliferation and reduced HSCs, while MYC deficiency causes accumulation of HSCs(A. Wilson et al., 2004). Moreover, hematopoietic cells at different stages may require different levels of MYC, as overexpression of MYC in mature cells from lymphoid or myeloid lineages results in lymphoma or leukemia in mice model and MYC overexpression blocks differentiation in mature hematopoietic cells(Baena, Ortiz, Martínez-A, & Moreno de Alborán, 2007; Laurenti et al., 2008; Reavie et al., 2010; A. Wilson et al., 2004). Thus, the expression level of MYC is critical for the

balance between self-renewed survival and differentiation in hematopoietic cells.

1.3.3 MYC and cancer

MYC has been a well-recognized proto-oncogene for over 30 years(Cole & Henriksson, 2006). MYC was found to be altered in different types of cancer through genetic rearrangement, amplification, and dysregulated transcription(Boxer & Dang, 2001; Nesbit, Tersak, & Prochownik, 1999). The increased expression of MYC could be found in about 70% of human cancers(Nilsson & Cleveland, 2003). Continued expression of c-MYC is critical to the initiation and progression of different cancers, such as breast cancer, non-small cell lung cancer, and hematological malignancies, etc.(J. M. Adams et al., 1985; Felsher & Bishop, 1999; Jain et al., 2002; Leder, Pattengale, Kuo, Stewart, & Leder, 1986)

In Burkitt lymphoma, the t (8; 14) translocation results in a fusion gene of c-MYC and immunoglobulin, which leads to aberrant expression of c-MYC(Carè et al., 1986; Erikson, Ar-Rushdi, Drwinga, Nowell, & Croce, 1983). In CML that is characterized by the existence of BCR-ABL fusion gene, c-MYC could be up-regulated by BCR-ABL and this up-regulation is important to the oncogenic effect of BCR-ABL(Melo & Barnes, 2007; Sawyers, Callahan, & Witte, 1992; S. Xie, Lin, Sun, & Arlinghaus, 2002). Another study also showed that c-MYC may be relevant to the progression of CML(Handa et al., 1997). In AML, due to the

diversity of this disease, only a few studies have been performed to evaluate the c-MYC expression level. Although amplification of c-MYC could be observed, it could not ensure an increase in the mRNA level(Bruyère, Sutherland, Chipperfield, & Hudoba, 2010; S. Lee et al., 2009; Paulsson et al., 2003; Stasik et al., 2010). But evidence showed that some important recurrent translocations in AML, including AML1-ETO, PLZF-RARa, and PML-RARa, induce leukemogenesis through activation of MYC(Müller-Tidow et al., 2004; K. L. Rice et al., 2009). More importantly, ectopically expressed c-MYC could rapidly induce AML in mice without additional anti-apoptotic mutations(Luo et al., 2005). A large scale ChIP study had also demonstrated that the occupancy by MYC at target promoters and the ratio of MYC and MIZ1 are important for the regulation of target genes in MYC-transformed tumor cells(Walz et al., 2014).

1.4 TXNIP

Thioredoxin-interacting protein (TXNIP) was first identified in the AML cell line HL60 in 1995(K. S. Chen & DeLuca, 1995). As it was found to be up-regulated by 1, 25-dihydroxyvitamin D3, it is also termed Vitamin D up-regulated Protein 1 (VDUP-1). It has been identified as a negative regulator of thioredoxin (TRX) activity through direct binding to the catalytic active center of reduced TRX(Junn et al., 2000; Nishiyama et al., 1999). TRXs are a family of proteins containing a catalytic site that is able to undergo reversible oxidation,

which allows TRX to reduce oxidized proteins using electrons from NAPDH(Buchanan, Schurmann, Decottignies, & Lozano, 1994; Holmgren, 1995; Nakamura, Nakamura, & Yodoi, 1997). The TRXs are responsible for maintaining an appropriate level of oxidative products during metabolism. When TXNIP inhibits TRX, the redox regulation loses control and affects the downstream pathways including inflammation, apoptosis, etc.(Mustacich & Powis, 2000).

1.4.1 TXNIP in glucose metabolism

An important upstream regulator of TXNIP is the glucose flux(N. Wu et al., 2013). Increased glucose uptake leads to increased glycolytic intermediates, which stimulate the binding of the MondoA: MLX complex to the carbohydrate response element (ChoRE) at the promoter region of TXNIP, which activates the transcription of TXNIP(Minn, Hafele, & Shalev, 2005). The increased TXNIP expression causes inhibition of the glucose transporter Glut1, resulting in suppressed glucose uptake(Parikh et al., 2007). Therefore, the glucose uptake and TXNIP expression form a negative feedback loop to maintain the energy homeostasis.

1.4.2 TXNIP and inflammation

TXNIP also plays a role in inflammation(T. Lane, Flam, Lockey, & Kolliputi, 2013). Zhou *et al* found that TXNIP could interact with NOD-like receptor protein 3 (NLRP3) and activate the inflammasome in pancreatic cells(R. Zhou, Tardivel, Thorens, Choi, & Tschopp, 2010). In unstressed cells, TXNIP binds to TRX1 and remains at inactive state, while under oxidative stress, the cumulative reactive oxygen spices (ROS) induces TRX1-TXNIP dissociation and enhances TXNIP-NLRP3 association. The assembly of the NLRP3 inflammasome activates caspase-1 and promotes IL-1β production(Sokolovska et al., 2013).

1.4.3 TXNIP and apoptosis

Besides TRX1, TXNIP is also able to bind to TRX2. In insulin secreting beta cells, TXNIP translocates into the mitochondria and interacts with TRX2 under oxidative stress(Saxena, Chen, & Shalev, 2010). The competitive binding of TXNIP releases apoptosis signal-regulating kinase 1 (ASK1) from TRX2 and leads to its activation. Activated ASK1 causes phosphorylation and activation of downstream targets including JNK and p38 MAPK, finally resulting in apoptosis(Hsieh, Kuro-o, Rosenblatt, Brobey, & Papaconstantinou, 2010; Sarker et al., 2003; Tobiume et al., 2001).

1.4.4 TXNIP as a tumor suppressor

TXNIP is recognized as a tumor suppressor because of its proliferation inhibition and pro-apoptotic function. The expression of TXNIP is greatly reduced in multiple types of cancers including breast cancer, lung cancer, gastric cancer, colon cancer, and hematopoietic malignancies(Cadenas et al., 2010; Kopantzev et al., 2008; Kwon et al., 2010; J.-H. Lee et al., 2010; Takahashi et al., 2002; J. Zhou & Chng, 2013).

1.4.4.1 TXNIP in solid tumor

In breast cancer, Cadenas *et al* reported that the cellular TXNIP level was positively related to the metastasis-free interval and induction of human epidermal growth factor receptor 2 (HER2) in breast cancer cell line caused a dramatic decrease in TXNIP expression level(Cadenas et al., 2010). Either the low TXNIP level or the combination with a high MYC level could be used as a prognostic factor in patients with triple negative breast cancer and is closely related with metastasis-free survival(Shen et al., 2015). This prognosis factor also has a strong relation with overall survival in tumors with p53 mutations. In non-small cell lung carcinomas, the TXNIP expression level is lower compared to the TXNIP level in adjacent normal tissues(Kopantzev et al., 2008). In lung large-cell carcinoma, a high level of TXNIP expression is associated with good prognosis(da Motta, De Bastiani, Stapenhorst, & Klamt, 2015). In a microarray analysis in gastric cancer tissues, 85% of the cancer cells showed decreased TXNIP levels and patients with a high TXNIP level and

low TXN level had much better prognosis while the adverse group had worse prognosis(Lim et al., 2012). Immunohistochemistry demonstrated that TXNIP expression is associated with a lack of lymph node involvement, no perineural invasion and moderate tumor differentiation. The TXNIP level was also reduced in colon cancer and treatment with suberoylanilide hydroxamic acid (SAHA) and 5-fluorouracil could induce the expression of TXNIP(Takahashi et al., 2002). In mouse model with deficient TXNIP, the incidence rate of hepatocellular carcinoma is as high as 40%(Sheth et al., 2006).

1.4.4.2 TXNIP in hematopoietic malignancies

In patients with diffuse large B-cell lymphoma (DLBCL), decreased expression of TXNIP usually associates with poor response to chemotherapy treatment such as glucocorticoid(C. Li et al., 2012; Tome et al., 2005). Further studies conducted in a murine T-cell lymphoma cell line and in patients with childhood acute lymphoblastic leukemia showed that TXNIP was up-regulated after glucocorticoid treatment either in cell line or in patients(Z. Wang et al., 2006). These results suggest that TXNIP may be an important mediator of glucocorticoid-induced apoptosis.

In AML, *Zhou et al* found that TXNIP level is much lower in either AML cell lines or patient cells, compared to that of healthy controls(J. Zhou et al.,

2011). Furthermore, treating cells with DZNep, an inhibitor of DNA metheyltransferase EZH2, could restore the expression of TXNIP, which indicates that TXNIP could be regulated through epigenetic mechanism.

Although the anti-tumor effect of JQ1 has been validated in multiple types of cancers, the details of the mechanism of JQ1 has not been fully understood. In different types of cancers, MYC depletion was observed after JQ1 treatment and thus became the main focus in researches of JQ1. The cell cycle arrest caused by MYC repression was proved to be an important mechanism of JQ1's anti-tumor effect. However, although MYC has been shown to be involved in cell survival, arrest in cell cycle usually does not directly lead to cell death. In a study in AML stem cells, researchers reported that JQ1 is able to induce cell death directly in leukemic stem cells, which suggests that other mechanisms may be also involved in the effect of JQ1. In this study, we aimed to solve the problem whether JQ1 induces cell death in AML, and if so, what the underlying mechanism is.

Through the cell growth assay and flow cytometry assay, I found that JQ1 not only inhibited the growth of AML cells, but also led to cell death. Further western blot and caspase activity assays confirmed that JQ1 induces cell death in AML cells through activating the intrinsic apoptotic pathway. Through analysis of published gene expression profile data, I identified TXNIP as a potential mediator in JQ1's cell death effect. qRT-PCR and western blot validated the up-regulation of TXNIP after JQ1 treatment, while knock-down experiments demonstrated TXNIP plays a role in JQ1-induced apoptosis. Flow

cytometry assay and rescue assay confirmed that ROS production is not the key mechanism of TXNIP induced apoptosis, and p38 MAPK pathway was shown to be the activated after JQ1 treatment, and treatment with phosphor-p38 inhibitor partially rescued the cell death effect caused by JQ1. Together, these results suggest p38 MAPK activation is an important mediator of JQ1-induced apoptosis in AML cells.

The mechanism of the up-regulation of TXNIP was also carefully studied. Epigenetic mechanism like histone demethylation through EZH2 was excluded as EZH2 level was not affected by JQ1. Through searching literatures and available data, we hypothesized that MYC is able to repress TXNIP expression through regulating miRNAs from the miR-17-92 cluster. This hypothesis was confirmed by the up-regulation of miR-17-92 transcripts and mature miRNAs upon JQ1 treatment. The direct targeting of 3’UTR of TXNIP by miRNAs was confirmed by luciferase assay with vectors containing either wild type or mutant TXNIP 3’UTR.

Chapter 2: Materials and methods

2.1 Cell culture and cryopreservation of cell lines

AML cell lines OCI-AML2 and OCI-AML3 cell lines were purchased from American Type Culture Collection (ATCC) and maintained in Minimum Essential Medium α (MEM α) with 20% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin antibiotics. All other AML cell lines used in this thesis (including MOLM-14, KG1, KG1a, Kasumi-1 and MV4-11) were purchased from ATCC and maintained in Roswell Park Memorial Institute (RPMI) -1640 with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin antibiotics. During cryopreservation, cells were suspended at 5 M/mL in complete medium with 10% FBS and 10% Dimethyl sulfoxide (DMSO). After being kept in -80 °C refrigerator overnight, cells were transferred into liquid nitrogen tank for long-term preservation.

2.2 Drug treatment

JQ1 was dissolved in dimethyl sulfoxide (DMSO) at 10 mM and stored in -20 °C refrigerator. A working stock was diluted at 1 mM. Cultured cells were washed with phosphate buffered saline (PBS) for three times and resuspended in appropriate culture media. Then cells were treated with JQ1 before harvested by centrifugation and washed with PBS for three times. Cell pellets were used for followed studies stored at -80 °C refrigerator.

MAPK inhibitor SB203580 was used in rescue experiments. The powder form of this inhibitor was dissolved in DMSO at 10 mM and stored in -20 °C

refrigerator. Cultured cells were washed with PBS for three times and resuspended in culture media. MAPK inhibitor or DMSO was added at the concentration of 25 µM into the cells 1 hour before JQ1 treatment. After treatment for 24hr or 48hr, cells were harvested for FACS analysis.

2.3 Cell growth assay

The relative numbers of cells under JQ1 treatment was measured using CellTiter-Glo Luminescent Cell Viability Assay (Promega). The experiments were performed according to the manufacturer's instruction. Briefly, cells were seeded in 96-well plate at the concentration of 2000 cells/well. After JQ1 treatment, 50 µL of CellTiter-Glo reagent was added to each well and mixed for 2 min on an orbital shaker to induce cell lysis. The plate was incubated at room temperature for 10 min to stabilize luminescent signal prior to recording luminescence using GloMax 96 Microplate Luminometer (Promega).

2.4 RNA extraction, cDNA conversion and quantitative Real-time PCR

The RNA extraction was performed according the manufacturer's protocol. Briefly, cell pellets were harvested and washed with PBS. For 5 million cells, 350 µL RLT buffer was used to resuspend the cell pellets and homogenization was accomplished by vortexing for 1 min. After homogenization, 1 volume of 70% ethanol was added into the lysate and mixed by pipetting. The lysate was then transferred into an RNeasy mini spin column sitting in a 2-mL collection tube, and centrifuged for 15 sec at 10000 g. The flow-through was

then discarded and 700 µL RW1 buffer and 500 µL RPE buffer were used to wash the column sequentially. After washing, the column was centrifuged for 1 min at 10000 g in a fresh collection tube. Finally, 10 µL EB buffer was used to elute the RNA from the column into a fresh microcentrifuge tube. The concentration of isolated RNA was determined using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific).

The complementary DNA (cDNA) were then synthesized using the iScript cDNA synthesis kit (Bio-Rad). For each reaction, the following system was used: 1 µg RNA, 4 µL reverse transcriptase, and 15 µL RNase-free water. The mixtures were proceeded to the reverse transcription in 200 µL PCR tubes according to following protocol:

25°C, 5 min;

42°C, 30 min;

85°C, 5 min.

The cDNA was kept at -20°C.

The real-time quantitative PCR was performed in Applied Biosystems 7300 Real-Time PCR System using iTaq Universal SYBR Green Supermix (Bio-Rad). The reaction system contained 10ul Supermix, 2 µL cDNA template, 0.5 µL primers, 7.5 µL nuclease-free water. The following protocol was used in the real-time PCR:

50°C, 2 min,

95°C, 10 min;

(95°C, 15 sec, 60°C, 1 min) X 40 cycles. The results were analyzed using the 7300 Real-Time PCR machine (Applied Biosystems) and Prism 5 (GraphPad software).

Table 4 List of real time PCR primers

Name	Sequence
MYC qPCR primer	For: 5’-AATGAAAAGGCCCCCAAGGTAGTTATCC-3’ Rev: 5’-GTCGTTTCCGCAACAAGTCCTCTTC-3’
TXNIP qPCR primer	For: TCATGGTGATGTTCAAGAAGATC Rev: ACTTCACACCTCCACTATC
miR-17-92 qPCR primer	For: CTGTCGCCCAATCAAACTG Rev: GTCACAATCCCCACCAAAC
GAPDH qPCR primer	For: GTATTGGGCGCCTGGTCAC Rev: CTCCTGGAAGATGGTGATGG

2.5 microRNA extraction and quantification

For microRNAs extraction, cells were washed with PBS for 3 times and pellets were collected by centrifugation. Cells pellets were lysed using QIAzol lysis buffer (QIAGEN) and homogenized at room temperature for 5 minutes. For 700 µL QIAzol lysis buffer, 140 µL of chloroform was added and mixed by vortexing. The mixture was then centrifuged at 12 000 g, 4°C for 20 minutes after incubating on ice for 5 minutes. The upper aqueous phase containing RNA was transferred into a new centrifuge tube carefully without disturbing the lower organic phase. One and a half volume of 100% ethanol was added and mixed by vortexing. The mixture was then transferred into an RNeasy mini spin column and centrifuged at 12 000 g for 15 seconds. The flow-through was discarded and the column was washed using 700 µL Buffer RWT, 500 µL Buffer RPE, and 500 µL Buffer RPE, subsequently. After that, the

column was placed on a new collection tube and centrifuged at 12 000 g for 2 minutes to dry up the ethanol. The microRNAs was eluted using 10 µL RNase-free water into a new centrifuge tube. The concentration was measured using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

The quantification was performed using TaqMan microRNA assays. First, cDNA was synthesized using TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific). MicroRNAs were diluted and mixed in a 15 µL system according to the following recipe, 0.15 µL 100mM dNTPs, 1 µL MultiScribe™ Reverse Transcriptase, 1.5 µL 10✕ Reverse Transcription Buffer, 0.19 µL RNase Inhibitor, 4.16 µL Nuclease-free water, 5 µL 2 ng/µL miRNA sample, 3 µL 5 X RT primer. The reverse transcription was performed using the following program on a Bio-rad T100 thermal cycler, 16°C, 30 min; 42°C, 30 min; 85°C, 5min; 4°C, forever.

After reverse transcription, the product was mixed in following system, 1ul TaqMan MicroRNA Assay (20X), 1.33 µL RT-PCR product, 7.67 µL Nuclease-free water, 10 µL TaqMan 2✕ Universal PCR Master Mix. The qPCR was performed using the following program on a Applied Biosystems 7300 Real-Time PCR Machine, 95°C, 5 min; 95°C, 15s; 60°C, 1 min; 40 cycles. The results were analyzed using GraphPad Prism 5.

2.6 Protein extraction and Western Blot

Cultured cells were collected by centrifugation and washed with PBS for three times. Cell pellets were resuspended in NP-40 lysis buffer containing 20mM Tris pH8, 10% glycerol, 150mM NaCl, 0.1% NP-40, 0.1mM EDTA and 1 X protease inhibitor and 1 X phosphatase inhibitor. The cells were kept on ice

for 15 minutes to completely lyse the cells and then centrifuged at 12500 g, 4°C for 30 min. After centrifugation, the supernatants containing whole cell protein were transferred into a new microcentrifuge tube.

The concentrations of extracted proteins were then quantified using Bradford protein assay. Briefly, 1 μL of protein was diluted into 10 μL using milliQ water. Then 200 mL Bradford solution (Bio-Rad) was added and mixed. The absorbance at 595 nm was measured using GeneQuant 1300 spectrophotometer (GE Healthcare). The absolute concentrations of proteins were calculated based on a standard curve regressed from results of bovine serum albumin (BSA) at a serially diluted concentration from 1 mg/uL to 0.125 mg/uL.

After quantification, the 2 X SDS sample buffer (Bio-Rad) containing 5 % 2- Mercaptoethanol (Bio-Rad) was added into respected protein samples at 1: 1 ratio. The mixtures were then boiled at 95 °C for 5 min to denature the proteins.

The SDS-polyacrylamide gels were casted into two layers, upper one is stacking layer containing 4% (w/v) acrylamide-bisacrylamide (Bio-Rad) and lower one is resolving gel containing 7.5% -12% (w/v) acrylamide-bisacrylamide (Bio-Rad), depending on the size of target protein.

The protein samples and protein ladder were then loaded into the gel and the running buffer containing 25 mM Tris pH8.3 (Bio-Rad), 190 mM Glycine(Bio-Rad), and 0.1% SDS (Bio-Rad) was added into the rack. Electrophoresis was then carried out at 60 V for 15 min, followed by 120 V for

1 hour. After electrophoresis, the gel containing proteins was cut and PVDF membrane (Bio-Rad) was also cut at the same size. The transfer rack was carefully assembled to avoid bubbles in the sequence of one layer of cotton, one layer of filter paper, protein gel, PVDF membrane, one layer of filter paper, one layer of cotton. The rack was immersed in pre-chilled transfer buffer containing 25 mM Tris pH8.3 (Bio-Rad), 190 mM glycine (Bio-Rad), and 20% methanol. The proteins on the gel were then transferred to PVDF membrane using electrophoresis at 400 mA for 90 min in the 4 °C cold room. The PVDF membrane containing proteins was then blocked with 5% non-fat milk in phosphate buffered saline with 0.01% Tween-20 (Bio-Rad) (PBST) for 1 hour.

The PVDF membrane was then washed three times with PBST for 30 min and incubated in primary antibodies diluted in 5% non-fat milk in 0.01% PBST at 1: 1000 ratio for 1 hour at room temperature or overnight at 4°C. After incubation, the membrane was washed with 0.01% PBST three times for 30 min. The second antibodies were diluted in 5% non-fat milk in 0.01% PBST at 1: 5000 ratio and incubated with membrane for 1hr at room temperature followed by three times washing with 0.01% PBST for 30 min. The membrane was then dried up using tissue paper and the enhanced chemiluminescence (ECL) reagent was mixed at 1:1 ratio before applied to respective size of target protein on the membrane. The chemiluminescent signal was captured either using films exposure or CCD imager (ImageQuant LAS 500).

The antibodies used in this thesis were listed in Table 5.

Table 5 List of antibodies

Antibody	Company	Catalog number
MYC (9E10)	Santa Cruz Biotechnology	sc-40
TXNIP	MBL International	K0205-3
β-actin HRP	Santa Cruz Biotechnology	sc-47778
Ezh2	Cell Signaling Technology	3147
Cleaved PARP	Cell Signaling Technology	9541
Caspase-3	Cell Signaling Technology	9661
Caspase-9	Cell Signaling Technology	9501
phospho-p38	Cell Signaling Technology	9211
p38	Cell Signaling Technology	9212
Goat anti-rabbit IgG HRP	Santa Cruz Biotechnology	sc-2030
Goat anti-mouse IgG HRP	Santa Cruz Biotechnology	sc-2005

2.7 DNA purification

DNA purification was performed using the QIAGEN minElute PCR purification kit according to the manufacture's protocol. Briefly, 1 volume of PCR products or elution products from Chromatin Immunoprecipitation were mixed with five volume of PB buffer through vortexing. The mixture was applied to a MinElute column and centrifuged at maximum speed for one minute. The flow-through was discarded and the column was washed using 750 µL of PE buffer and centrifuged at 12 000g speed for one minute. The flow-through was discarded and the empty column was centrifuged for 2 min at maximum speed before placed on a clean 1.5 mL microcentrifuge tube. 10

µL EB buffer was added to the center of the membrane on the column. The column was centrifuged at maximum speed for 2 min. The elution containing DNA was collected in microcentrifuge tube.

2.8 Caspase activity assay

The activities of caspase-3 and caspase-7 were measured using Caspase-Glo 3/7 assay (Promega). The experiments were performed according to the manufacturer's instruction. Briefly, cells were seeded in 96-well plate at the concentration of 2000 cells/well. After JQ1 treatment, 50 µL of Caspase-Glo 3/7 reagent was added for each well and mixed for 30 min on plate shaker at room temperature to induce cell lysis. The lysates were then transferred into white-walled 96-well plate prior to recording luminescence using GloMax 96 Microplate Luminometer (Promega).

2.9 Flow cytometry assay

Flow cytometry assay was employed to examine the apoptosis of cell lines. Cells were harvested and washed with PBS for 3 times and resuspended in 100 µL 1 X binding buffer. For 1 X 10^6 cells, 1 µL propidium iodide (PI) and 1.5 µL FITC-Annexin V was added and incubated in the dark at room temperature for 30 min to stain the cells. After staining, 400 µL 1 X binding buffer was added into each sample and the cells were filtered into 5ml FACS tubes (BD Falcon) through a cell strainer to get a single cell suspension. The FITC and Propidium Iodide (PI) fluorescence signals of stained cells were then captured

using BD LSRII (BD Bioscience). The results were analyzed by FlowJo and Prism 5 (GraphPad software).

2.10 Chemical transformation of *Escherichia coli*

The PCR products or purified plasmids were transformed into *E.coli* for expansion. Briefly, the chemically-competent *Escherichia coli* strain MAX Efficiency DH5α obtained from Life technologies were thawed on ice. For 50 µL of bacteria, 500 ng of plasmid or 5 µL of PCR products were added. The mixture was kept on ice for 30min before heat shock for 90 seconds in 42°C water bath. After heat shock, 800 µL of Lysogeny broth (LB) culture media was added and the mixture was shaken at 200 g 37°C for 40 min to recover the *E. coli* cells. Then the cultured bacterial cells were spread on LB plates containing Ampicillin or Kanamycin based on the resistance gene encoded by the plasmid. The LB plates were cultured at 37°C overnight and single colonies were picked to continue culture in LB media containing respective antibiotics for further extraction of plasmids.

2.11 Plasmid extraction

Mini plasmid extraction for validation was performed using E.Z.N.A.® Plasmid DNA Mini Kit I (Omega Bio-Tek) according to the manufacturer's protocol. Bacteria cells were harvested by centrifugation at 10 000 g for 1 min at room-temperature and the supernatant was discarded. For less than 5 mL bacterial culture, 250 uL Solution I /RNase A was added and homogenized by vortexing. Mixture was incubated at room temperature for 3 min after adding 250 uL Solution II and mixing. After a clear lysate was obtained, 350 uL

Solution III was added and the solution was mixed thoroughly and immediately before proceeding to centrifuge at 12000 g for 10 min. The supernatant was then transferred into a HiBind DNA Mini Column and centrifuged at 12000 g for 1 min. The column was then washed with 500 uL HB Buffer and 700 uL DNA Wash Buffer followed by centrifugation at 12000 g for 2 min to dry the column matrix. The plasmid DNA was then eluted into a microcentrifuge tube using 20 uL Elution Buffer and centrifuged at 12000 g for 1 min. The plasmid concentration was then measured using NanoDrop 2000 Spectrophotometer.

Large amount of plasmid extraction was performed using QIAGEN Plasmid Maxi kits (QIAGEN) according the manufacturer's protocol. First, freshly cultured bacteria cells were harvested by centrifugation at 6000 x g for 15 min at 4°C. The pellet was resuspended in P1 buffer. For 100 mL of bacteria cells, 10 mL P1 buffer was used. Then 10 mL P2 lysis buffer was added and mixed thoroughly by vigorously inverting the tubes, and incubated at room temperature for 5min. Next 10 mL of pre-chilled P3 Buffer and mixed immediatedly and thoroughly by vigorously inverting. The mixture was incubated on ice for 20 min to achieve neutralization. The mixture was then centrifuged at 20000 x g for 30 min at 4°C twice, and the precipitate was discarded and the supernatant containing plasmid DNA was applied to a QIAGEN-tip pre-equilibrated using 10 mL Buffer QBT. The column was emptied by gravity flow. After that, the QIAGEN-tip was washed using 30 mL of Buffer QC twice and the DNA was eluted using 15ml of Buffer QF into a 50 mL Falcon tube. 10.5 mL room-temperature isopropanol was added into the

elution and mixed thoroughly to precipitate the plasmid DNA and the tube was then centrifuged at 15000 x g for 30 min at 4°C. The supernatant was carefully decanted and the DNA pellet was washed using 5 mL of room-temperature 70% ethanol and centrifuged at 15000 x g for 15 min at 4°C. The supernatant was decanted and the DNA pellet was air-dried for 2 hours at room-temperature and dissolved in 200 – 500 µL of TE buffer, pH8.0. The concentration of isolated plasmid DNA was measured using NanoDrop 2000 Spectrophotometer (Thermo Scientific).

2.12 Chemical transfection and electroporation

Transfection of 293T cells was accomplished using jetPRIME transfection reagent (Polyplus-transfection). Cells were seeded in 10 cm petri dish at 70% confluence one day before transfection. One the day of transfection, for each 10 cm plate, 10 µg plasmid DNA or 20 nM siRNA were mixed with 500 µL jetPRIME buffer and 20 µL jetPRIME reagent and incubated at room temperature for 10 min. Then the mixture was added into cells dropwise. After incubation of desired time, RNA or protein was isolated to perform following analysis.

Suspension cells were transfected by electroporation using Neon transfection system according to the manufacturer's instruction. The Neon device and Neon Pipette Station were set up according to the instruction. The Neon Tube was filled with 3 mL Electrolytic Buffer E2. Cells growing at Log phase were harvested and washed three times with PBS and resuspended in 110 µL Resuspension Buffer R at a final density of 5 X 10^7 cells/ mL prior to mixing with plasmid DNA at 10 µg / 1 X 10^6 cells or siRNA at 25 nM. The mixture was

then transferred into the 100 µL Neon tips using Neon pipette and the Neon pipette was installed on the Neon tube. Electroporation was then performed using pre-optimized protocol. After electroporation, the cells were cultured in respective culture media and harvested at desired time point.

2.13 Chromatin immunoprecipitation (ChIP)

The ChIP experiments were performed according to a protocol inside the lab. Briefly, cells at log phase were used. Formaldehyde was added to a final concentration of 1% and the mixture was shaken gently at room temperature for 10 min. Then glycine was added to a final concentration of 0.125 M to stop the cross-linking reaction. After shaken at room temperature for 15 min, the cells were harvested by centrifugation at 2000 g for 5 min at 4°C and then washed with PBS.

Table 6 Buffers used in ChIP experiment

Buffers	recipes
FA cell lysis buffer	10 mM Tris-HCl pH8, 0.25% Triton-X100, 10 mM EDTA, 100mM NaCl
1% SDS FA lysis buffer	50 mM HEPES-KOH pH7.5, 1% Triton-X100, 2 mM EDTA, 150 mM NaCl, 0.1% NaDOC, 1% SDS
0.1% SDS FA lysis buffer	50 mM HEPES-KOH pH7.5, 1% Triton-X100, 2 mM EDTA, 150 mM NaCl, 0.1% NaDOC, 0.1% SDS
NP40/LiCl	10 mM Tris pH8, 0.25M LiCl, 1 mM EDTA, 0.5% NP40, 0.5% NaDOC
ChIP elution buffer	50 mM Tris-HCl, 10 mM EDTA, 1% SDS

Cell pellets were resuspended in pre-chilled FA lysis buffer (20 million cells in 12 mL buffer) with EDTA-free protease inhibitor and PMSF (1: 1000). The mixture were then rotated for 15 min at 4°C and then centrifuged at 800 g for 10 min at 4°C. The supernatant was discarded carefully.

1% SDS FA lysis buffer was pre-thawed at 37°C to dissolve the SDS precipitate and cooled down on ice. EDTA-free protease inhibitor and PMSF (1: 1000) were then added to the 1% SDS FA lysis buffer. The cell pellets were resuspended in 1% FA lysis buffer (20 millon cells in 12 mL buffer). The suspension were rotated for 15 min at 4°C and then centrifuged at 12000 g for 20 min at 4°C. The supernant were discarded carefully.

Cell pellets were resuspended in pre-chilled 0.1% SDS FA lysis buffer (20 million cells in 12 mL buffer) with EDTA-free protease inhibitor and PMSF (1: 1000). The mixture were then rotated for 15 min at 4°C and then centrifuged at 800 g for 10 min at 4°C. The supernatant was discarded carefully. The pellets containing nuclei were resuspended in 0.1% SDS FA lysis buffer (20 million cells in 1 mL buffer) and homogenized by pipetting. The samples were used for sonication or stored at -80°C.

The homogenized samples were then transferred into a BD FACS tube and sonicated using a proper condition that varies among different cell lines. After sonication, the samples were centrifuged at 12000 g for 30 min at 4°C. The supernatant were then transferred into a new centrifuge tube and used for followed experiments or stored at -80°C.

The sonication results were examined using DNA electrophoresis. 5 μL sonicated samples and 5 μL RNase A were added into 45 μL 0.1% SDS FA lysis

buffer. The mixture was incubated at 37°C for 30 min before adding 5 µL protease K and then incubated at 62°C for 2 hours. The size of decrossed-linked DNA was checked using electrophoresis on 2% agarose gel. The DNA fragments should be between 200 -1000 bp.

Dynabeads protein G from Lifetech were used for the immunoprecipitation. Magnetic beads were homogenized by vortexing. The slurry was washed by PBS and then placed on the magnetic stand to remove the supernatant. For 100 µL slurry, 200 µL PBS was used to resuspend the magnetic beads into 50% slurry. The slurry was used to pre-clear chromatin and to bind antibodies. For 50 µL of slurry, 5 µg primary antibody and 450 µL PBS were added. The mixture was vortexed and rotated at 4°C for 2 hours. For 100 µL slurry, 400 µL sonicated sample was added and vortexed and rotated at room temperature for 2 hours. The magnetic beads bound with antibody were washed with PBS. The supernatant was removed using magnetic stand (Invitrogen). The pre-cleared samples were centrifuged at 12000 g for 10 min at 4°C. The supernatant was collected. For each sample, 10 µL pre-cleared chromatin sample was collected as input. Each viral of magnetic beads was resuspended in 500 µL pre-cleared chromatin sample and rotated at 4°C overnight for immunoprecipitation.

The immunoprecipited magnetic beads were collected by centrifugation at 4000 g for 10 min at 4°C. The supernatant was removed using the magnetic stand. The beads were washed twice using 1 mL 0.1% SDS FA lysis buffer and rotated for 5 min at room temperature. The supernatant was removed using the magnetic stand and beads were resuspened in 1 mL NP40/LiCl buffer. The

beads were rotated for 5 min at room temperature and the supernatant was removed using the magnetic stand. The beads were resuspened in 1 mL TE buffer and rotated for 5 min at room temperature. The supernatant was removed using the magnetic stand. The beads were resuspended in 100 µL elution buffer. The input of precleared chromatin was also thawed and each 10 µL elution buffer was added into 90 µL elution buffer. For each 100 µL elution buffer, 5 µL protease K wash added. The mixture were shaken at 600 g for 2 hour at 62°C. The DNA contained in the supernatant was extracted using the QIAGEN minElute PCR purification kit (QIAGEN). The DNA was quantified using real time qPCR with primers targeting TXNIP promoter region.

2.14 Luciferase assay

The culture media of 293T cells was removed and cells were washed with PBS carefully to avoid dislodging them. Passive lysis buffer (PLB) (Promega) was diluted from 5X stock and added into the culture dish. For a 10 cm dish, 1 mL of PLB was used. The cultured cells were lysed on an orbital shaker at room temperature for 15 min. The cell lysates were transferred into a microcentrifuge tube. The protein concentrations were measured using Bradford assay as mentioned above in Chapter 2.6. 20 µL protein and 100 µL of luciferase assay reagent (LAR) were mixed in a new microcentrifuge tube and the luciferase activities were measured using a GloMAX 20/20 singe tube

luminometer. The relative luciferase activities were normalized using the protein concentrations.

2.15 Statistical analysis

Experimental data are presented as the mean ± standard error (SEM). All statistical analyses were performed using analysis of a two-tailed Student's t test with GraphPad Prism 5. Differences were considered statistically significant when P values were less than 0.05. * indicates $P<0.05$; ** indicates $P<0.01$; *** indicates $P<0.001$;

Chapter 3 Results

3.1 JQ1 inhibits cell growth in AML cell lines

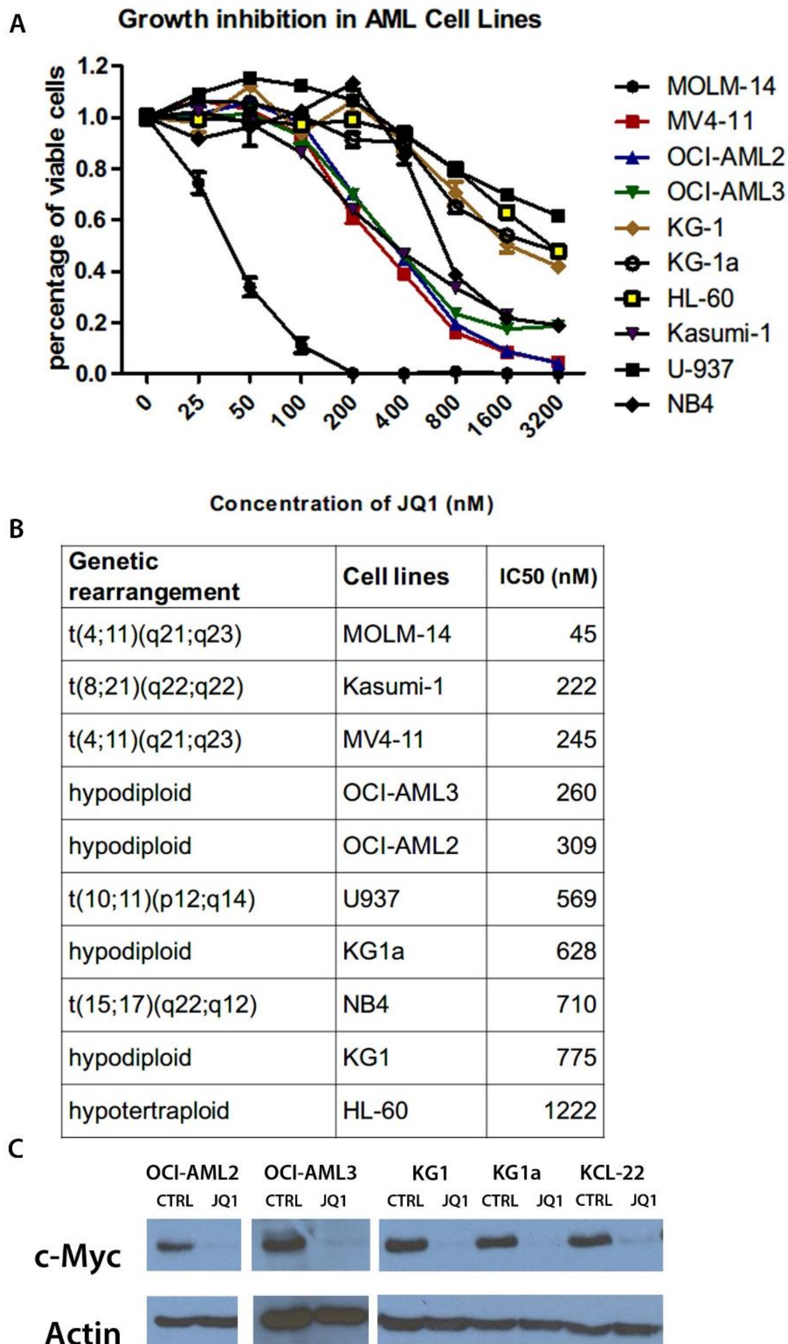
JQ1 has been reported to repress cell proliferation in many different types of cancer, such as NUT-midline carcinoma, multiple myeloma, Burkitt's lymphoma, acute lymphoblastic leukemia, and acute myeloid leukemia(Filippakopoulos et al., 2010; Mertz et al., 2011; Toyoshima et al., 2012; Zuber et al., 2011). The anti-growth effect of JQ1 has also been reported in solid tumors like glioblastoma, neuroblastoma, prostate carcinoma, and non-small cell lung cancer.(Asangani et al., 2014; Z. Cheng et al., 2013; Puissant et al., 2013; Shimamura et al., 2013). Most of these studies suggest that one of the main therapeutic mechanisms is via the suppression of MYC. However, JQ1 treatment induces significant changes in gene expression. It is likely that other mechanisms may be involved and differing mechanisms may exist in different cell types and diseases.

To further investigate the effect of JQ1 in AML, we first checked if JQ1 could impair the growth of AML cell lines using CellTiter-Glo assay (Promega). After treatment with JQ1 for 48 hours, all AML cell lines tested showed significant growth inhibition, which demonstrated the anti-growth effect of JQ1 in AML (Fig. 3.1A). Furthermore, this inhibition is dose-dependent, suggesting a specific effect of JQ1 on AML cell lines. The IC_{50} of each cell line was calculated according to the definition of the US FDA using GraphPad Prism (Fig. 3.1B). It is clear that the IC_{50}s of most cell lines were in the nanomolar range, which indicates that JQ1 is potent in AML and is of

potential clinical utility. It is therefore important to study and further elucidate the mechanisms of JQ1-mediated cell death in AML.

Interestingly, it seems that the sensitivity to JQ1 does not depend on the karyotype of AML cells. AML cell lines harboring different genetic alterations, such as chromosome rearrangement and genetic mutations including NPM1 and FLT3, showed similar responses to JQ1 treatment. For example, MV4-11 harbors MLL-AF4 translocation and is very sensitive to JQ1 treatment. OCI-AML2 and OCI-AML3 have similar IC_{50} compared with MV4-11, but only the latter harbors the NPM1 mutation.

This is worth noticing because in a previous study of another BRD4 inhibitor, i-BET151, it was reported that the anti-proliferation effect of i-BET151 depends on the MLL translocation status(Dawson et al., 2011). Only AML cells with MLL translocations are sensitive to i-BET151 treatment. This seemingly contradictory result suggests that JQ1 and i-bet151 may have different mechanisms of action, despite both targeting BRD4.



Genetic rearrangement	Cell lines	IC50 (nM)
t(4;11)(q21;q23)	MOLM-14	45
t(8;21)(q22;q22)	Kasumi-1	222
t(4;11)(q21;q23)	MV4-11	245
hypodiploid	OCI-AML3	260
hypodiploid	OCI-AML2	309
t(10;11)(p12;q14)	U937	569
hypodiploid	KG1a	628
t(15;17)(q22;q12)	NB4	710
hypodiploid	KG1	775
hypotertraploid	HL-60	1222

Figure 3.1 The anti-growth effect of JQ1 in AML cell lines. (A) Cell growth analysis using CellTiter-Glo (CTG) assay. The cells were treated with JQ1 at gradient concentration for 48 hours and the cell viability was measured using CellTiter-Glo. The growth rates were normalized to cells without treatment. (B) The chromosomal abnormalities and IC50 of each cell line. IC50 was calculated using GraphPad Prism 5. (C) Western blot results showing the MYC level change after JQ1 treatment. Cells were treated at respective IC50 concentration for 48 hours before they were lysed for Western blot analysis. Antibodies against MYC and actin were used to detect the protein levels.

MYC has been identified to be a major downstream target of JQ1 in different cancer types (Delmore et al., 2011; Mertz et al., 2011; Ott et al., 2012). It is reported that JQ1 could repress MYC expression through inhibiting the pro-transcription activity of BRD4. Therefore, it is necessary to examine the MYC level change upon JQ1 treatment.

Cells were treated with JQ1 and the protein levels of MYC were detected using Western blot analysis. All cell lines tested have high level of MYC expression, a known hallmark of AML, before JQ1 treatment. Consistent with previous publications, MYC expression was significantly inhibited by JQ1 as MYC proteins were completely depleted after treatment (Fig. 3.1C).

To summarize, these results demonstrated that JQ1 could inhibit the proliferation of most AML cell lines, regardless of their chromosome status, which is in contrast with the mechanism of i-BET151. In addition, Western blot results showed that MYC were depleted after JQ1 treatment, which is reported to be the main reason for the cell growth inhibition caused by JQ1 in many types of cancer. However, whether the JQ1-induced MYC depletion has any other effects in AML, besides cell cycle arrest, remains unclear and needs further study.

3.2 JQ1 induces apoptosis in AML cell lines by activating caspase pathway

The anti-growth effect of JQ1 has been reported in many different kinds of cancer. JQ1 could induce cell cycle arrest through inhibiting MYC expression

in AML, multiple myeloma, and lymphoma.(Delmore et al., 2011; Mertz et al., 2011; Zuber et al., 2011)

Besides controlling cell cycle and proliferation, MYC also plays an important role in cell survival. Therefore, it would be interesting to explore whether JQ1 has anti-survival effect in cancer cells, which would add more potential to this promising drug as cell-killing effect is critical in the elimination of non-dividing cancer stem cells, which are often causes of relapse.

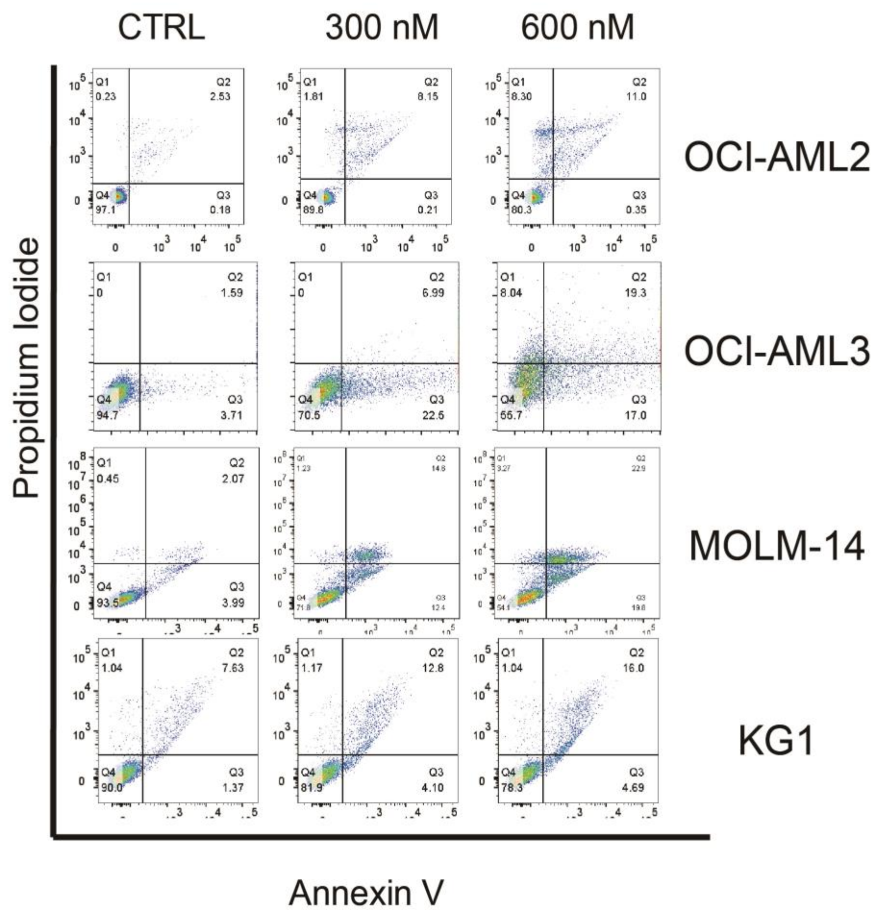
However, there are only a few reports about the role of JQ1 in cell death induction. Mertz et al reported that JQ1 is able to induce apoptosis in multiple myeloma cell line LP-1(Mertz et al., 2011), while Delmore et al found that JQ1 only led to a modest induction of apoptosis in another multiple myeloma cell line MM.1S(Delmore et al., 2011). Ott et al reported that JQ1 induced cell death in B-ALL cells(Ott et al., 2012), and Emadali et al also showed that JQ1 could enhance the cell death induced by Rituximab in resistant B-cell lymphoma cell lines mediated by CYCLON(Emadali et al., 2013). In AML, JQ1's ability in inducing cell death was also poorly studied. Only two studies have shown JQ1 is able to induce cell death while no mechanism was explored(H. Herrmann et al., 2012; Stewart, Horne, Bastow, & Chevassut, 2013). Considering the role of MYC in maintaining cell viability and mediating cell death, it is of great importance for us to explore the anti-survival effect of JQ1 in AML.

I first checked whether JQ1 treatment could lead to cell death in AML cell lines using flow cytometry assay. Four AML cells lines, OCI-AML2, OCI-AML3,

MOLM-14, and KG1 were treated with JQ1 and stained with Propidium iodide and Annexin V-FITC before they were measured using a BD LSR II flow cytometer. The data were analyzed using FlowJo software.

Fig. 3.2A shows the cell populations stained with Propidium iodide (PI) and Annexin V. The Annexin V positive populations are considered cells undergoing apoptosis and the percentage of apoptotic cells were summarized in Fig. 3.2B. The results showed that JQ1 is able to induce strong apoptosis in all four AML cell lines tested, and this apoptotic effect of JQ1 is also dose-dependent.

A



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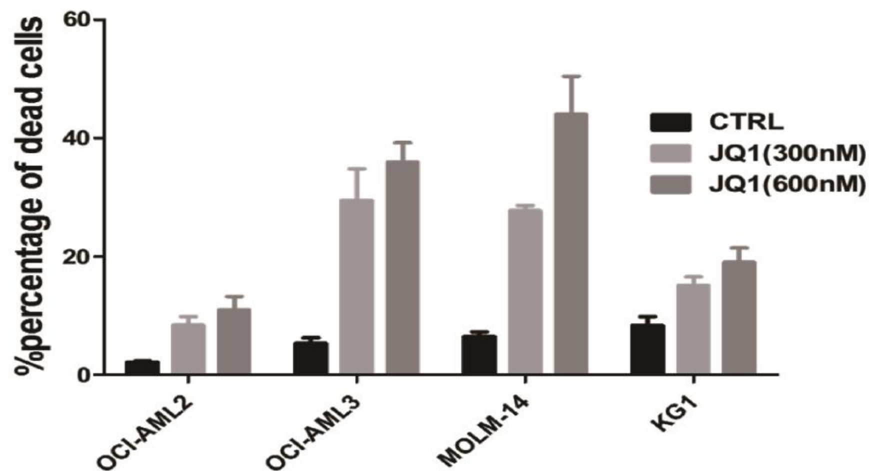

Figure 3.2 Flow cytometry assay showing JQ1-induced apoptosis in AML cell lines. (A) Scatter plots showing the PI staining and Annexin V staining in JQ1 treated AML cell lines. Cells were treated with JQ1 at various concentrations for 48 hours before they were stained with PI and Annexin V. The cells were then analyzed using a BD LSR II flow cytometer. (B) Histogram of percentages of apoptotic cells after JQ1 treatment in AML cell lines. The percentages of Annexin V + cells were shown.

These findings demonstrate the anti-survival effect of JQ1 in AML and also indicate that besides cell cycle arrest, apoptosis also plays a role in the anti-tumor effect of JQ1. The results also prove that JQ1 induces programmed cell death in AML cells, which could be related to its gene-specific effects.

As the apoptotic effect of JQ1 is rarely mentioned previously, it is important to further dissect the mechanisms of apoptotic cell death.

3.3 JQ1 activates intrinsic apoptotic pathway

To further investigate how JQ1 induced apoptosis in AML, the protein levels of Poly ADP ribose polymerase (PARP) were examined. PARP responses to DNA damage and mediates DNA repair in normal cells. During programmed cell death, PARP protein is cleaved by caspase-3, resulting in an inactive form, which blocks DNA repair and finally leads to apoptosis. Therefore, PARP cleavage is commonly regarded as a sign of an activated caspase pathway.

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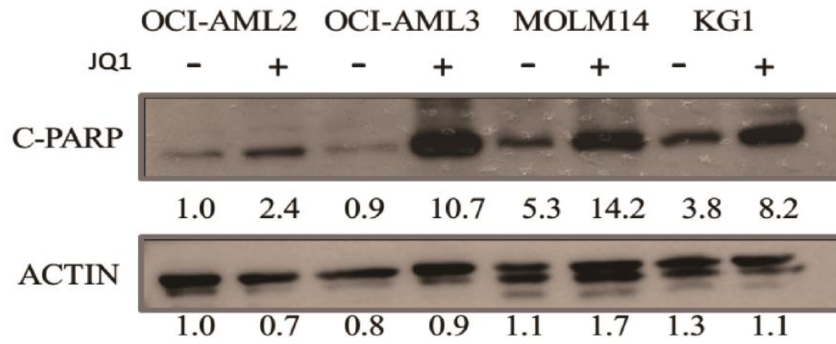

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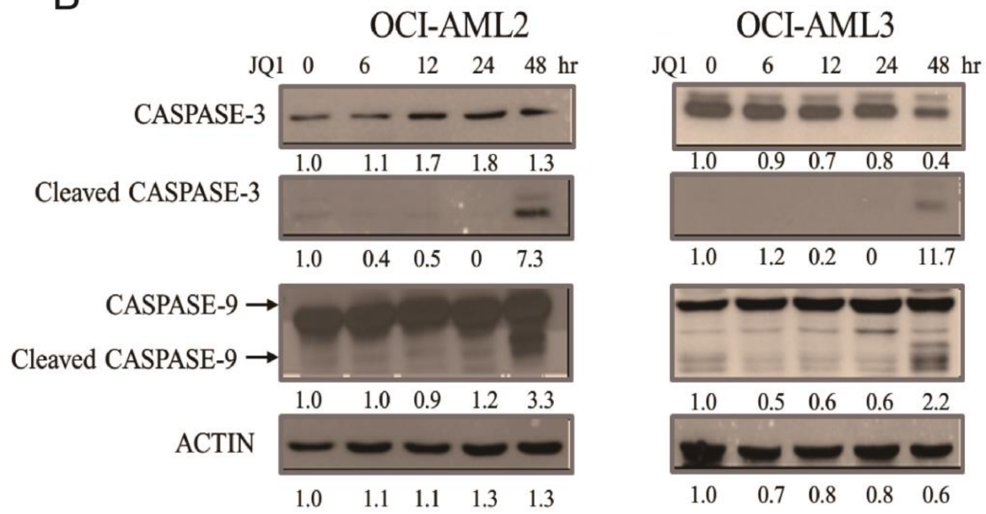

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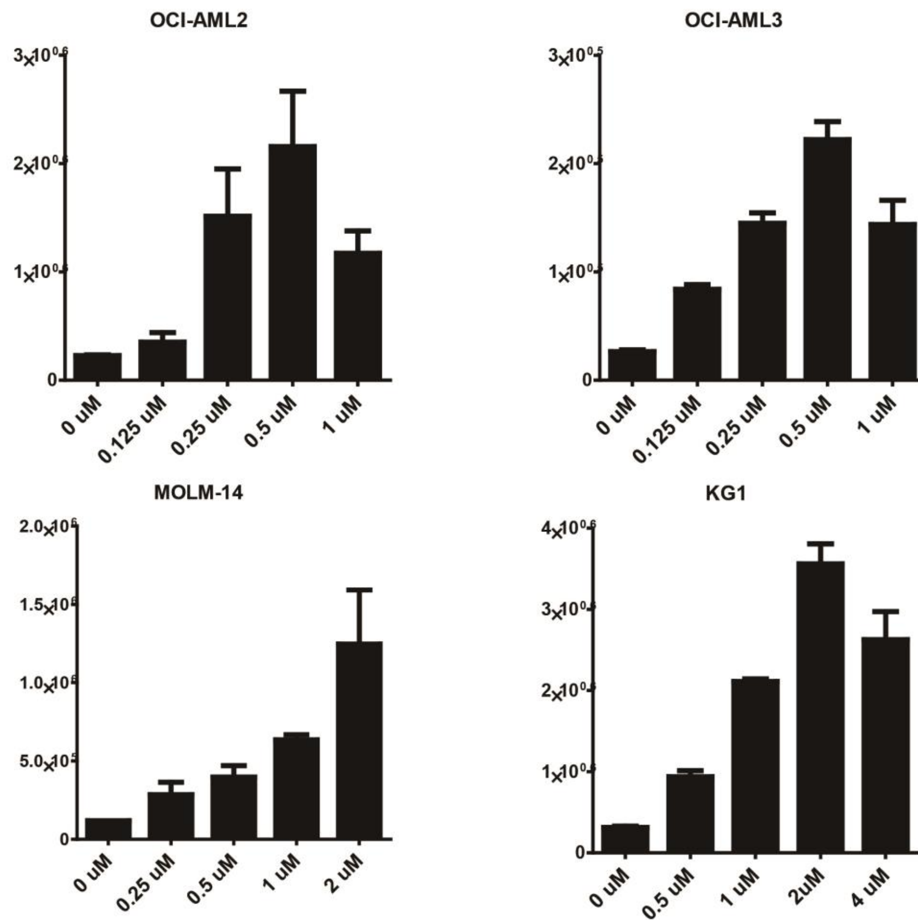

Figure 3.3 JQ1 activates intrinsic apoptosis pathway. (A) The western blot results of cleaved PARP after JQ1 treatment. Cells were treated with JQ1 at respective IC_{50} for 24 hours. (B) The western blot results of caspase-3 and caspase-9. Cells were treated with JQ1 at respective IC_{50} and harvested at different time points before lysed for western blot. Antibodies against full length and cleaved caspase 9, full length and cleaved caspase 3, and actin were used to detect protein levels. (C) Caspase activity assay of dose-dependent JQ1 treated AML cell lines. Cells were treated at respective dose for 48 hours before adding Caspase-Glo 3/7. The mixture was then analyzed using a GloMax-Multi+ Microplate Multimode Reader. For each concentration, triplicate samples were prepared. The relative activities were normalized to culture medium.

As shown in Fig. 3.3A, the cleavages of the PARP protein were observed in all cell lines tested after JQ1 treatment, which indicates the activation of the caspase pathway. The caspase proteins were detected using western blot.

The results clearly showed that Caspase -9 and its downstream target, Caspase -3, were cleaved after JQ1 treatment for 48 hours, demonstrating the activation of the intrinsic apoptosis pathway (Fig. 3.3B).

To further validate the activation of the apoptotic pathway, caspase activities were also examined using Caspase-Glo 3/7 assay as described in Chapter 2.8. The results showed that the activities of Caspase -3 and Caspase -7 increased in a dose-dependent manner after JQ1 treatment for 48 hours in AML cells (Fig. 3.3C). In OCI-AML2, OCI-AML3, and MOLM-14, the cells treated with the highest concentration (1uM, 1uM, and 4uM, respectively) of JQ1 showed relatively decreased caspase activity. This might be due to excessive cell death upon high concentration of JQ1 treatment.

Taken together, these results demonstrate that JQ1 leads to the activation of the intrinsic apoptosis pathway in AML cells, which finally leads to apoptosis. This induction of caspase activation is both dose- and time-dependent.

3.4 TXNIP is an important mediator in JQ1-induced apoptosis

We have found JQ1 could induce cell death through the intrinsic apoptosis pathway. However, the key mediator of JQ1-activated apoptosis in AML remained unclear.

Several groups have performed mRNA microarray in JQ1-treated cell lines(Mertz et al., 2011; Zuber et al., 2011). Through studying these published gene expression profile (GEP) data, we identified a set of genes whose expressions were consistently changed upon JQ1 treatment (Fig. 3.4). Among these genes, Thioredoxin-interacting protein (TXNIP) caused our attention as

it has also been identified to be the mediator of apoptosis caused by another small molecule drug, DZNep.

TXNIP has long been known as a mediator of glucose uptake and the redox control system. More importantly, our research group has previously reported that this gene is a tumor suppressor and induces apoptosis in AML (J. Zhou et al., 2011). As the major function of BRD4 is activating transcription, there are fewer genes that were up-regulated upon JQ1 treatment compared to those that are down-regulated. Thus, the up-regulation of TXNIP after JQ1 treatment is worthy of further investigation.

Lower levels of TXNIP, has been found in different types of cancer, compared to normal tissue, including hepatocellular carcinoma, breast cancer, bladder cancer and leukemia(J. Zhou & Chng, 2013). In leukemia, our research group has found that TXNIP expression is much lower in AML cell lines and primary AML patient cells than its expression in healthy controls. More importantly, overexpression of TXNIP was shown to induce apoptosis in AML cells(J. Zhou et al., 2011) and in T-lymphoma cells(C. L. Chen et al., 2008). Others had also reported that TXNIP was the key target gene involved in glucocorticoid-induced apoptosis in acute lymphoblastic leukemia(Z. Wang et al., 2006). So it is of great interest for us to study the role of TXNIP in JQ1-induced apoptosis in AML.

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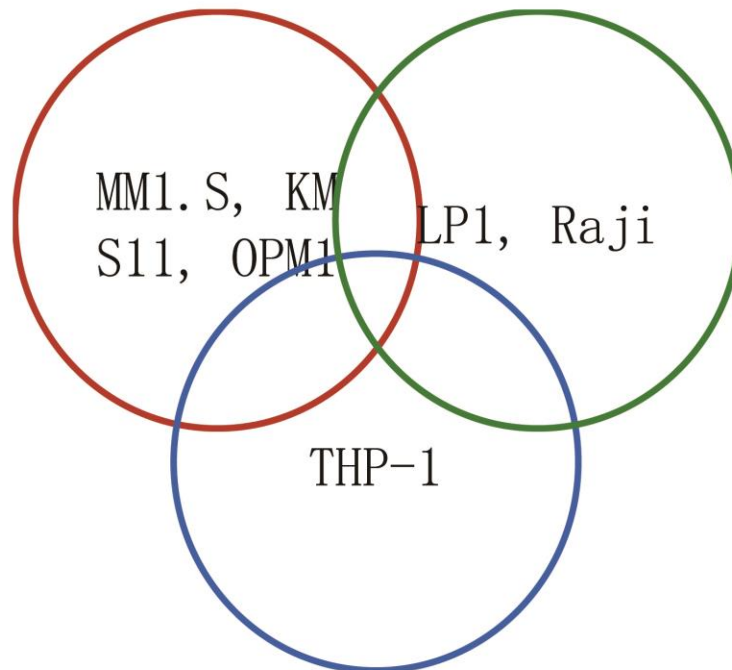


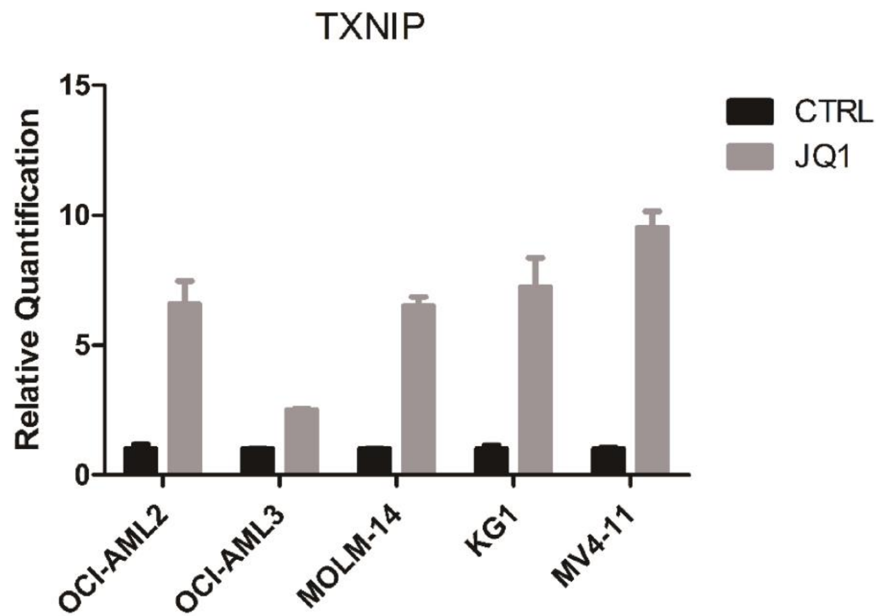
B

Up-regulated genes

BMPR2	involved in endochondral bone formation and embryogenesis
C1orf63	Arginine/Serine-Rich Protein 1
CALCOCO1	coactivator for aryl hydrocarbon and nuclear receptors
HEXIM1	general RNA polymerase II transcription inhibitor
HIST2H2BF	responsible for the nucleosome structure of the chromosomal fiber in eukaryotes
ITFG3	Integrin Alpha FG-GAP Repeat Containing 3
KIAA0913	Zinc Finger, SWIM-Type Containing 8
MAP2	involved in microtubule assembly
PAG1	involved in the regulation of T cell activation
SAT1	catalyzes the acetylation of spermidine and spermine
TMEM2	Transmembrane Protein 2
TXNIP	act as an oxidative stress mediator by inhibiting thioredoxin activity
YPEL1	associated with DiGeorge syndrome on chromosome 22
ZFYVE16	regulate membrane trafficking in the endosome
ZSWIM6	Zinc Finger, SWIM-Type Containing 6

Figure 3.4 Gene expression analysis from GEP data. (A) Three studies used in analysis. (B) Genes commonly up-regulated in all data sets.

A



B

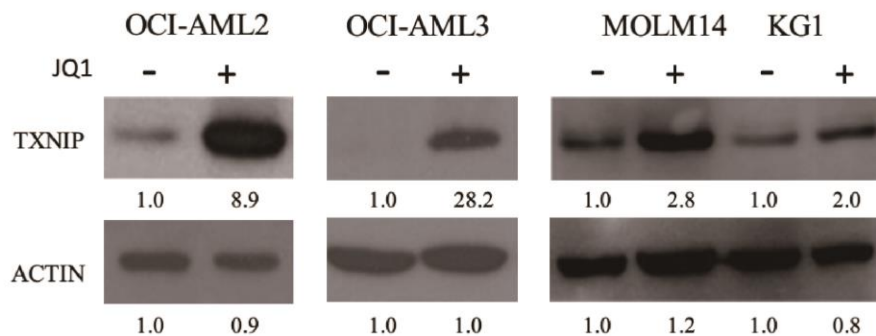
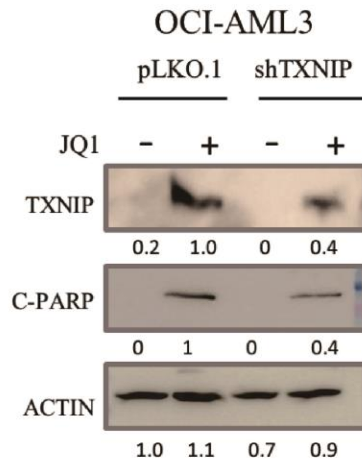


Figure 3.5 TXNIP is up-regulated by JQ1 treatment and mediates JQ1 induced apoptosis. The real time qPCR and western blot results showing TXNIP mRNA (A) and protein (B) level after JQ1 treatment. Cells were treated with JQ1 at respective IC_{50} for 24 hours and lysed for RNA and protein extraction. cDNA were prepared using reverse transcription PCR. TXNIP mRNA levels were measured using real time qPCR. Triplicates were performed for each sample. Mean and standard error was shown. TXNIP protein levels were measured using western blot.

The expression levels of TXNIP before and after JQ1 treatment were examined using real time qPCR and western blot. The results show that TXNIP

expression was dramatically increased at both mRNA and protein levels upon JQ1 treatment, which validates the observation from the GEP data (Fig. 3.5).

A



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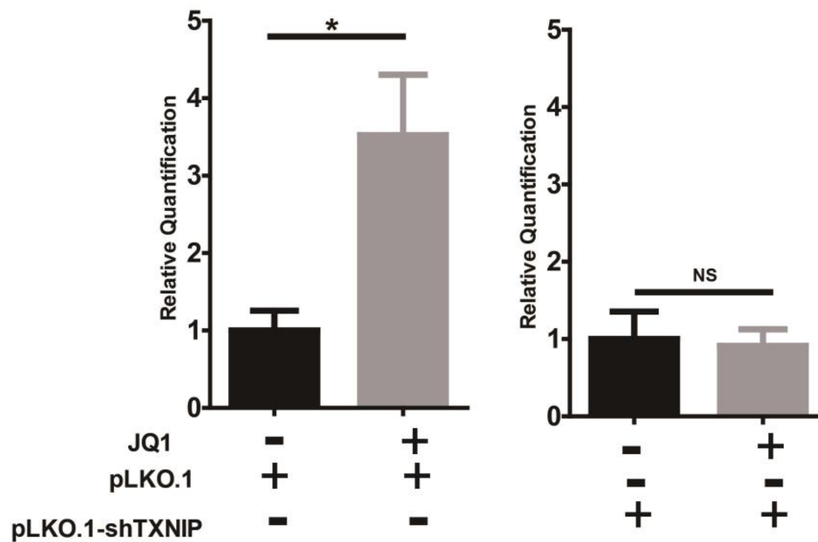


Figure 3.6 Inhibition of TXNIP partially rescued apoptosis induced by JQ1 in AML cells. (A) Western blot results showing the TXNIP and cleaved PARP level. (B) Relative quantification of apoptotic cell percentage. Cells stained with FITC-Annexin V and PI were measured using flow cytometry and results were analyzed with Flowjo. Triplates were performed and standard errors were shown as error bar.

To study whether TXNIP plays a role in JQ1-induced apoptosis, OCI-AML2 cells were transfected with shRNAs targeting TXNIP, followed by JQ1

treatment. The level of apoptosis was examined using western blot and flow cytometry. The results are shown in Fig. 3.6. From the results, it is clear that knocking down TXNIP rescued the apoptosis caused by JQ1 in AML cells, showing a significant lower level of cleaved PARP and no change on apoptotic cell percentages.

As TXNIP has been demonstrated to induce apoptosis in AML cells, the up-regulation of TXNIP might also be involved in the JQ1-induced apoptosis.

3.5 JQ1 induces apoptosis through elevated TXNIP and inhibition of mitochondrial pathway

Although we have found that JQ1 might induce apoptosis through up-regulation of TXNIP, how the up-regulated TXNIP induces apoptosis in AML remains unclear. It is well known that TXNIP negatively regulates thioredoxin and perturbs the redox control system. Also, in a previous study using DZNep, a small molecular inhibitor of EZH2, our lab found that elevated TXNIP level could increase the reactive oxygen species (ROS) production in AML cell lines and patient cells, leading to apoptosis(J. Zhou et al., 2011). I therefore investigated whether the same mechanism also mediates JQ1 induced apoptosis.

First, the localizations of TXNIP before and after JQ1 treatment were examined using western blot (Fig. 3.7). Apparently, most TXNIP located in the cytoplasmic compartment instead of nucleus, either with or without JQ1 treatment, which implicates a major role of TXNIP in the cytoplasm.

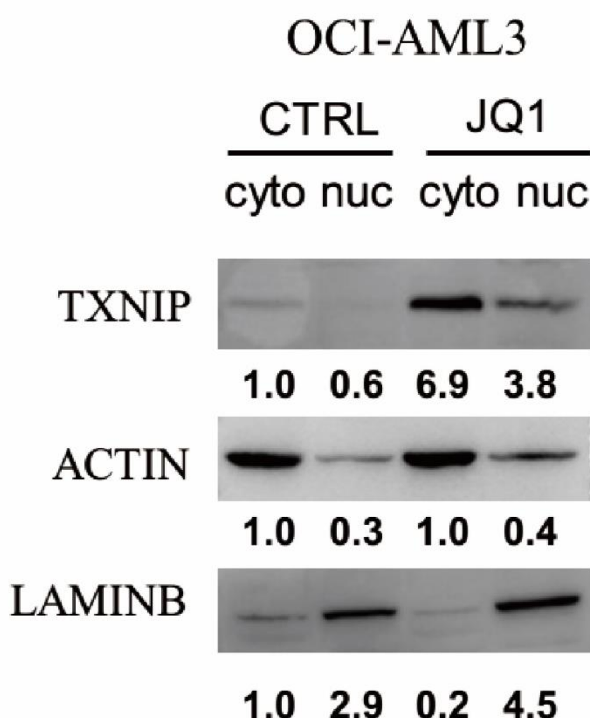


Figure 3.7 location of TXNIP in AML cells. The location of TXNIP protein was determined with western blot. Cells were treated with JQ1 for 48 hours before cytoplasmic and nuclear proteins were extracted. Markers for cytoplasm and nucleus were used in western blot.

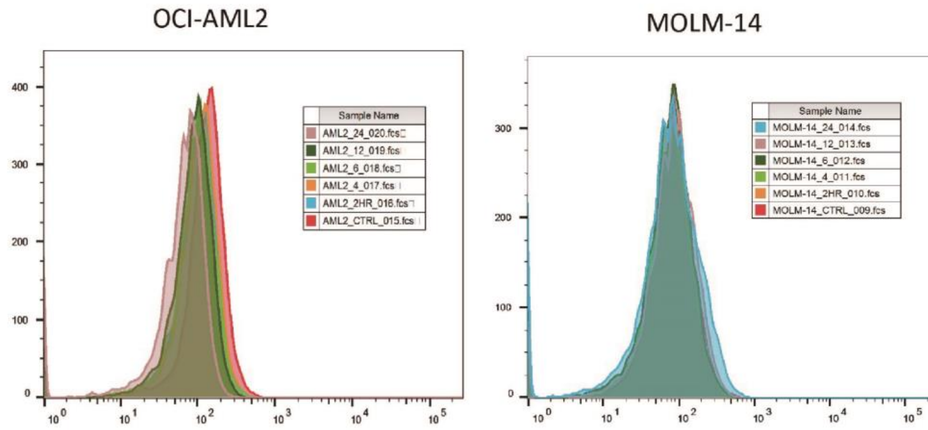
OCI-AML2 and MOLM-14 cells were treated with JQ1 at the respective IC_{50} and cells were collected at different time points. The cells were stained with H2-DCFDA and analyzed using flow cytometry (Fig. 3.8A). The mean values of ROS level were shown in Fig. 3.8B. These results show that the ROS level decreased in OCI-AML2, and slightly increased in OCI-AML3, after JQ1 treatment, which differs from the results after DZNep treatment.

To confirm these results, the ROS scavenger, N-acetyl-L-cysteine(NAC), which has been shown to be able to rescue the apoptosis caused by DZNep treatment, was used to rescue the JQ1-induced apoptosis.

The cells were pre-treated with NAC prior to adding of JQ1 and analyzed by flow cytometry. However, results showed that NAC treatment has no

significant effect on the percentage of apoptotic cells, indicating that NAC failed to rescue the apoptosis induced by JQ1 (Fig. 3.8C and Fig.3.8D).

A



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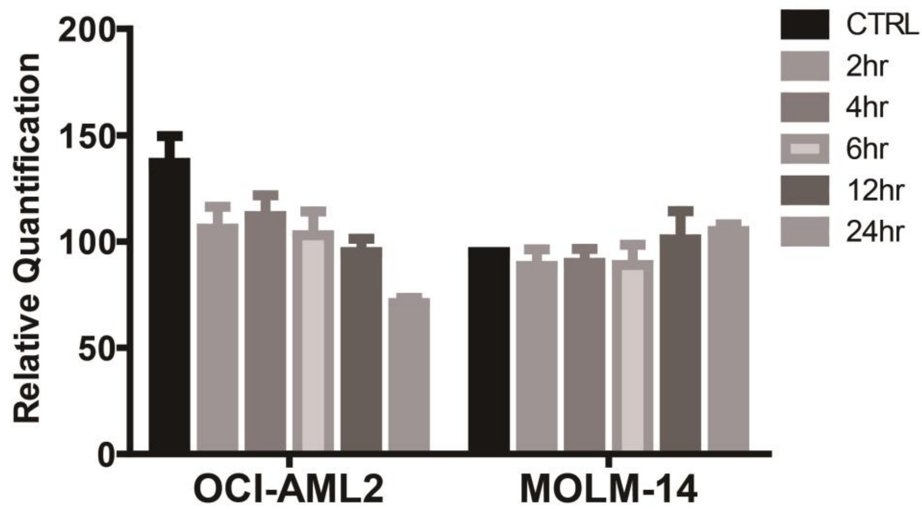


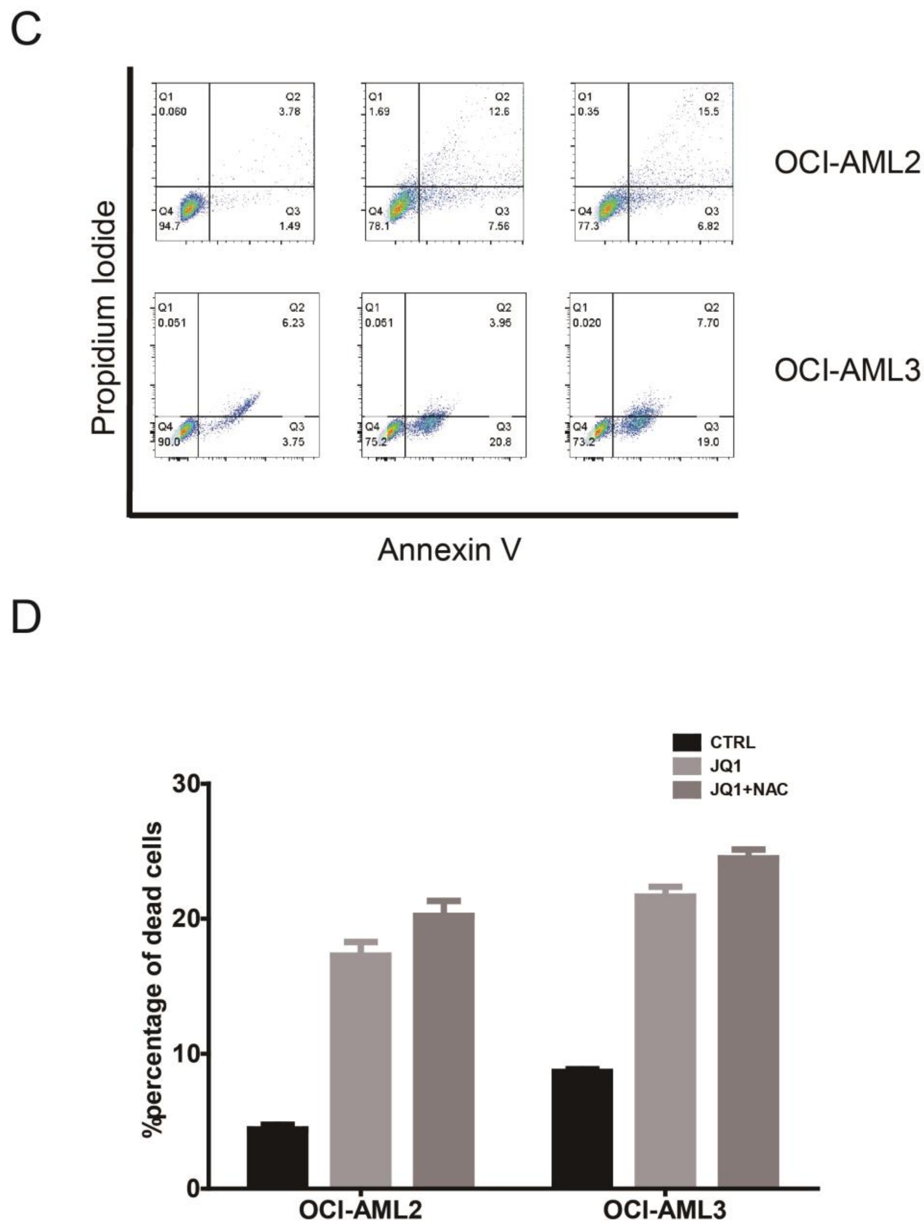


Figure 3.8 ROS is not the main factor in JQ1 induced apoptosis (A) Flow cytometry results showing the ROS level change in OCI-AML2 and MOLM-14 cells. Cells were treated with JQ1 at respective IC_{50} and harvested at different time points. The cells were washed and stained with H2-DCFDA and analyzed using flow cytometry. The ROS levels were detected using a BD LSR II flow cytometer. (B) The relative ROS levels in OCI-AML2 and MOLM-14 cells after JQ1 treatment. (C)(D)Change on dead cell percentages after JQ1 and NAC treatment in OCI-AML2 and OCI-AML3 cells measured by flow cytometry. Cells were pretreated with NAC or DMSO for 1 hour before JQ1 was added. Cells were harvested after 48 hours and the apoptosis were analyzed using PI and Annexin V staining.

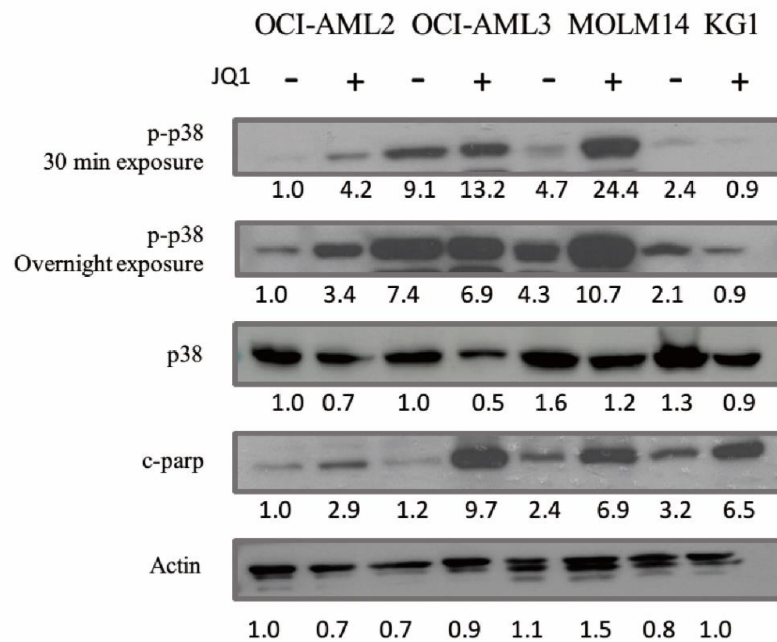
These results showed that elevated ROS level is not the key reason of JQ1 induced cell death and TXNIP might execute its function in a different way comparing to DZNep treatment.

TXNIP has been reported to inhibit the activities of two members of the thioredoxin family, TRX1 and TRX2(Saxena et al., 2010). TRX1 mainly exists in the cytoplasm and plays a central role in mediating oxidative stress. The binding of TXNIP to TRX1 could abolish its function and lead to ROS accumulation. However, the other thioredoxin, TRX2, mainly exists in the mitochondria and controls the mitochondrial mediated apoptosis pathway(Lu & Holmgren, 2014). As ROS increase was not observed after JQ1 treatment, I hypothesized that TRX2 may be involved in apoptosis caused by JQ1.

In human pancreatic beta cells, TXNIP has been shown to be able to mediate ceramide-induced apoptosis(C. L. Chen et al., 2008). Under ceramide treatment, TXNIP relocates from the nucleus into the mitochondria and binds to TRX2, resulting in activation of the p38 MAPK pathway(C. L. Chen et al., 2008). Thus, we examined whether the p38 MAPK level was affected by JQ1 treatment.

The phospho-p38 levels were checked using western blot. The results show a significantly increased phospho-p38 level after JQ1 treatment while the p38 levels decreased slightly (Fig. 3.9A). To further validate the role of phospho-p38 in JQ1 induced apoptosis, a phospho-p38 inhibitor, SB203580 was used to inhibit the function of phospho-p38. Co-treatment with JQ1 and SB203580

showed a significant reduction in apoptosis, suggesting phospho-p38 may be an important mediator of JQ1-induced apoptosis (Fig. 3.9B).



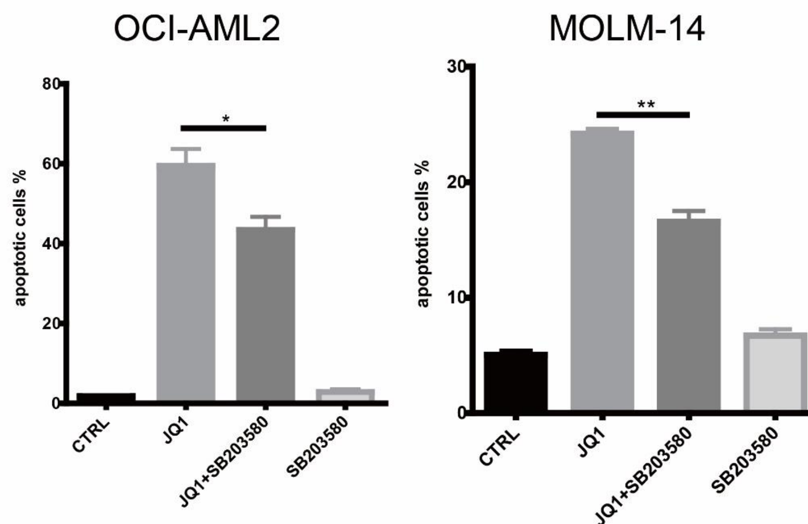


Figure 3.9 Elevated TXNIP induces apoptosis through p38 MAPK pathway (A) Western blot results showing the phospho-p38 levels before and after JQ1 treatment. Cells were treated with JQ1 for 24 hours and lysed for protein extraction. The phospho-p38 and p38 were blotted using anti-phopho-p38 and anti-p38 from Cell Signaling Technology. (B) p38 MAPK inhibitor failed to rescue cells from JQ1 induced apoptosis. Cells were treated with DMSO, or JQ1 alone, or combination of JQ1 and SB203580, or SB203580 for 24 hours and analyzed with flow cytometry. The percentages of Annexin V positive cells were shown.

To summarize, unlike DZNep, jQ1 elevates TXNIP proteins and induces apoptosis mainly through TRX2-p38 MAPK pathway. The activation of p38 MAPK pathway is also consistent with the activation of the intrinsic apoptotic pathway, which has been shown in Chapter 3.2.

3.6 TXNIP is a downstream target of MYC

We have demonstrated that TXNIP might be involved in JQ1-induced apoptosis in AML, however, the details of the mechanism of TXNIP upregulation during this process remains to be answered. As a lot of factors, including glucose flux, oxidative stresses, and ceramide treatment, have been shown to induce TXNIP expression in different cell models, it is important to study whether TXNIP is a specific downstream target or a general response to cellular stress under JQ1 treatment(Chai et al., 2012; C. L. Chen et al., 2008; Hui et al., 2008). Therefore, further investigation is required to elucidate the regulation of TXNIP in AML.

As mentioned earlier in Chapter 1, MYC has been reported as an important downstream target of BRD4 and decrease after JQ1 treatment. This has also been confirmed to be true in AML by my own results shown in Chapter 2.1. Being a key transcription regulator, MYC is known to control multiple cellular processes through its regulatory network including p21, p15, E2F, HDAC2 and so on(Meyer & Penn, 2008). Therefore, I proceeded to elucidate whether MYC is responsible for the up-regulation of TXNIP.

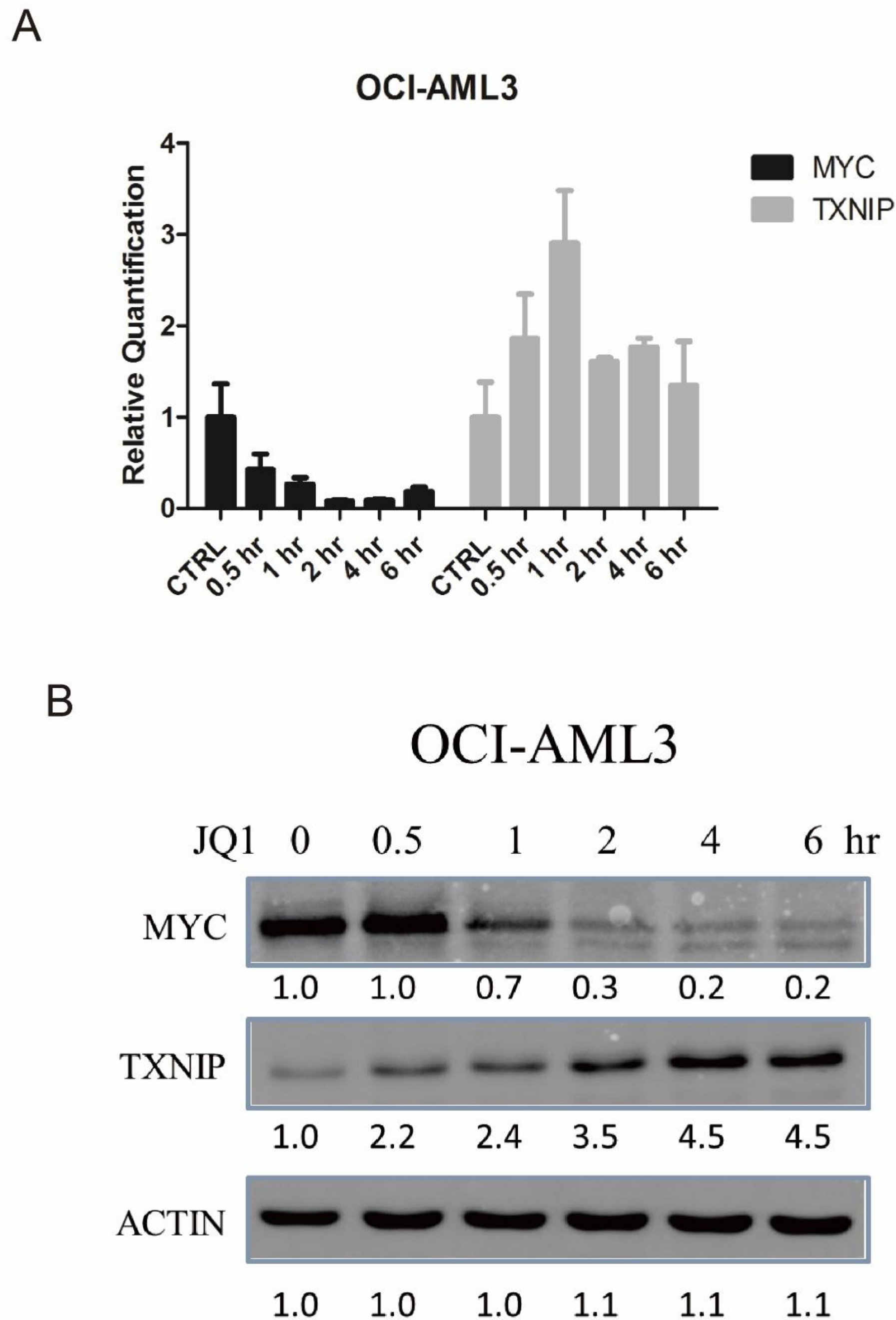


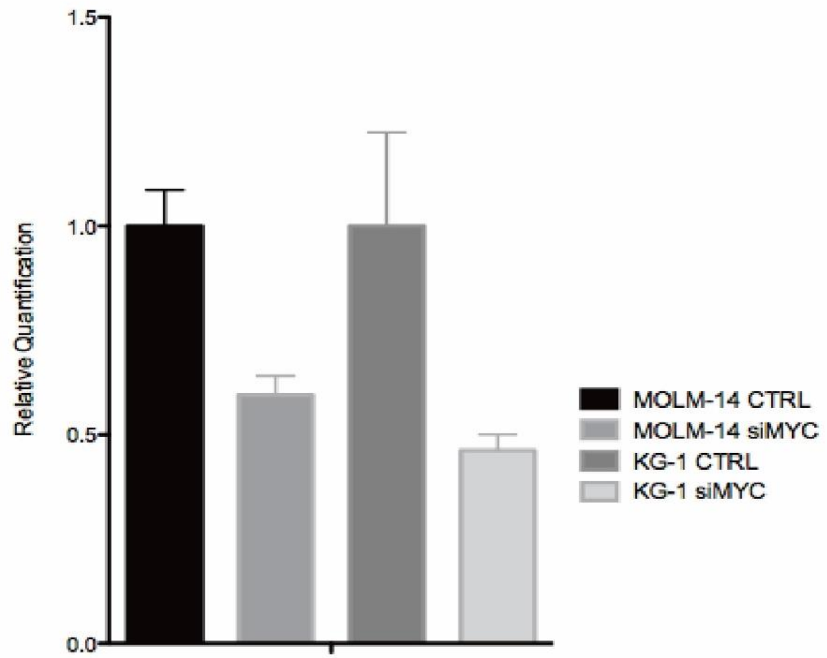
Figure 3.10 MYC and TXNIP levels changed rapidly after JQ1 treatment. (A) Quantitative RT-PCR results showing the mRNA level of MYC and TXNIP before and after JQ1 treatment. The mRNA levels were normalized to endogenous GAPDH. Triplicates were performed for each sample. Mean and standard error was shown. (B) Western blot results showing the protein levels of MYC and TXNIP before and after JQ1 treatment.

The mRNA and protein levels were checked after a short time (within 6 hours) treatment of the OCI-AML3 cells with JQ1. The expression pattern shows that

mRNA and protein levels of MYC and TXNIP were both changed promptly upon JQ1 treatment, which indicates that TXNIP up-regulation might be a specific downstream target of JQ1 instead of a general response to cellular stress (Fig. 3.10). However, it is not clear whether the reduction of MYC is prior to the change of TXNIP from these results. Another report also indicated that MYC itself is also a partner of BRD4, and its function was also depleted upon JQ1 treatment. Therefore, it is not easy to determine whether MYC and TXNIP levels changed chronologically or not from above experimental data.

To confirm the relation between MYC and TXNIP, siRNAs targeting MYC were used to knock down MYC expression in AML cells. The mixture of four siRNAs were transfected into AML cells and the mRNA levels of MYC and TXNIP were examined using qRT-PCR.

A



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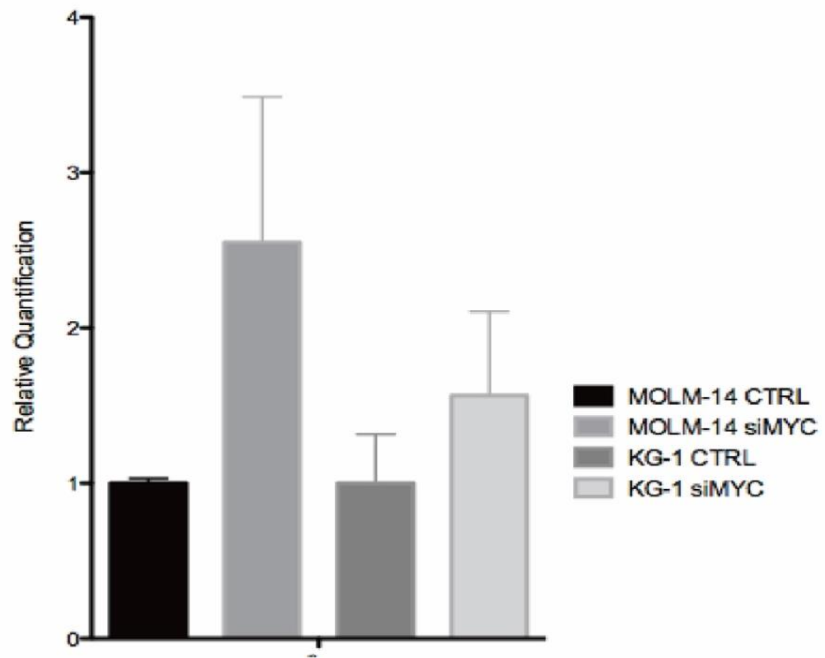


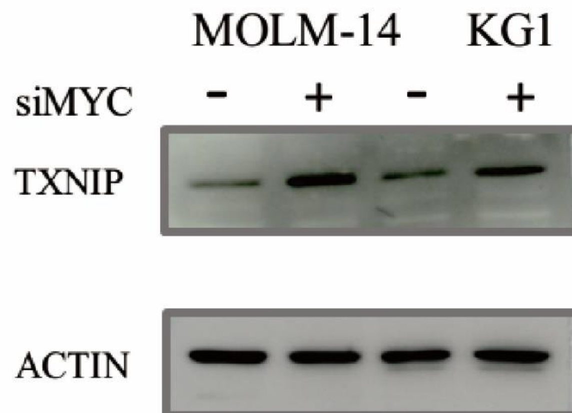
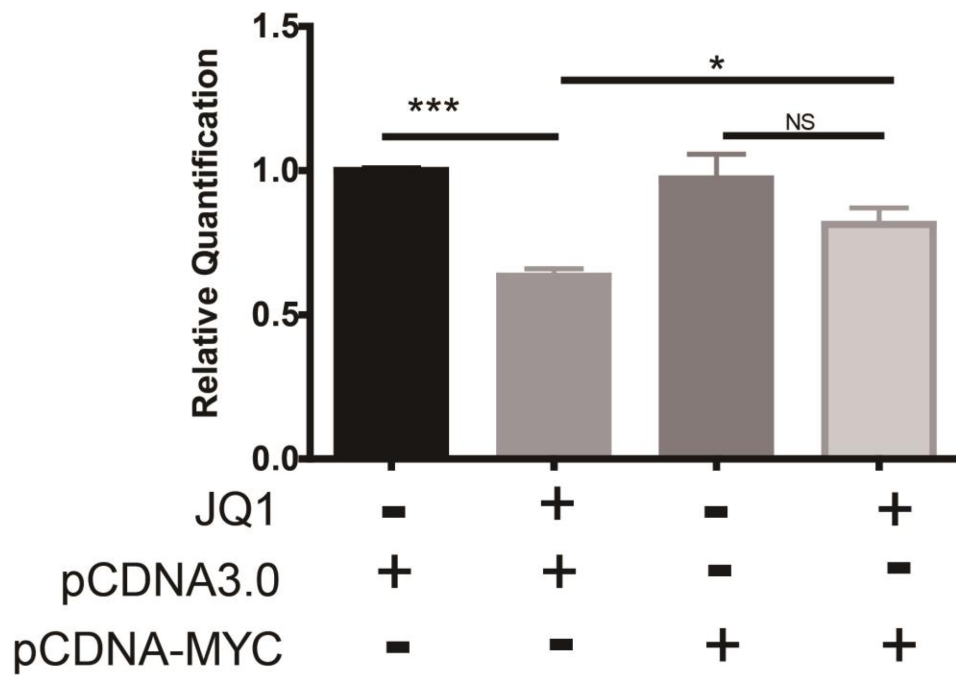
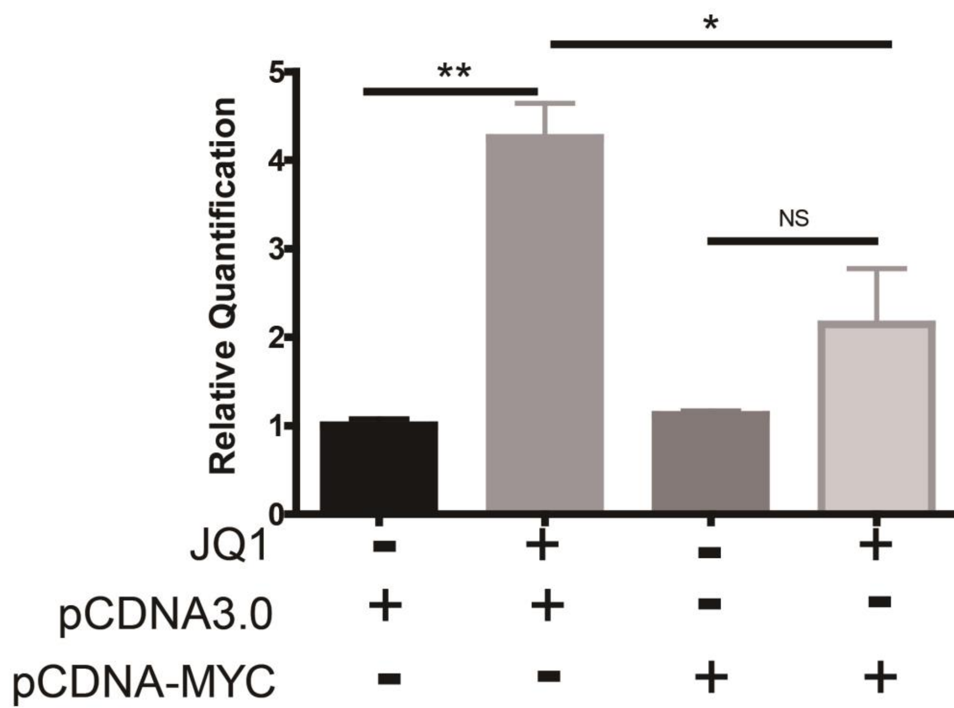


Figure 3.11 Knock down of MYC in AML cell lines increased expression of TXNIP. (A) Quantitative RT-PCR results showing MYC (A) and TXNIP (B) levels before and after MYC siRNAs transfection in MOLM-14 and KG1 cells. Cells were transfected with siRNAs targeting MYC using NEON transfection system. The mRNA levels of MYC were detected using real time qPCR and normalized to endogenous GAPDH. Triplicates were performed for each sample. Mean and standard error was shown. (C) Western blot results showing the TXNIP level change after MYC siRNAs transfection in MOLM-14 and KG1 cells.

The changes of MYC and TXNIP mRNA level after MYC knock down were shown in Fig. 3.11A and Fig.3.11B. Obviously, after knocking down MYC, the mRNA level of TXNIP increased significantly in both cell lines, which indicated that MYC might be the upstream regulator of TXNIP in AML. The same observation was also confirmed by western blot results (3.11C).


A
MYC
Relative Quantification
1.5
1.0
0.5
0.0
***
*
NS
JQ1 - + - +
pCDNA3.0 + + - -
pCDNA-MYC - - + +


B
TXNIP
Relative Quantification
5
4
3
2
1
0
**
*
NS
JQ1 - + - +
pCDNA3.0 + + - -
pCDNA-MYC - - + +

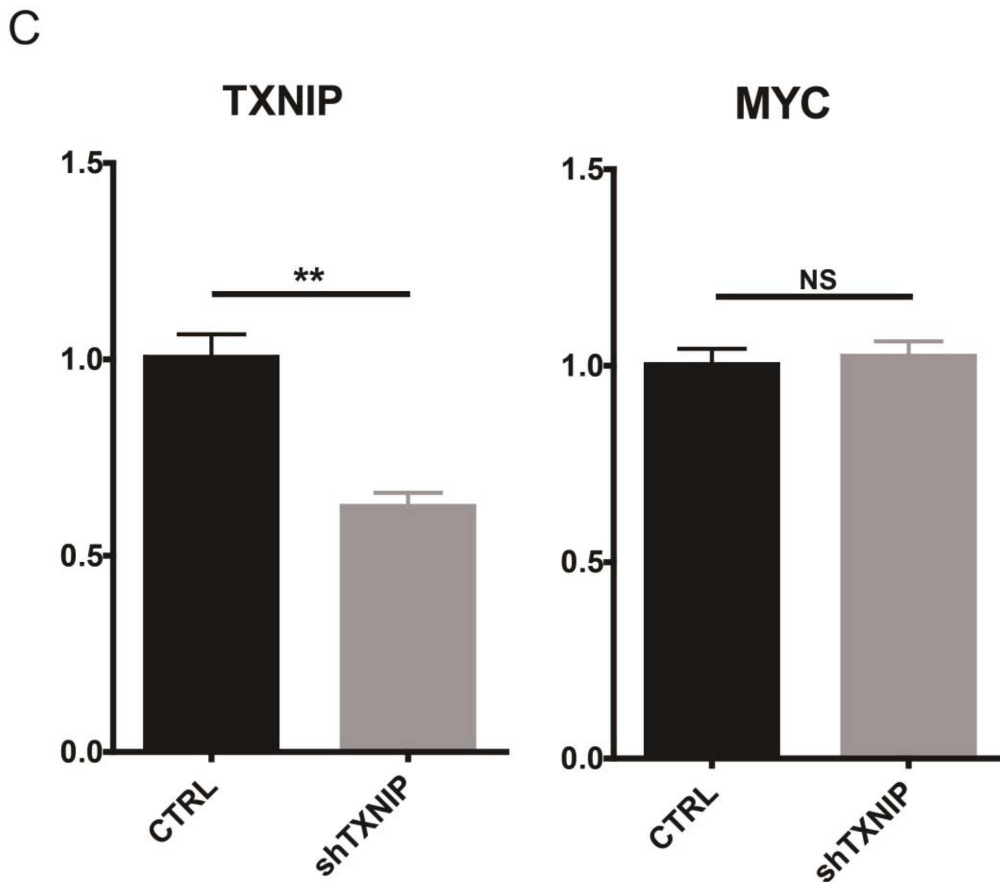


Figure 3.12 Ectopically expressed MYC rescued the MYC depletion by JQ1 treatment. Cells were transfected with pCDNA-MYC using NEON transfection system and treated with JQ1 for 24 hours. The mRNA levels of MYC (A) and TXNIP (B) were detected using real time qPCR. Cells were either transfected with pCDNA3.0 or pCDNA3.1-MYC vectors, followed by JQ1 treatment. Triplicates were performed for each sample. Mean and standard error was shown. (C) TXNIP and MYC mRNA levels after knocking down TXNIP with shRNAs in KG1 cells. Cells were transfected with either pLKO.1 or pLKO.1-shTXNIP vectors for 24 hours before mRNA levels were examined using qRT-PCR. Triplicates were performed for each sample. Mean and standard error was shown.

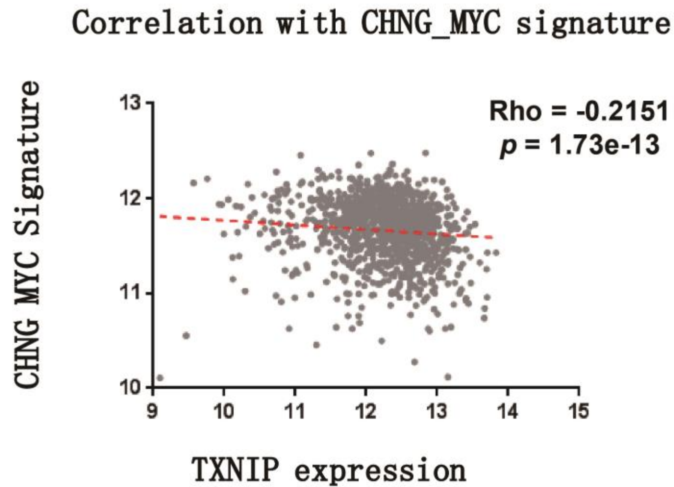
To further validate these results, KG1 cells were transfected with a MYC-expressing plasmid and then treated with JQ1. The mRNA level of MYC and TXNIP were examined using real time qPCR. The results showed that ectopically expressed MYC not only rescued the decreased MYC expression after JQ1 treatment, but also largely inhibited the increase of TXNIP, which strongly supported the idea that MYC regulates the expression of TXNIP (Fig. 3.12A and Fig. 3.12B).

To validate that TXNIP expression was controlled by MYC, KG1 cells were transfected with shRNAs targeting TXNIP, and the MYC level was examined. The results showed that knocking down of TXNIP did not affect the MYC level at all, which demonstrated that MYC is the upstream regulator of TXNIP (Fig. 3.12C).

To further investigate the relationship of MYC and TXNIP in AML patients, the correlation between MYC activation and TXNIP expression was analyzed using a MYC signature generated by our previous study in the expression profile of a cohort of more than 1000 AML patients. The result of spearman's correlation test was shown in Figure 3.13A. It shows that an inverse correlation may exist between the expression of TXNIP and the MYC signature in AML patients. This result provides evidence for the importance of studying MYC and TXNIP relationship in AML.

The connection between TXNIP expression and clinical outcome of AML patients was also studied. Using a cohort data from Oncomine, I found that patients who are alive at 3 years after diagnosis have much higher TXNIP level while those dead have lower TXNIP level (Figure 3.13B). This finding indicates that TXNIP expression level in AML patients may be related to the prognosis of AML patients, thus should be further studied.

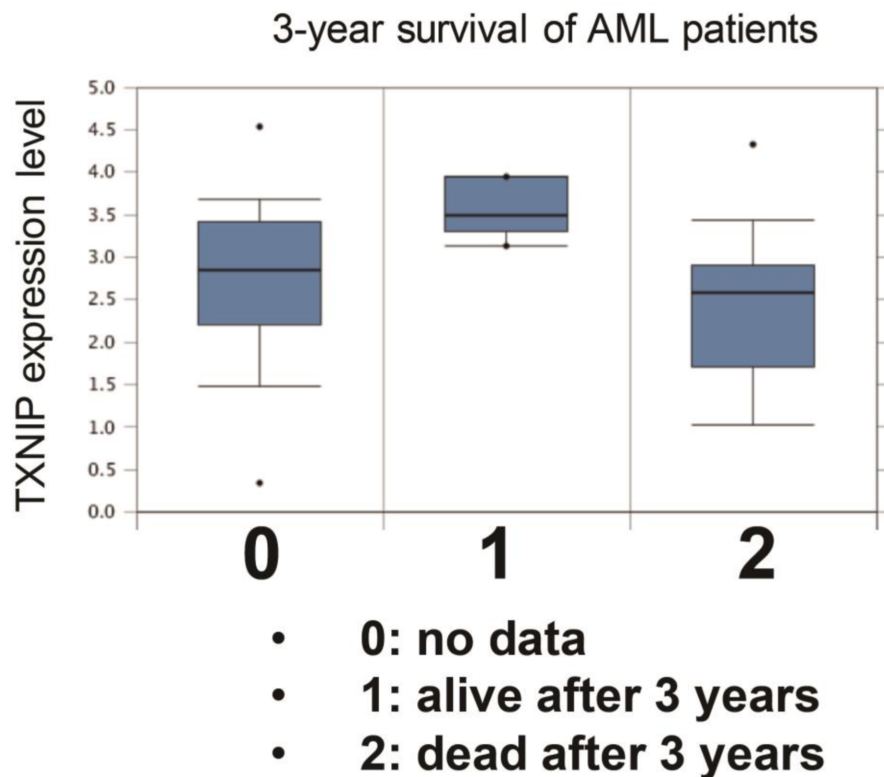
A



AML TXNIP

(n = 1149, 201010_s_at)

B



- 0: no data
- 1: alive after 3 years
- 2: dead after 3 years

Figure 3.13 The correlation between MYC and TXNIP levels in AML patients' samples and the correlation between TXNIP levels and survival of AML patients. (A) The expression levels of TXNIP and MYC signature were analyzed using spearman's correlation test. The Spearman's rank correlation coefficient and the Spearman's Correlation Coefficient p-value

are shown. (B) TXNIP expression levels in AML patients alive or dead 3 years after diagnosis. Data were obtained from Oncomine.

Taken together, these results showed that TXNIP might be a novel downstream target of MYC in AML cells and MYC could not only mediate the inhibition of proliferation through cell cycle arrest, but also regulate the cellular response to JQ1-induced apoptosis.

3.7 MYC regulates TXNIP indirectly through miRNAs

We have demonstrated the important regulatory role of MYC in TXNIP expression. However, the detailed mechanism of how MYC represses the expression of TXNIP is still unknown and needs to be investigated.

As a master regulator of multiple cellular processes such as cell cycle progression, apoptosis and so on, MYC often acts as a transcription activator and initiates the transcription of many downstream targets together with other transcription factors. MYC could also cooperate with other partners like MIZ1, binds to the gene promoters and executes a repressive role in gene regulation(Peukert et al., 1997; Si, Yu, Zhang, & DeWille, 2010)

To explore whether MYC could repress the expression of TXNIP through direct binding to the TXNIP promoter, the promoter sequence of TXNIP was extracted from Transcriptional Regulatory Element Database (TRED) and analyzed for potential transcription motifs. However, no MYC binding sites like E-box could be identified, indicating that direct suppression through promoter binding might not be the way that MYC regulates TXNIP expression.

Enhancer of zeste homolog 2 (EZH2) is a histone methyltransferase enzyme and is able to catalyze the addition of methyl groups to histone H3 at lysine 27, which is a marker of heterochromatin and gene silencing. As an important component of the Polycomb Repressive Complex 2 (PRC2), EZH2 acts as an important epigenetic mediator of gene transcription and could maintain a transcriptionally repressive state in the cell. It is over-expressed in multiple types of cancer and therefore identified as a target in cancer treatment(Pereira et al., 2010; J. A. Simon & Lange, 2008; Jin-zhi Tan, Yan, Wang, Jiang, & Xu, 2014).

In our previous study of a small molecular EZH2 inhibitor, 3-deazaneplanocin A (DZNep), we found that EZH2 could bind to the promoter region of TXNIP and repress the expression of TXNIP through histone H3 tri-methylation at lysine 27(J. Zhou et al., 2011). Another study in our lab also reported MYC could regulate the expression of EZH2 in lymphoma cells(J. Yan et al., 2013). These findings supported a hypothesis that JQ1 regulates TXNIP through the MYC-EZH2-TXNIP axis.

If this hypothesis is true, we should expect a decrease in the EZH2 level after JQ1 treatment. Therefore, the expressions of EZH2 were examined in AML cells treated with JQ1. However, the western blot results showed that there is no significant change in protein levels of EZH2 in OCI-AML2 and OCI-AML3 cells treated by JQ1, which is in contrast to the hypothesis that EZH2 is the regulator of TXNIP under JQ1 treatment (Fig. 3.14). Therefore, EZH2 may not be the intermediate player between MYC and TXNIP in AML cells treated with JQ1.

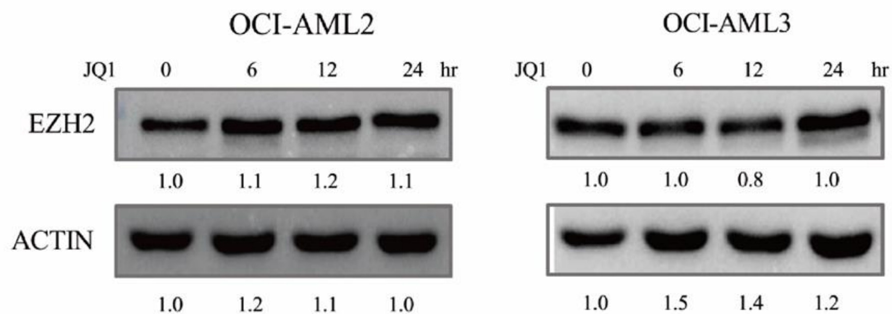


Figure 3.14 EZH2 level is not affected by JQ1 treatment. EZH2 protein levels after JQ1 treatment in OCI-AML2 and OCI-AML3. Cells were treated with JQ1 and collected at respective time points. Protein levels of EZH2 were analyzed using western blot.

Besides direct regulation, MYC is also known to control lots of genes indirectly through regulating the expression of a variety of microRNAs and subsequently affecting the downstream targets, which consist of an important part of the MYC regulatory network. It has been shown that MYC could repress the expression of let-7, miR-15a, miR-34a, amongst others and enhance the expression of the miR-17-92 cluster, including miR-17, miR-18a, and miR-20 (Salvatori et al., 2011; X Zhang et al., 2012; Xinwei Zhang et al.,

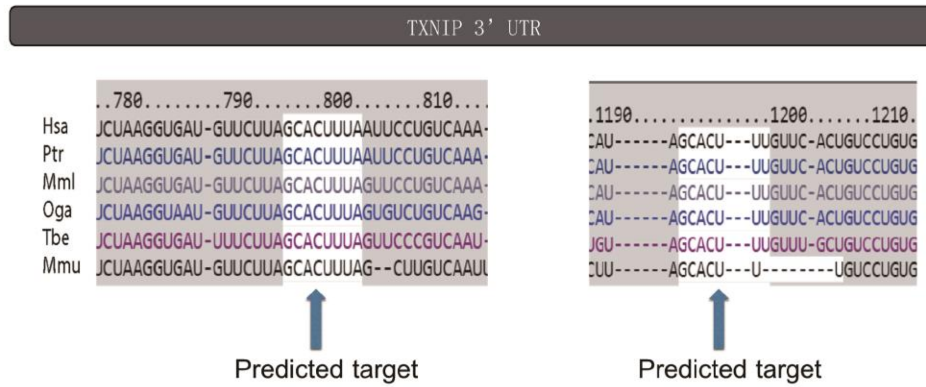
2012)(Wong et al., 2010). Li et al reported that MYC could suppress specific target genes through miR-17-92 to maintain survival and other properties of cancer cells and enforced expression of miR-17-92 could prevent cell death under MYC suppression(Y. Li, Choi, Casey, Dill, & Felsher, 2014). More importantly, miR-17 has been reported to target TXNIP in senescent fibroblast cells(Zhuo et al., 2010). Therefore, we hypothesize that TXNIP might also be under the regulation of MYC through the miR-17-92 cluster.

The 3'UTR of TXNIP was analyzed for miRNA binding sites and among all the six miRNAs from miR-17-92 cluster (miR-17, miR-18a, miR-19a, miR-19b-1, miR-20a, miR-92a-1), the 3'UTR of TXNIP contains two highly conserved binding sites of two of these miRNAs, miR-17 and miR-20a, as predicted by TargetScan (Fig. 3.15A).

Two cell lines, OCI-AML2 and OCI-AML3, were treated with JQ1 for 6 hours and the levels of miR-17-92 mRNA at different time points were checked using real time PCR. It was found that the mRNA level of miR-17-92 decreased significantly after JQ1 treatment, which is consistent with the changes on MYC (Fig. 3.15B).

After confirming the mRNA level of miR-17-92, the mature miRNA levels were also examined. Both of the two miRNAs targeting TXNIP, miR-17 and miR-20a, were down-regulated after JQ1 treatment, which provided further evidence for our hypothesis that MYC controls expression of TXNIP through these miRNAs (Fig. 3.15C).

A



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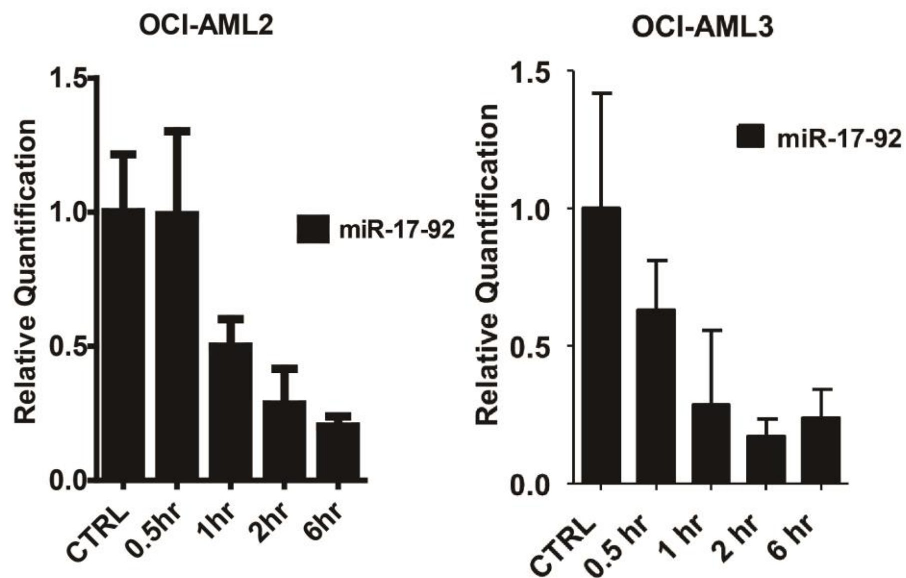

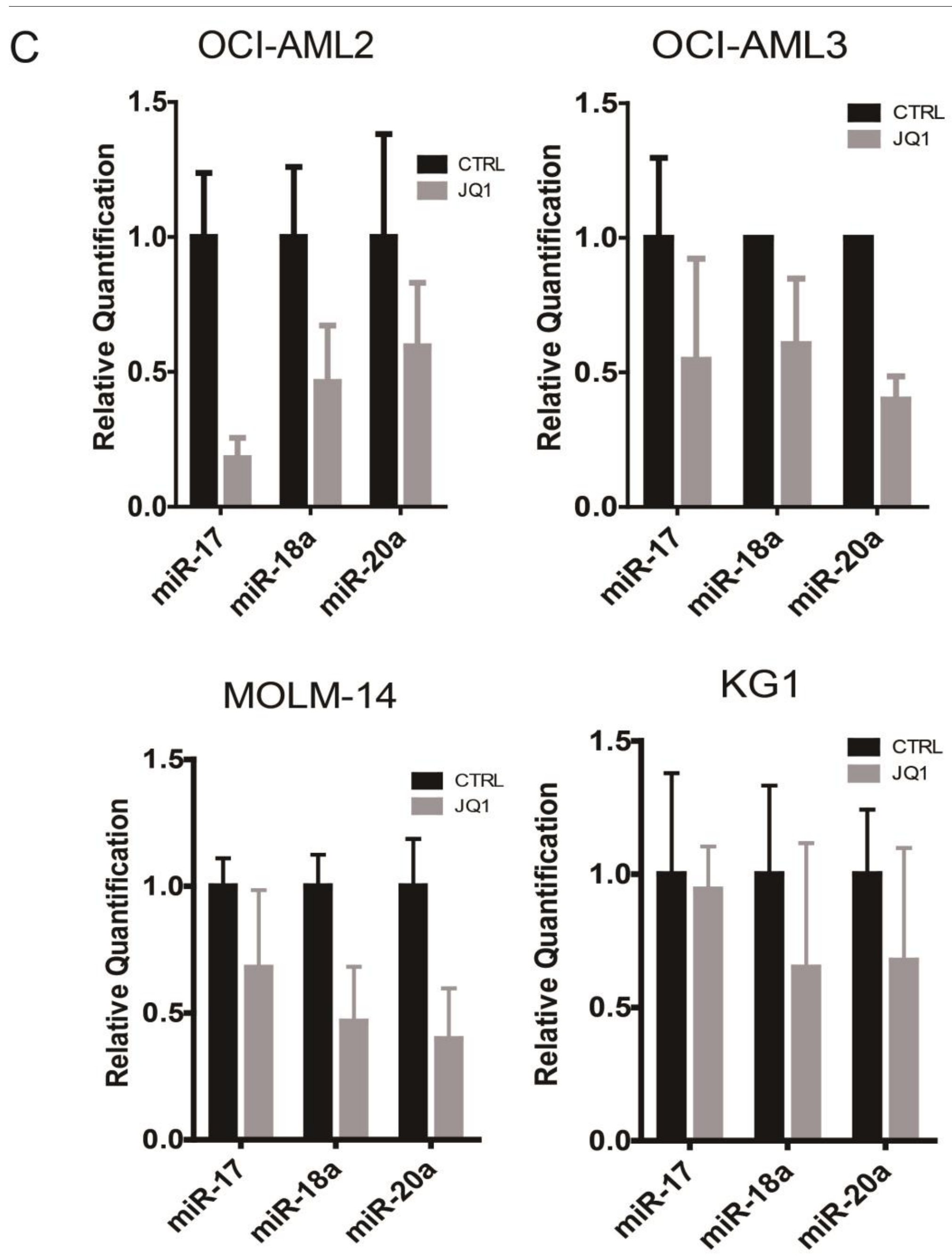

Figure 3.15 The transcript level of miR-17-92 and mature miRNAs decreased after JQ1 treatment and MYC knock down. (A) 3'UTR of TXNIP was shown and predicted to be containing two target sites of miRNAs from miR-17-92 cluster. The prediction was performed using TargetScan 7.0. (B) JQ1 treatment repressed expression of miR-17-92 rapidly. Cells were treated with JQ1 at respective IC_{50} and harvested at respective time points then lysed for RNA extraction. cDNA were prepared using reverse transcription PCR. miR-17-92 levels were detected using real time qPCR and normalized using endogenous GAPDH. Triplication was performed for each sample. Mean and standard error was shown. (C) The expressions of mature miRNAs from miR-17-92 cluster were inhibited by JQ1 treatment. Cells were with JQ1 at respective IC_{50} for 24 hours and lysed for miRNA extraction. The cDNAs were synthesized using respective miRNA RT primers. The mature miRNAs levels were detected using Taqman microRNA assay with respective miRNA probes. U6 snRNA levels were used as endogenous control.

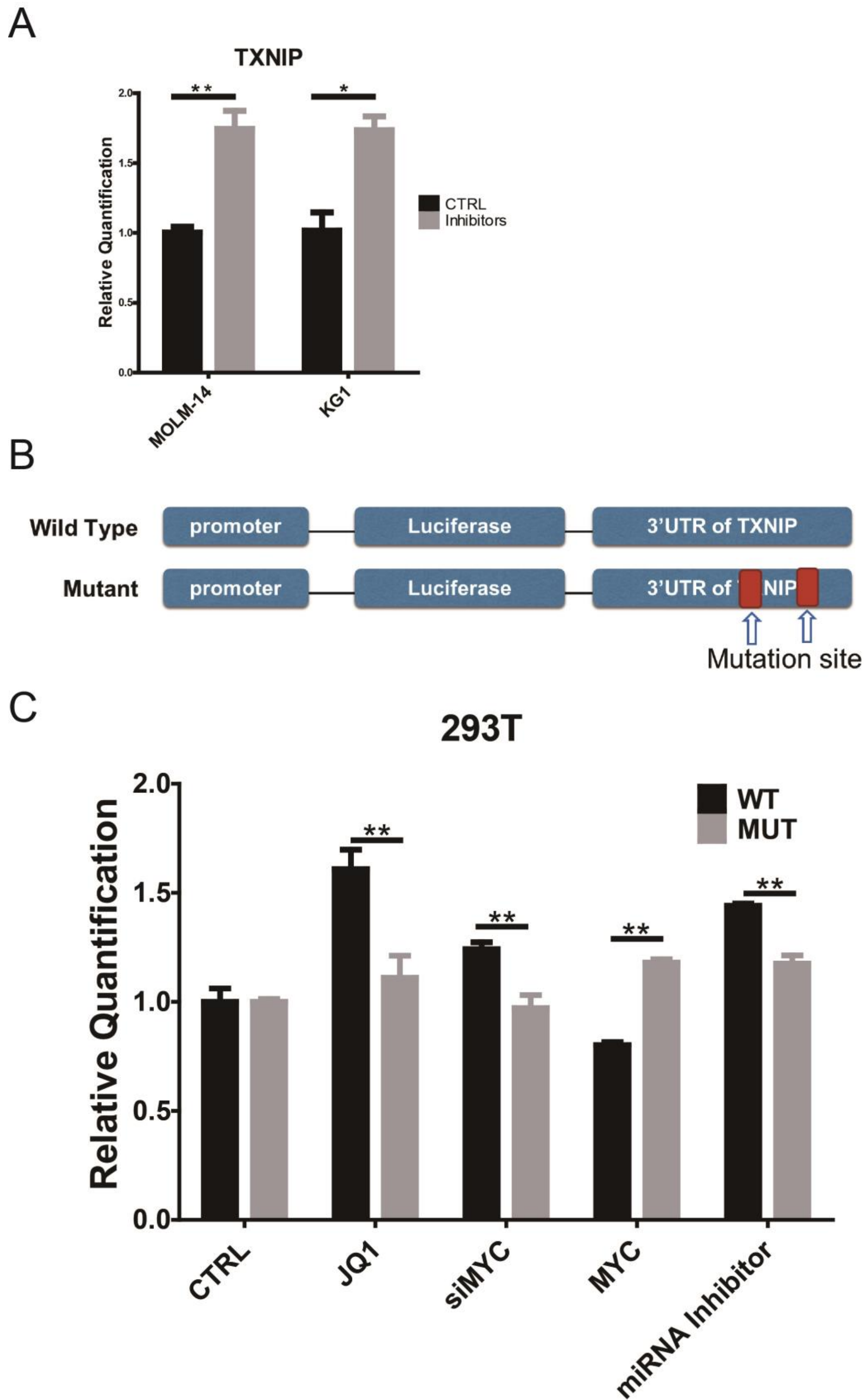

Figure 3.16 The miRNAs target TXNIP directly. (A) Inhibition of miRNAs increased TXNIP expression. MOLM-14 and KG1 cells were transfected with miRNA inhibitors using NEON transfection system. The cells were harvested 24 hours later and lysed for RNA extraction. The expression level of TXNIP was determined using real time qPCR and normalized using

endogenous GAPDH. Triplicates were performed for each sample. Mean and standard error was shown. (B) Vectors used in luciferase assay with wild type (WT) or mutant (mut) 3'UTR of TXNIP. 3'UTR of TXNIP was cloned into pMirTarget luciferase reporter vector. Mutant 3'UTR was generated by using site-directed mutation on the miRNAs target sites. (C) miRNAs directly target TXNIP 3' UTR. 293T cells were transfected using pMirTarget plasmids containing clones of either wild type TXNIP 3'UTR region or mutated 3'UTR of TXNIP. Cells were then treated with JQ1 at 2 μM, or transfected with siRNA targeting MYC, or pCDNA-MYC, or miRNA inhibitors, for 24 hours and lysed for protein using Passive Lysis Buffer. The protein concentration were determined using Bradford. The luciferase activities were measured using a GloMax 20/20 Single Tube Luminometer and normalized to total protein concentration.

To find out whether these miRNAs could regulate the expression of TXNIP, miRNA inhibitors were transfected into MOLM-14 and KG1 cells and the level of TXNIP were examined. Fig. 3.16A shows that inhibition of miR-17, miR-18 and miR-20 significantly increased the mRNA level of TXNIP, which provides strong evidence for the hypothesis that these miRNAs could regulate TXNIP expression.

To validate the direct targeting on TXNIP by these miRNAs, luciferase assay was carried out using plasmids carrying clones of either wild type or mutant TXNIP 3'UTR (Fig. 3.16B). These plasmids were transfected into 293T cells. The cells were then treated with JQ1, or transfected with siRNA targeting MYC, or MYC expressing vector, or miRNA inhibitors. The relative luciferase activities were shown in Fig. 3.16C. It clearly shows that the JQ1 treatment, siRNA transfection, and miRNA inhibitors significantly increased the luciferase activities, while cells transfected with MYC expressing vector showed decreased luciferase activities. However, in cells transfected with mutant TXNIP 3'UTR vector, the effects of different treatments were significantly abolished. Taken together, these results proved the indirect regulation of TXNIP through miRNAs by MYC.

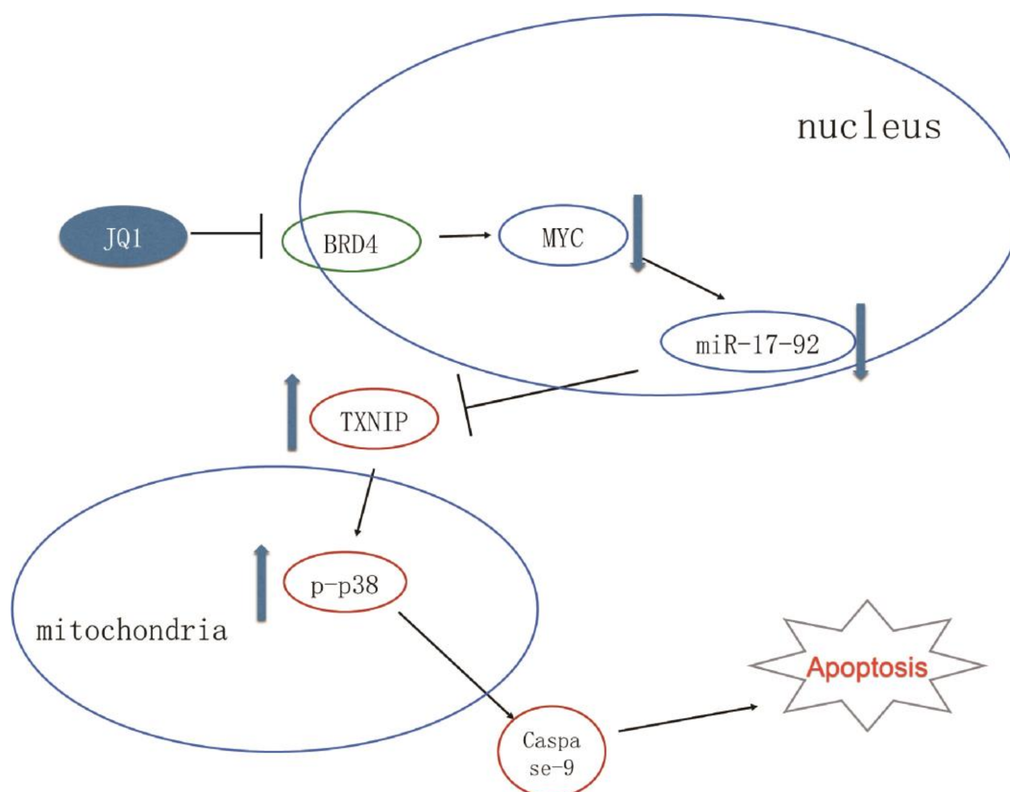

Figure 3.17 Model for JQ1-induced cell death in AML.

3.8 Summary

Target therapy has been proved to be an effective therapy in treating cancer while no much available approaches to target caner related genes in AML. As the discovery of JQ1, a small molecule drug targeting BRD4, it has been found to also largely repression the expression of MYC. In AML, the anti-tumor effect of JQ1 has been demonstrated. However, the detailed mechanism of JQ1 induced cell death have not been revealed yet.

In this study, I found that besides inhibiting cell growth, JQ1 also induces cell death in AML cells. Through further dissection, I found that the intrinsic apoptosis pathway was activated after JQ1 treatment. Furthermore, through literature review and carefully study of the GEP data, I identified TXNIP, a redox regulator, plays an important role in JQ1 induced apoptosis in AML. Unlike in our previous report about another drug DZNep, TXNIP induces apoptosis upon JQ1 treatment through activating the p38 MAPK pathway in mitochondria instead of elevating ROS production.

The mechanism of TXNIP up-regulation upon JQ1 treatment was also studied. Knock down and rescue assay demonstrated that MYC is the up-stream regulator of TXNIP. microRNA assays and luciferase assays confirmed that miR-17-92 cluster is the key mediator in the MYC-regulated TXNIP expression. The model I proposed as shown in Fig. 3.17.

Chapter 4 Discussion

4.1 Targeting MYC in AML

As one of the most studied oncogene, MYC has always been the focus of targeted therapy. While numerous methods have been developed to target MYC in cancer therapy, including antisense oligonucleotides, RNA interference, targeting the protein stability, disrupting the MYC/MAX heterodimer, and small molecules blocking transcription, translation, and post-translation, none of them showed adequate inhibitory effectiveness compared to the new BRD4 inhibitor, JQ1(Hurley, Von Hoff, Siddiqui-Jain, & Yang, 2006; Ponzielli, Katz, Barsyte-Lovejoy, & Penn, 2005; Prochownik, 2004; Vita & Henriksson, 2006). As a novel small molecular drug, JQ1 showed great repressive effect on MYC, albeit through indirect targeting. As a driver oncogene, inhibition of MYC is easily proved to be effective in MYC-driven cancers like Burkitt lymphoma. Although MYC translocation or mutation was not the main factor in AML oncogenesis, ectopically expressed MYC has been demonstrated to induce leukemogenesis in mice models while repression of MYC showed impaired tumor growth(Delgado & León, 2010; Luo et al., 2005). These evidence provide the rationale of targeting MYC in AML.

In this study, the effect of JQ1 was demonstrated in AML and the detailed mechanism was explored. As expected, JQ1 showed promising effect in AML, not only through the well-studied cell cycle arrest but also through inducing cell death. These results not only proved the effectiveness of targeting MYC through epigenetic mechanism, but also demonstrated the complex network of MYC. While the role of MYC has been well analysed in multiple reviews,

the connection between MYC and apoptosis has remained controversial. Overexpressed MYC is able to induce apoptosis through repression of the BCL-2 family or induction of ARF, while deficient MYC has also been shown to induce cell death, which indicates the importance of surpassing the surveillance within normal cells in MYC-driven oncogenesis(Dansen et al., 2006; C M Eischen et al., 2001; Christine M Eischen et al., 1999; Finch et al., 2006). In this study, we showed that targeting MYC in AML reactivates a potent tumor suppressor, TXNIP, the expression of which is often depleted in AML.

4.2 TXNIP as a tumor suppressor in AML

The expression level of TXNIP is much higher in hematopoietic cells compared to other tissues, while in hematopoietic malignancies, the TXNIP level was much lower compared to normal cells, which indicates the importance of TXNIP in hematopoiesis and hematopoietic tumorigenesis(C. Li et al., 2012). HSCs have a high TXNIP expression level, which is found to be associated with the self-renew and differentiation of HSCs(Jung & Choi, 2014). TXNIP controls the fate of HSCs through regulating the response to cellular ROS stress. In HSCs, deficient TXNIP is associated with a higher level of ROS, which differs from its normal function of inhibiting TRX and increasing ROS production. TXNIP-deficient mice also exhibit apoptosis of HSCs induced by the increased ROS level and impaired hematopoiesis(Jung et al., 2013). The role of TXNIP in hematopoiesis provides hints for its involvement in hematopoietic malignancies.

In AML, Zhou *et al* has reported the low expression of TXNIP in either AML patient samples or AML cell lines, which is further confirmed by this study(J. Zhou et al., 2011). Loss of TXNIP expression, whether during differentiation from HSCs or during transformation to leukemic cells, might be an important step in the tumorigenesis of AML, as a high level of TXNIP usually associates with apoptosis.

Different functions of TXNIP have been reported in different cell types and through different mechanisms. Although a lot of work is still required to uncover the different roles of TXNIP under various circumstances, it is clear that TXNIP is able to induce apoptosis in multiple cell types. Increased ROS and activated ASK1-MAPK pathways have been reported to be downstream of TXNIP(C. L. Chen et al., 2008; J. Zhou & Chng, 2013). Interestingly, both ROS and p38 MAPK also have been known to be the regulator of TXNIP, forming a feed-back regulatory loop(Spindel, World, & Berk, 2012). These findings add more details to the already complicated picture of the TXNIP network.

In this study, elevation of TXNIP level induces apoptosis in AML, and reinforces the tumor suppressor role of TXNIP. Despite its well-known role in control of ROS homeostasis, TXNIP also has redox-independent functions, such as activating the ASK1-MAPK pathway(Spindel et al., 2012). In this study, increased TXNIP might not lead to cell death through disruption of ROS production but through interaction with the TRX2 in mitochondria and activating the downstream pathway.

The localization of TXNIP has not been well studied either. It has been reported that TXNIP has transcription repressor activity, which indicates nuclear location, and TRX1 inhibitory activity, which suggests a cytoplasmic location(Saxena et al., 2010; J. Zhou & Chng, 2013). In pancreatic cells, the translocation of TXNIP from nuclear to mitochondria is found to be related to oxidative stress induced apoptosis. However, we found that in AML, the majority of TXNIP resides in cytoplasmic components under normal conditions, indicating the mitochondrial pathway might be more involved in TXNIP function in hematopoietic cells compared to in pancreatic beta cells.

4.3 Relationship between MYC and TXNIP

The relationship between MYC and TXNIP has not been explored before. In this study, it has been demonstrated that MYC controls TXNIP expression through microRNAs, which has been well-known as an important epigenetic regulatory mechanism. Different microRNA targets have been reported to mediate MYC functions in many aspects, including cell cycle, proliferation, differentiation, tumorigenesis, angiogenesis, and metastasis(Jackstadt & Hermeking, 2014). However, the involvement of microRNA between MYC and apoptosis has not been well-recognized. In this study, MYC was found to regulate apoptosis through the miR-17-92 cluster, which has long been known as a downstream target of MYC. A genome-wide study also showed that MYC maintains the survival, proliferation, and neoplastic state in hematopoietic malignancies through regulating the miR-17-92 cluster(Y. Li et al., 2014). Recently, another group has reported that MYC could repress the expression of TXNIP and the expression levels of MYC and TXNIP are inversely

correlated in triple negative breast cancer patients(Shen et al., 2015). These findings are consistent with and corresponding to our own results, indicating the importance of microRNA in the regulatory network of MYC not only in hematopoietic malignancies but also in solid tumors.

My findings further proved the anti-tumor role of TXNIP and demonstrated the rationality of reactivating TXNIP in AML as a therapeutic approach.

4.4 Future directions

In this study, the apoptosis-inducing ability of JQ1 was demonstrated in AML cells and the details of the mechanism were elucidated. However, knocking-down of TXNIP and treatment with p38 MAPK inhibitor only partially rescued the apoptosis caused by JQ1, suggesting other mechanisms may also be involved in JQ1-induced apoptosis in AML. Therefore, I think following experiments should be performed to strength the findings make in this study.

First, the effectiveness of the combination of JQ1 and other target drugs should be tested in AML. As JQ1 inhibits gene expression through targeting the epigenetic “reader” BRD4, it would be interesting to study whether other drugs targeting epigenetic regulators would have a synergetic effect with JQ1. For example, DZNep, a small molecule drug targeting the histone methyltransferase EZH2, has been reported to induces apoptosis in AML cells through up-regulating TXNIP. So it would be interesting to see whether co=treatment of JQ1 and DZNep could induces stronger cell death effect in AML cells.

Another interesting direction is, since TXNIP has been proved to play a role in apoptosis in AML and the expression level was associated with survival of AML patients, it would be interesting to study the importance of TXNIP in the leukemogenesis and resistance of AML cells. By employing the advanced gene manipulation technology CRISPR, AML cells with TXNIP knock out could be generated and therefore tested for resistance of different chemotherapy and target therapy drugs.

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