REGULATION OF NUCLEAR-FACTOR KAPPA B SIGNALING PATHWAY BY JUMONJI-DOMAIN CONTAINING PROTEIN 8

YEO KOK SIONG

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

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YEO KOK SIONG

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REGULATION OF NUCLEAR-FACTOR KAPPA B SIGNALING PATHWAY BY JUMONJI-DOMAIN CONTAINING PROTEIN 8

ABSTRACT

Tumor Necrosis Factor (TNF)-induced signaling mediates pleiotropic biological consequences including inflammation, immunity, cell proliferation and apoptosis. Misregulation of TNF signaling has been attributed as one of the leading causes of chronic inflammatory diseases and cancer. Jumonji domain-containing protein 8 (JMJD8) belongs to the JmjC family. However, only part of the family members has been described as hydroxylase enzymes that function as histone demethylases. Here, JMJD8 was demonstrated to regulate TNF-induced NF-κB signaling positively. Silencing the expression of JMJD8 using RNA interference (RNAi) significantly suppressed the TNF-induced expression of several NF-κB-dependent genes. Moreover, knockdown of JMJD8 expression reduced RIP ubiquitination, IKK kinase activity, delays IκBα degradation and subsequently blocks nuclear translocation of p65. JMJD8 deficiency also enhances TNF-induced apoptosis. Furthermore, bioinformatics analysis and immunofluorescence microscopy were employed to examine the physiological properties of JMJD8. Immunofluorescence microscopy and immunoprecipitation demonstrate that JMJD8 localizes to endoplasmic reticulum (ER) and forms dimers or oligomers *in vivo*, respectively. Protease protection assay further shows that JMJD8 localized specifically to the ER lumen. In addition, potential JMJD8-interacting proteins that are known to regulate protein complex assembly and protein folding are identified. Taken together, these findings indicate that JMJD8 functions as a positive regulator of TNF-induced NF-κB signaling and JMJD8 represents the first JmjC domain-containing protein found in the lumen of endoplasmic reticulum.

Keywords: JMJD8, TNF, NF-κB

REGULASI ISYARAT NUKLEAR-FAKTOR KAPPA B OLEH PROTEIN MENGANDUNGI DOMAIN JUMONJI 8

ABSTRAK

Isyarat yang diaruh oleh TNF mempengaruhi kesan biologi pleiotropik termasuk keradangan, imuniti, percambahan sel dan apoptosis. Regulasi isyarat TNF yang tidak teratur dikaitkan sebagai penyebab utama penyakit keradangan kronik dan kanser. Protein mengandungi domain Jumonji 8 (JMJD8) tergolong di dalam keluarga protein JmjC. Tetapi, hanya sebahagian daripada ahli keluarga protein tersebut dihuraikan sebagai enzim hidroxylase yang berfungsi sebagai pendementilan histon. Di sini, JMJD8 ditunjukkan mengawal selia secara positif isyarat NF-κB yang diaruh oleh TNF. Penyahaktifan gen JMJD8 dengan menggunakan gangguan RNA (RNAi) menyebabkan sekatan kepada ekspresi beberapa gen yang bergantung kepada NF-κB yang diaruh oleh TNF. Tambahan pula, penyahaktifan gen JMJD8 mengurangkan ubikuitinasi RIP serta aktiviti kinase IKK, menyebabkan kelewatan dalam degradasi IκBα dan seterusnya menghalang translokasi p65 ke dalam nukleus. Pengurangan JMJD8 juga meningkatkan apoptosis yang diaruh oleh TNF. Sebaliknya, analisa bioinformatika dan mikroskopi immunofluoresensi digunakan untuk memeriksa sifat-sifat fisiologi JMJD8. Mikroskopi immunofluoresensi dan pengimunomendakan menunjukkan bahawa JMJD8 disasarkan ke retikulum endoplasma and berupaya untuk bentuk dimer atau oligomer *in vivo*. Selanjutnya, kajian perlindungan protease menunjukkan bahawa JMJD8 disasarkan khusus di lumen retikulum endoplasma. Di samping itu, protein yang berpontensi berinteraksi dengan JMJD8 dikenal pasti dan dikenali untuk mengawal pemasangan kompleks protein dan lipatan protein. Hasil kajian ini menunjukkan bahawa JMJD8 berfungsi sebagai pengawal positif kepada isyarat NF-κB yang diaruh oleh TNF dan JMJD8 merupakan protein yang mengandungi domain JmjC yang pertama dijumpa dalam lumen retikulum endoplasma.

Kata Kunci: JMJD8, TNF, NF-κB

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α	:	Alpha
&	:	And
β	:	Beta
℃	:	Degree Celcius
δ	:	Delta
ε	:	Epsilon
γ	:	Gamma
κ	:	Kappa
μ	:	Micro
%	:	Percentage
ζ	:	Zeta
AGC	:	Automatic gain control
AIF	:	Apoptosis inducing factor
AnkR	:	Ankyrin repeats
AP-1	:	Activator protein-1
BAFF		B-cell activating factor
BCL-3	:	B-cell lymphoma 3
BCR	:	B-cell receptor
BIR	:	Baculovirus IAP repeat
BIRC	:	Baculoviral IAP repeat-containing protein
BMM	:	Bone marrow macrophages
BMP	:	Bone morphogenetic protein
BRMS1	:	Breast cancer metastasis suppressor 1
c-Abl	:	Cellular-Abelson murine leukemia tyrosine kinase
CARD	:	Caspase recruitment domain
CC	:	Coil-coiled
CD40	:	Cluster of differentiation-40
CHX	:	Cyclohexamide
cIAP	:	Cellular inhibitor of apoptosis
CKII	:	Casein kinase II

COMMD1	:	COMM domain-containing 1
COX2	:	Cytochrome c oxidase subunit 2
CUE	:	Coupling of ubiquitin conjugation to endoplasmic reticulum degradation
CYLD	:	Cylindromatosis
DD	:	Death domain
DIAP2	:	*Drosophila* IAP
DIF	:	Differentiation inducing factor
DNA	:	Deoxyribonucleic acid
DR3	:	Death receptor 3
DSBH	:	Double-stranded β helix
DTT	:	Dithiothreitol
DUB	:	Deubiquitinase
eCFP	:	Enhanced cyan fluorescent protein
EDTA	:	Ethylenediaminetetraacetic acid
EEA1	:	Early endosome antigen 1
ER	:	Endoplasmic reticulum
ERK	:	Extracellular signal-regulated kinase
et al.	:	Et alia (and others)
FADD	:	FAS-associated death domain
FBXL11	:	F-box and leucine-rich repeat protein 11
FDR	:	False discovery rate
FIH	:	Factor inhibiting HIF
g	:	Gram
GLP	:	G9a-like protein
GO	:	Gene ontology
GRR	:	Glycine-rich region
GST	:	Glutathione S-transferase
h	:	Hour
HA	:	Hemagglutinin
HCD	:	Higher-energy collisional dissociation
HDAC1	:	Histone deacetylase 1

HDAC3	:	Histone deacetylase 3
HEK293T	:	Human embryonic kidney 293T
HIF1AN	:	Hypoxia-inducible factor 1-alpha inhibitor
HIV	:	Human immunodeficiency virus
HLH	:	Helix-loop-helix
HOIL1	:	Heme-oxidized IRP2 ubiquitin ligase 1
HOIP	:	HOIL-1-interacting protein
HSP90α	:	Heat shock protein 90α
HSPBAP1	:	Heat shock protein-associated protein 1
IAA	:	Iodoacetamide
IAP	:	Inhibitor of apoptosis
ICAM	:	Intercellular adhesion molecule 1
IFNB	:	Interferon β
IKK	:	IκB kinase
IL-1	:	Interleukin 1
IL-1β	:	Interleukin 1β
IL-2	:	Interleukin 2
IL-8	:	Interleukin 8
JAMM	:	JAB1/MPN/MOV34 metalloenzyme
JmjC	:	Jumonji C terminal
JmjN	:	Jumonji N terminal
JMJD8	:	Jumonji domain-containing protein 8
JNK	:	c-Jun N-terminal kinases
KCl	:	Potassium chloride
kDa	:	Kilo Dalton
KDM	:	Lysine demethylase
KMT	:	Lysine methyltransferase
l	:	Liter
LTR	:	Long terminal repeat
LPS	:	Lipopolysaccharide
LUBAC	:	Linear ubiquitin chain assembly complex

LZ	:	Leucine zipper
M-MuLV	:	Moloney murine leukemia virus
MAP	:	Mitogen-activated protein
MAP3K7	:	Mitogenic-activated protein (MAP) kinase kinases 7
mg	:	Miligram
$MgCl_2$	:	Magnesium chloride
min	:	Minute
MINA53	:	Myc-induced nuclear antigen, 53 kDa
MKK	:	MAP kinase kinase
ml	:	Mililiter
MS	:	Mass spectrometry
MTDH	:	Metadherin/Lyric
NaCl	:	Sodium chloride
NBD	:	NEMO-binding domain
NEMO	:	NF-κB essential modifier
NES	:	Nuclear export signal
NFAT	:	Nuclear factor associated with activated T-cells
NFATc1	:	Nuclear factor of activated T-cells 1
NF-κB	:	Nuclear factor kappa B
NK	:	Natural killer
NLS	:	Nuclear localization signal
NO66	:	Nucleolar protein 66
NSD1	:	Nuclear receptor-binding SET domain-containing protein
Nw	:	Nuclear wash
O-GlcNAc	:	O-linked β-N-acetylglucosamine
OGT	:	β-N-acetylglucosaminyltransferase
OTU	:	Ovarian tumor proteases
PAMPs	:	Pathogen-associated molecular patterns
PARP1	:	Poly [ADP-ribose] polymerase 1
PBS	:	Phosphate buffer saline
PCR	:	Polymerase chain reaction

PDLIM2	:	PDZ and LIM domain 2
PEST	:	Proline-, glutamate-, serine-, and threonine-rich
PHD	:	Plant homeodomain
PKC	:	Protein kinase C
Plk1	:	Polo-like kinase 1
PMSF	:	Phenylmethane sulfonyl fluoride
PP5	:	Protein Phosphatase 5
ppm	:	Part per million
PRMT	:	Protein arginine methyltransferase
RCAS1	:	Receptor-binding cancer antigen expressed on SiSo cells
RHD	:	Rel homology domain
RHIM	:	RIP homotypic interaction motif
RING	:	Really interesting new gene
RIP1	:	Receptor-interacting serine/threonine-protein kinase 1
RNAi	:	RNA interference
rpm	:	Resolution per minute
SAM	:	S-adenosylmethionine
$SCF^{\beta\text{-TRCP}}$	:	Skp, Cullin, F-box containing complex, beta-transducin repeat containing protein
SD	:	Standard deviation
SET7/9	:	SET domain-containing protein 7/9
SETD6	:	SET domain containing 6
SHARPIN	:	SHANK associated RH domain interactor
SIRT1	:	Sirtuin 1
SNF2H	:	Sucrose nonfermenting protein 2 homolog
SODD	:	Silencer of death domains
SRD	:	Signal receiving domain
SUMO	:	Small ubiquitin-related modifier
TAB	:	TAK1 binding protein
TACE	:	TNFα-converting enzyme
TAD	:	Transcriptional activation domains
TAK1	:	Transforming growth factor-β (TGF-β)-activated kinase 1

TCR	:	T-cell receptor
TFA	:	Trifluoroacetic acid
TLR4	:	Toll-like receptor 4
TM	:	Transmembrane
TNF	:	Tumor necrosis factor
TNFAIP3	:	Tumor necrosis factor alpha-induced protein 3
TNFR1	:	Tumor necrosis factor receptor 1
TRADD	:	TNFR1-associated death domain
TRAF2	:	TNF receptor-associated factor 2
TWY5	:	tRNA wybutosine-synthesizing protein 5
UBA	:	Ubiquitin-associated domain
UCH	:	C-terminal hydrolase
ULD	:	Ubiquitin-like domain
USP	:	Ubiquitin-specific protease
ZNF	:	Zinc finger

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CHAPTER 1: INTRODUCTION

1.1 Introduction

The tumor necrosis factor (TNF) superfamily consists of 19 ligands and 29 receptors with diverse physiological functions (Aggarwal et al., 2012). Among the family members, TNFα and tumor necrosis factor receptor 1 (TNFR1) is the most well-characterized ligand and receptor, respectively. As a pleiotropic pro-inflammation cytokine, TNFα regulates many biological processes namely inflammation, immunity, cell proliferation, and apoptosis (Hehlgans & Pfeffer, 2005; Wertz, 2014). Stimulating cells with TNFα activates NF-κB and Mitogen-activated protein (MAP) kinases, including Extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinases (JNK). In the TNFR1 signaling, engagement of TNFα with TNFR1 leads to the recruitment of the TNFR1-associated death domain (TRADD) protein. TRADD subsequently serves as a platform for the recruitment of TNF receptor-associated factor 2 (TRAF2) protein, the death domain kinase RIP1 or associate with FAS-associated death domain (FADD) protein and caspase 8 after dissociated from TNFR1. While the association of FADD with TRADD triggers the apoptosis program, binding of TRAF2 and RIP1 to TRADD activates NF-κB and JNK (Brenner et al., 2015; Micheau & Tschopp, 2003).

NF-κB consists of five members including p65 (also known as RelA), RelB, cRel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2), which can form either homo- or heterodimers (Hayden & Ghosh, 2008, 2014). In resting cells, NF-κB is sequestered in the cytoplasm and bound to its inhibitor, IκB family members. Upon stimulation, IκB is phosphorylated by an upstream kinase complex consists of IκB kinase (IKK) α, IKKβ and NEMO which leads to its degradation via the ubiquitin-proteasome pathway. Free NF-κB is then translocated into the nucleus to activate its target genes (Hayden & Ghosh, 2008, 2014; Silverman & Maniatis, 2001). Although the activity of NF-κB is primarily regulated by its translocation into the nucleus, post-translational modifications of the NF-

κB protein have distinct functional significances in regulating the activity of NF-κB protein. Recently, many post-translational modifications such as acetylation, phosphorylation, ubiquitination and methylation of the NF-κB members have been shown to regulate the NF-κB activities (Carr et al., 2015; Ea & Baltimore, 2009; Perkins, 2006). For example, previous studies showed that methylation of p65 at lysine 37 (K37) by a methyltransferase, SET domain-containing protein 7/9 (SET7/9), modulates its function (Ea & Baltimore, 2009), acetylation of p65 at K218 and K221 inhibits IκB binding and enhances DNA binding (Chen et al., 2002), and acetylation of p65 at K122 and K123 inhibits its transcriptional activation activity (Kiernan et al., 2003). These post-translational modifications are reversible. To date, only one group has reported that p65 is regulated by demethylase, namely F-box and leucine-rich repeat protein 11 (FBXL11) (Lu et al., 2009, 2010). However, it is unclear whether the NF-κB activity is also regulated by other demethylases.

Jumonji domain-containing (JMJD) proteins were first reported by Takeuchi's group (Takeuchi et al., 1995). There are currently more than 30 protein members identified in mammals that contain a Jumonji C (JmjC) domain (Yamane et al., 2006). Most of the JmjC domain-containing proteins are hydroxylase enzymes that function as demethylases (Tsukada et al., 2006). JmjC family members classified as histone demethylases usually contain known histone-binding domains such as Plant homeodomain (PHD) and Tudor domains (Shi & Whetstine, 2007). Many proteins in this family have been shown to be involved in cell development, differentiation and proliferation through regulating various signaling pathways. On the other hand, deregulation of JMJD proteins can lead to various human malignancies (Shi & Whetstine, 2007; Takeuchi et al., 1995). For example, Jumonji domain-containing protein 2C (JMJD2C) (also known as GASC1) is upregulated in squamous cell carcinoma (Yang et al., 2000) and it regulates cell proliferation (Cloos et al., 2006).

Jumonji domain-containing protein 8 (JMJD8) is a JmjC domain-only protein that contains a JmjC domain at 74-269 amino acid residues with no other recognizable protein domains. Recent studies have shown that JMJD8 involves in angiogenesis and cellular metabolism through interacting with pyruvate kinase M2 (Boeckel et al., 2016). Here, the role of JMJD8 in TNF signaling pathway was examined and JMJD8 was demonstrated to function as a positive regulator of TNF-induced NF-κB signaling. In addition, the subcellular localization, biophysical and biochemical properties of JMJD8 were also examined. JMJD8 was found to contain a signal peptide and mainly localized to the lumen of endoplasmic reticulum (ER). The signal peptide of JMJD8 is important for its ER localization as well as its dimerization or oligomerization. Furthermore, thirty-five potential JMJD8-interacting proteins were identified that may shed light into understanding the biological function of JMJD8.

1.2 Objectives

A previous finding from our group showing that methylation of p65 protein regulates its transcriptional activity (Ea & Baltimore, 2009) prompted us to evaluate whether demethylases are also involved in TNF-induced NF-κB signaling. A preliminary RNA interference (RNAi) screening of a group of Jumonji domain-containing proteins found that JMJD8, a JmjC domain-only protein may regulateTNF-induced NF-κB signaling. Silencing the expression of JMJD8 using RNAi greatly suppressed the TNFα-induced expression of several NF-κB-dependent genes. Furthermore, both IκBα degradation and nuclear translocation of p65 after TNFα stimulation were interfered, suggesting that JMJD8 plays a role in regulating NF-κB. Therefore, the objectives of this study are as below:

1. **To examine the role of JMJD8 in NF-κB signaling pathway**

   Given that silencing of JMJD8 interrupts the NF-κB activity, suggesting that it may be involved in the NF-κB signaling pathway. To test the involvement of JMJD8 in NF-κB pathway, the effects of JMJD8 knockdown observed in the preliminary results will be verified with multiple siRNA oligos in control and JMJD8 silenced cells to eliminate the possibilities of off-target event. Next, the mechanism of how JMJD8 affects the NF-κB activity will be determined by a series of biochemical tests.

2. **To study the biophysical and biochemical properties of JMJD8.**

   Briefly, the JMJD8 protein amino acid sequence will be analyzed and the domains of the protein will be predicted with bioinformatics tools. Next, the truncated as well as the wild type JMJD8 proteins fused with an enhanced cyan fluorescent protein (eCFP) tag will be expressed and their subcellular localization will be examined using an immunofluorescence assay and a confocal microscopy. Antibodies targeting specific organelle proteins will be used to determine the subcellular localization of JMJD8 in the cells. Lastly, immunoprecipitation and mass-spectrometry analysis will be conducted to identify the interaction partners of JMJD8.

The proposed experiments are anticipated to shed lights in understanding the involvement of demethylases in regulating NF-κB activity, as well as identify a new player that fine-tunes the transcriptional activity of NF-κB.

CHAPTER 2: LITERATURE REVIEW

2.1 The Nuclear Factor kappa B (NF-κB) signaling pathway

2.1.1 The discovery of NF-κB

NF-κB was discovered by David Baltimore and his co-worker 30 years ago based on a series of experiments that identified a DNA binding protein which binds specifically to conserved Deoxyribonucleic acid (DNA) sequences at the promoter of κ light-chain gene in B cells (Sen & Baltimore, 1986a). Since then, scientists have continuously been fascinated by the diverse functional roles of NF-κB, leading to many research publications detailing the complexity of NF-κB dynamics in the cells.

NF-κB was initially thought to be expressed only in B cells to regulate B cell maturation and development due to the failure to detect the DNA binding activity of NF-κB in other cell types using a high sensitive gel-shift assay (Baeuerle & Baltimore, 1988a, 1988b). However, NF-κB was later proven to be evolutionarily conserved across all cell types and even species (Ghosh et al., 1998) with the DNA binding ability of NF-κB in other cell types being masked by an inhibitor named inhibitor of kappa B (IκB) (Baeuerle & Baltimore, 1988a, 1988b).

Inside the cells, NF-κB usually stays in its latency state and responds rapidly upon stimulated. When a cell encounters inflammation or other challenges, it activates a signal cascade in an orderly manner to activate NF-κB. When the purpose of the stimulus is accomplished, the pathway returns to its latency states. For example, the cells from innate immunity that act as the first line of defense at a wound or infection site are activated through NF-κB. Once the threat is resolved, NF-κB will be reset to prevent extensive activation (Ben-Neriah, 2002; Zhang et al., 2017).

After years of studies, what began as a simple ligand and activator response has evolved to become a complex mechanism that involves many intermediate factors and processes such as protein-protein dimerization, phosphorylation, and ubiquitination

(Hayden & Ghosh, 2008, 2012, 2014). Subsequently, many inducers were found to activate individual pathways through NF-κB, which act as a central coordinator. Moreover, NF-κB was found to be not a single transcriptional protein but a family of 15 homo- or heterodimer complexes that derive from the combination of 5 individual monomers (Smale, 2012). To date, these extraordinary complexes have been shown to positively or negatively regulate hundreds of genes (http://www.bu.edu/nf-kb/gene-resources/target-genes/).

2.1.2 General features of NF-κB

NF-κB regulates hundreds of genes through a conserved palindromic DNA sequence 5'-GGGRNWYYCC-3', (N, any base; R, purine; W, adenine or thymine; Y, pyrimidine), also known as κB site which exists in the promoter or enhancer region of NF-κB regulated genes (Sen & Baltimore, 1986a). The five members of NF-κB (RelA/p65, RelB, c-Rel, p50, and p52) occur naturally as homo- or heterodimer that recognizes this κB site in the nucleus and regulates gene expression.

The NF-κB family members contain a Rel homology domain (RHD) that shares sequence homology with v-Rel oncogene (Hayden & Ghosh, 2008). The RHD consists of 300 amino acids that mediates specific DNA binding, protein dimerization and inhibitory protein binding processes (Hayden & Ghosh, 2008, 2012, 2014; Smale, 2012). The NF-κB solely works and functions in the nucleus. Thus it contains a nuclear localization signal (NLS) within the RHD (Figure 2.1).

The five NF-κB members can be divided into two different classes. The first class consists of p50 and p52, which are cleaved products from its precursor protein p105 (NF-κB1) and p100 (NF-κB2), respectively. The N-terminal of the precursor protein contains ankyrin repeats (AnkR) that are removed after post-translational modification to form mature protein. The second class of NF-κB, including p65, RelB and c-Rel, are produced

as mature proteins with transcriptional activation domains (TADs) that are important for recruiting transcriptional regulator and machinery (O'shea & Perkins, 2008). Moreover, NF-κB proteins have both permissive and repressive role in gene transcription. Permissive NF-κB proteins usually contain TAD and exist as heterodimers, whereas homodimers like p50:p50 and p52:p52 are repressive (Zhang et al., 2017).

Among the NF-κB family members, p65 and p50 heterodimers are the most well studied NF-κB proteins. With p65 deletion, mice exhibit embryonic lethality at E15 and E16 due to excessive hepatocyte apoptosis (Beg & Baltimore, 1996). The hepatocyte apoptosis arose from the sensitivity to TNFα, since removing this cytokine rescued the $p65^{-/-}$ mice from lethality (Doi et al., 1999). In contrast to p65, deleting p50 and its precursor p105 shows normal growth and no histopathological changes in mice. Although p50 and p105 have no essential roles in embryogenesis and development, it has been shown to exhibit numerous setbacks in immune responses. Mice with *NF-κB1* deletion show abnormal B-cell proliferation when induced with LPS as well as defective antibody production (Sha et al., 1995).

P65, which refers to its protein size, contains an N-terminal RHD that is essential for its dimerization, interaction with IκBα and DNA binding. Moreover, p65 possesses a NLS within the RHD and is crucial for NF-κB nuclear localization ability. Besides, p65 comprises of a C-terminal TAD which is necessary for transcriptional gene activation and to facilitate the recruitment of coactivator (Hayden & Ghosh, 2004; Napetschnig & Wu, 2013) (Figure 2.1). In contrast to p65, p50 is a cleaved product of its precursor protein p105 or NF-κB1 with 50 kDa in size. Similar to p65, p50 contains an N-terminal RHD, a NLS within the RHD, and a glycine-rich region (GRR), but p50 does not have a TAD (Hayden & Ghosh, 2004, 2008) (Figure 2.1).

Figure 2.1: The structural domains of RelA/p65 and p105/p50.
RelA/p65 contains an N-terminal Rel homology domain (RHD), a nuclear localization signal (N) within the RHD and a C-terminal transactivation domain (TAD). The precursor p105 contains an N-terminal RHD, a nuclear localization signal (NLS) within the RHD, a glycine-rich region (GRR), seven AnkRs (A) and a C-terminal death domain (DD).

2.1.3 The discovery of inhibitor of κB (IκB)

After the discovery of NF-κB, a protein regulating NF-κB or inhibitor of κB (IκB) was the next milestone of NF-κB research (Baeuerle & Baltimore, 1988a; Sen & Baltimore, 1986a, 1986b). At the beginning of this challenge, Baeuerle found that NF-κB was sequestered in the cytoplasm of unstimulated cells and it gains DNA-binding ability only in the existence of dissociation agent like sodium deoxycholate. This observation leads to the discovery of IκB.

IκB family proteins consist of IκBα, IκBβ, IκBε, B-cell lymphoma 3 (BCL-3), IκBζ, IκBNS and the C-terminal portion of p105 (IκBγ) and p100 (IκBδ). IκB family proteins contain five to seven AnkRs, which is a 33 amino acids motif that specifically interacts with the RHD of Rel proteins (Ghosh et al., 1998). The activation of cytoplasmic NF-κB requires phosphorylation of IκB proteins on conserved serine residues, which is also known as destruction box serine residues (DSGXXS) and that leads to the dissociation of the inhibitor from NF-κB. Phosphorylation of IκB granted NF-κB with a nuclear localization and DNA-binding ability (Baeuerle & Baltimore, 1988a, 1988b).

At first, phosphorylation of IκB was thought to be the key factor of releasing the transcriptional active NF-κB (Ghosh & Baltimore, 1990). Later, it was proven to be insufficient for the activation of NF-κB and additional IκB degradation is crucial for liberating NF-κB (Alkalay et al., 1995a; Beg et al., 1993; Chen et al., 1995b; DiDonato

et al., 1995; Finco et al., 1994). Moreover, studies revealed that IκB degradation was inhibited by a proteasome inhibitor. Subsequently, three groups of researchers showed that the signal-induced ubiquitination and proteasome-dependent degradation of IκB are important for the activation of NF-κB (Alkalay et al., 1995b; Chen et al., 1995b; Palombella et al., 1994).

In contrast to p65 deletion, IκBα$^{-/-}$ mice exhibit normal phenotypes but died after 7-10 days postnatally with severe inflammatory dermatitis and granulocytosis (Beg et al., 1995; Klement et al., 1996). In the absence of both p50/p105 and IκBα, the lifespan of the mice prolonged significantly to 3-4 weeks before the same phenotype of IκBα deletion re-emerges. This observation suggests that constitutively active NF-κB in the nucleus regulates the survival of neonatal IκBα$^{-/-}$ mice (Beg et al., 1995).

2.1.4 General properties of IκBα

IκBα, the key inhibitor of p65, comprises an N-terminal signal receiving domain (SRD), six AnkRs in the center and a C-terminal proline-, glutamate-, serine-, and threonine-rich (PEST) sequences (Jacobs & Harrison, 1998; Napetschnig & Wu, 2013) (Figure 2.2). The N-terminal signal receiving domain contains two critical serine residues (S32 and S36) for IKK kinase phosphorylation (Brown et al., 1995; Chen et al., 1995b). The AnkRs of IκBα consists of two α-helices and one β-loop, which are important for the interaction of IκBα with p65/p50 heterodimers. The AnkR 1 and 2 mask the NLS signal of p65, whereas AnkR 4-6 interact with p65/p50 RHD and dimerization interface (Huxford et al., 1998; Jacobs & Harrison, 1998). The C-terminal PEST of IκBα interacts directly with the N-terminal RHD of p65 and restricts DNA binding ability of p65 (Napetschnig & Wu, 2013).

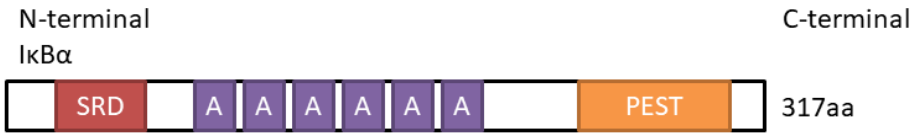


Figure 2.2: The structural domains of IκBα.
IκBα contains an N-terminal signal receiving domain (SRD), six AnkRs (A) in the center and a C-terminal proline-, glutamate-, serine-, and threonine-rich (PEST) sequences.

In TNF-treated cells, IκBα, which has a normal half-life of 2.5 hours, is rapidly phosphorylated and degraded within 1.5 minutes (Henkel et al., 1993). The phosphorylation of S32 and S36 is sufficient to target IκBα for degradation since the introduction of short phosphorylated peptides that mimic the phosphorylation-based motif (DpSGXXpS) of IκBα is able to block IκBα degradation *in vitro* and TNF-induced NF-κB translocation (Yaron et al., 1997). After phosphorylation of IκBα by IKK kinases, $SCF^{\beta\text{-}TRCP}$ complex (Skp, Cullin, F-box containing complex, beta-transducin repeat containing protein), an E3 ligase complex, recognizes phosphorylated IκBα and conjugates K48-linked ubiquitin chain at K21 and/or K22 of IκBα, which then leads to the proteasome-dependent degradation of IκBα (Scherer et al., 1995; Spencer et al., 1999; Strack et al., 2000; Winston et al., 1999). Thus, liberated NF-κB is free to translocate into the nucleus. Interestingly, transcription of IκB itself is regulated by NF-κB which in turns shuts down or negatively regulates NF-κB in a negative feedback loop (Brown et al., 1993; Sun et al., 1993).

Intriguingly, a structural study of the p65/p50 heterodimer and IκBα complexes shows that IκBα masks only the NLS of p65 but leave the NLS of p50 accessible for nuclear transportation machinery (Huxford et al., 1998; Jacobs & Harrison, 1998). Thus, the freely accessible NLS of p50 and the Nuclear export signal (NES) of IκBα control the NF-κB shuttling between nucleus and cytoplasm with the majority of NF-κB remains in the cytoplasm in a steady state (Huang et al., 2000; Johnson et al., 1999). This steady state is disturbed by TNF-induced IκBα degradation that unmasks the p65 NLS and promotes

p65/p50 translocation into the nucleus (Hayden & Ghosh, 2004). The canonical p65/p50 heterodimer recognizes a κB site via p65 and p50 binding to a 5'-GGPyN sequence with A: T base pairs in between them. However, p65/p50 have a flexible short linker region in their RHD that allows them to recognize a variation in κB site by adjusting and repositioning the interaction between the N-terminal RHD and DNA backbone (Hayden & Ghosh, 2004).

P50 is able to form a homodimer with transcriptional repressor effect since p50 does not possess a TAD domain and has no intrinsic ability to drive transcription (Elsharkawy et al., 2010; Hayden & Ghosh, 2004; Smale, 2012). In addition, p50 homodimer shares some similarity with the p65/p50 DNA binding site. Thus, it has been proposed that p50 homodimer modulates the activity of active p65/p50 by regulating the accessibility of the cognate κB elements (Hayden & Ghosh, 2008; Wan & Lenardo, 2009). Despite the p50 homodimer inhibitory effects, IκBα still plays the critical role in controlling the high-affinity DNA binding ability of p65/p50 heterodimer (Wan & Lenardo, 2009). Therefore, IκBα must be removed to allow p65/p50 to translocate into nuclear and provide p65/p50 full DNA binding affinity for transcriptional activation.

2.1.5 Canonical and noncanonical NF-κB pathways

NF-κB is a crucial signaling pathway in the mammalian cells mainly due to the various stimuli that lead to its activation. These include cell damage signal (reactive oxygen intermediates, ultraviolet light and free radical), infection (bacterial and viral), cytokines (TNF and Interleukin 1, IL-1) and others (http://www.bu.edu/nf-kb/physiological-mediators/inducers/) (Ghosh et al., 1998; Gilmore, 1999, 2006; Perkins, 2007). Different stimuli will employ different ways of NF-κB activation and thus, can be divided into canonical (classical) or non-canonical (alternative) pathways.

For canonical pathways, upon stimulation by TNFα via TNFR1, IL-1 via IL-1R or pathogen-associated molecular patterns (PAMPs) such as Toll-like receptor 4 (TLR4), a signaling cascade is activated, recruiting various protein adaptors and activate kinase complexes namely IKK. IKK will phosphorylate IκB proteins and leads to its degradation and freed NF-κB protein will then shuttle into nuclear for transcriptional activation of target genes (Karin, 1999). The classical IKK complexes consist of a regulatory subunit, namely "NF-κB essential modifier" or NEMO (also known as IKKγ, Fip-3 or IKKAP) and two catalytic subunits, namely IKKα and IKKβ. IKK kinase complex typically phosphorylates IκBα and leads to the activation and nuclear translocation of p65:p50 heterodimer (Hayden & Ghosh, 2012) (Figure 2.3).

The non-canonical pathway is usually cell type-specific. In the non-canonical pathway, NF-κB is activated by another class of TNF cytokine family, such as the Cluster of Differentiation-40 (CD40) ligand, B-cell activating factor (BAFF), or lymphotoxin-β. In contrast to the classical pathway that is mainly dependent on IKK complexes (NEMO, IKKα, and IKKβ), the alternative pathway employs IKKα only in a NEMO- and IKKβ-independent manner. This IKKα is activated by NF-κB interacting kinase (NIK) upon stimulation by ligand. Activated IKKα will phosphorylate p100 (precursor of p52), to generate p52:RelB heterodimers and induces p52:RelB nuclear translocation (Sun, 2011) (Figure 2.3).

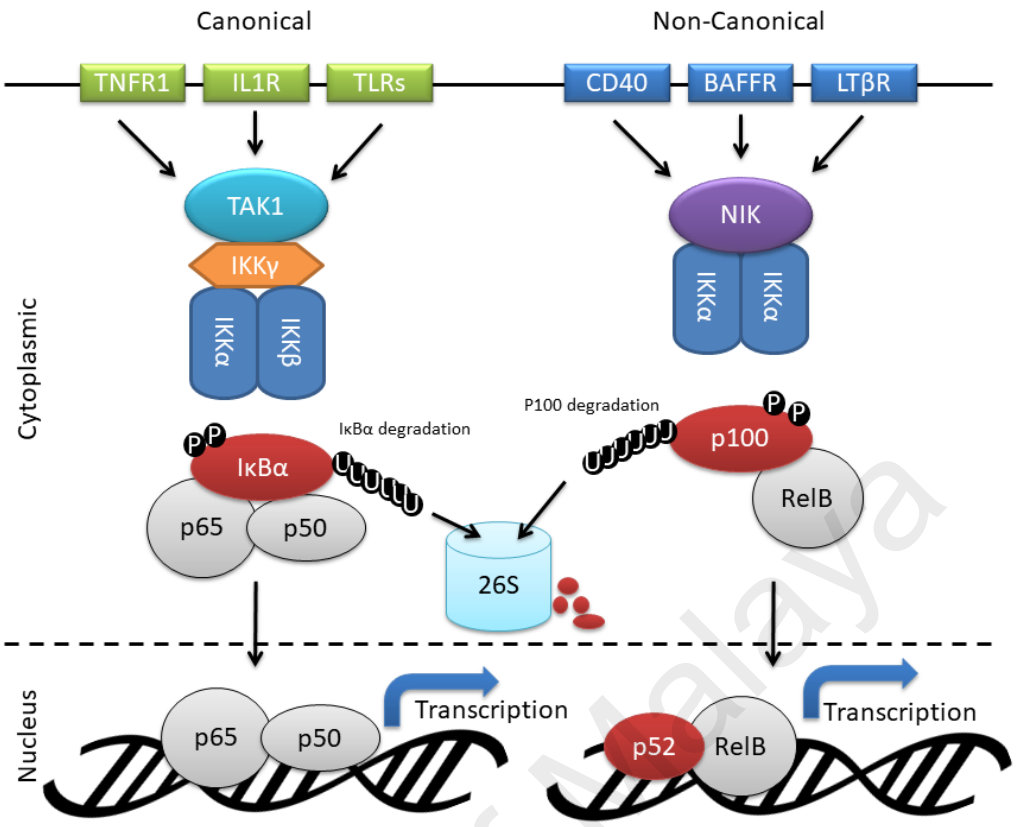


Figure 2.3: Canonical and noncanonical NF-κB signaling pathways.
The canonical pathway can be triggered by numerous cytokines via cytokine specific receptors such as TNFα to TNFR1, IL1β to IL1R or LPS to TLR4 which trigger the activation of a signaling cascade that leads to IKK complex activation by TAK1. IKK mediates IκBα phosphorylation followed by polyubiquitination mediated proteasomal degradation of IκBα. Freed NF-κB translocates into the nucleus and activates gene transcription. Noncanonical NF-κB pathway relies on ligands such as CD40, BAFF, or lymphotoxin-β that triggers the phosphorylation of IKKα homodimer by NIK and leads to the phosphorylation-dependent partial degradation of p100 to generate p52. Freed p52: RelB heterodimer will translocate into the nucleus to activate gene transcription. (P, phosphate; U, ubiquitin).

2.1.6 Biological role of NF-κB

NF-κB is a transcription factor that acts as a central coordinator in regulating multiple cellular processes including inflammation, immunity, cell growth or survival and development (Park & Hong, 2016). Since NF-κB controls more than hundreds of genes that modulate different cellular processes, it is critical for human health. Abnormality in NF-κB activity is closely related to multiple human diseases, such as autoimmune diseases, rheumatoid arthritis, atherosclerosis, inflammatory bowel diseases, multiple sclerosis, and cancer (Didonato et al., 2012; Hoesel & Schmid, 2013; Park & Hong, 2016). For example, NF-κB modulates the expression of genes that are involved in cell

proliferation and cell survival (Sen & Baltimore, 1986b). Many different type of tumor cells hijack the regulatory system of NF-κB and cause it to be constitutively active in order to switch on the cell proliferation and cell survival genes that in turn favor tumor proliferation and development (Park & Hong, 2016). Therefore, understanding the regulation of NF-κB is of considerable importance to control and treat diseases.

2.2 TNFα induced NF-κB signaling

TNF is closely associated with NF-κB as shown by the extensive research on the relationship between the two proteins. As early as year 1989, TNF was shown to activate NF-κB in the regulation of human immunodeficiency virus (HIV)-1 LTR (long terminal repeat) and Interleukin-2 (IL-2) receptor (Duh et al., 1989; Lowenthal et al., 1989; Osborn et al., 1989). It was not until much later that NF-κB was shown to modulate the expression of TNF when macrophage was treated with lipopolysaccharide (LPS) (Shakhov et al., 1990). Since then, the relationship between TNF and NF-κB was studied extensively. In a knockout study, TNF induced transcriptional activity is severely abrogated, and cell death is promoted when p65 is deleted (Beg & Baltimore, 1996; Beg et al., 1995; Doi et al., 1997). In fact, embryonic lethality is observed in the mice with knockout of p65, IKKβ or NEMO due to the severe cell death of hepatocytes, however, the lethality can be rescued when TNFR1 or TNF was deleted simultaneously (Alcamo et al., 2001; Doi et al., 1999; Li, 1999; Li et al., 1999; Rosenfeld et al., 2000). In brief, the entire pathway of TNF-induced NF-κB can be summarized in Figure 2.4.

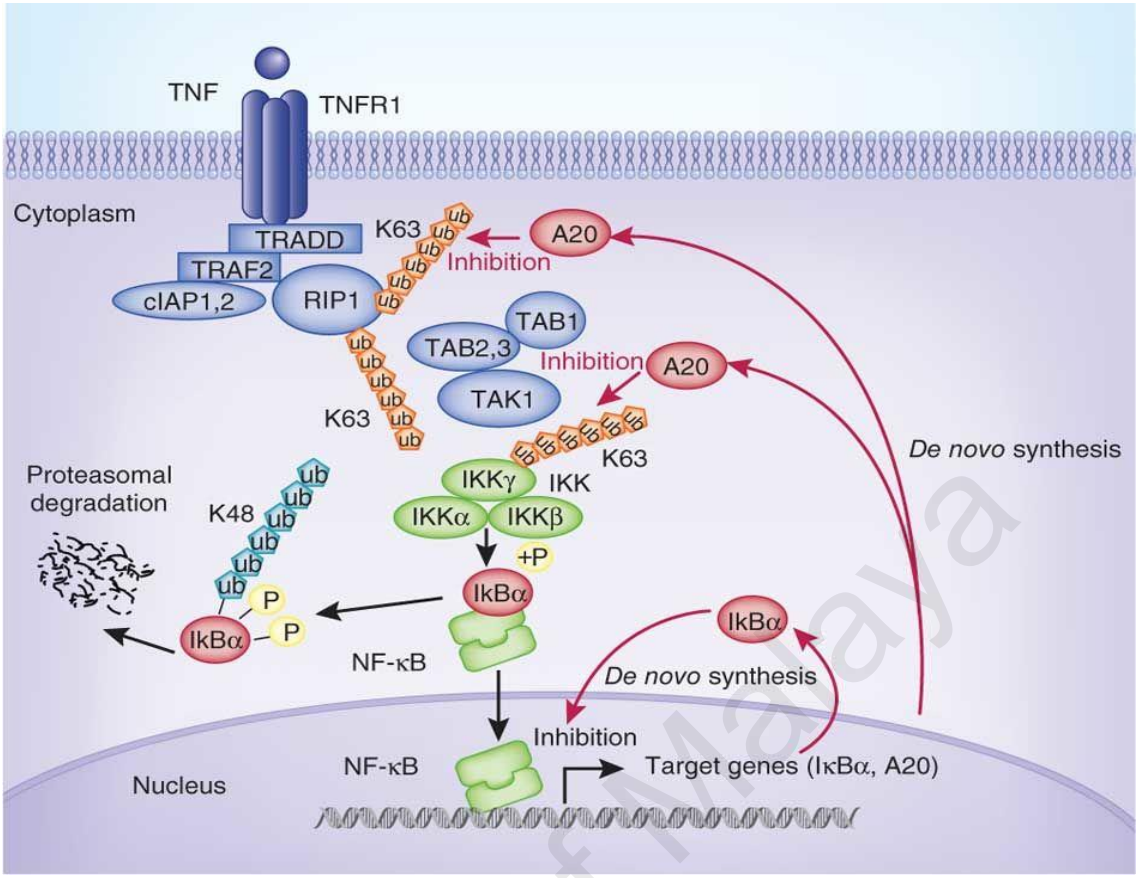


Figure 2.4: Schematic diagram of TNF-induced NF-κB.
Upon stimulation with TNFα, trimerized TNFR1 receptor complex recruits a series of proteins, such as TRADD, TRAF2, cIAPs, and RIP1, which leads to the activation of TAK1 and IKK complexes. Activated IKK complex phosphorylates IκBα, which triggers its K48-linked polyubiquitination and proteasomal degradation. Freed NF-κB translocates into nucleus and activates gene transcription. Activation of NF-κB leads to the activation of its negative regulators IκBα and A20. Resynthesized IκBα will translocate into nucleus and inhibit NF-κB function by shuttling it back to the cytoplasm. A20 is a deubiquitinase that negatively regulates polyubiquitination chain and shuts down the NF-κB activation. (Adapted by permission from Ruland (2011)).

2.2.1 TNFα ligand and receptor

Tumor necrosis factor (TNF) was first described in 1975 by Carswell as an inducible endotoxin molecule that was capable of inducing necrosis in the tumor *in vitro* (Carswell et al., 1975). Later in the mid-1980s, two different TNFs were cloned and characterized biochemically by Aggarwal and coworkers (Aggarwal et al., 1984, 1985, 2012; Pennica et al., 1984). Later, it was named TNFα and TNFβ based on their sequence homology. TNF is a well-characterized cytokine that plays an essential role in the immune system. More than a decade of research on TNF, demonstrated that it is a central player for more than just pro-inflammatory function but also for cell-cell communication, differentiation,

and cell death. The TNF superfamily comprises of 19 ligands and 29 receptors. Every member of TNF superfamily has a diverse function in the human body (Aggarwal et al., 2012). Among all the members of the TNF superfamily, TNFα remains one of the most established and characterized cytokines.

TNFα, also known as cachectin or differentiation inducing factor (DIF), is produced by activated monocytes or macrophages, activated NK and T cells and non-immune cells, such as epithelial and fibroblast cells (Falvo et al., 2010; Tsai et al., 1996). It is expressed as a trimeric type II transmembrane protein with about 26 kDa in mass, sometimes referred to as pro-TNF. Its production is regulated by NF-κB, c-Jun, activator protein-1 (AP-1) and nuclear factor associated with activated T-cells (NFAT) proteins (Tsai et al., 1996). Pro-TNF is further processed or cleaved by a metalloprotease named TNFα-converting enzyme (TACE, also known as ADAM17), to become a soluble form of TNF with a molecular mass of approximately 17 kDa (Black et al., 1997; Moss et al., 1997) (Figure 2.5). The soluble form of TNF is then circulated in human plasma and works as a cytokine in the endocrine system.

Both soluble and transmembrane TNF are capable of binding to its transmembrane receptors, such as TNFR1 (also known as p55/p60) and TNFR2 (also known as p75/p80) (Tartaglia et al., 1991). However, transmembrane TNF binds preferably to TNFR2 (Grell et al., 1995). TNFR1 is constitutively expressed in most of the cell types at low levels, while TNFR2 is only expressed in certain cell types (Carpentier et al., 2004). TNF binding to both TNFR1 and TNFR2 is capable of activating NF-κB. However, the resulting signaling cascade differs between TNFR1 and TNFR2. Due to the ubiquitous expression of TNFR1 in most of the cells, this study was limited to TNFR1-induced NF-κB signaling.

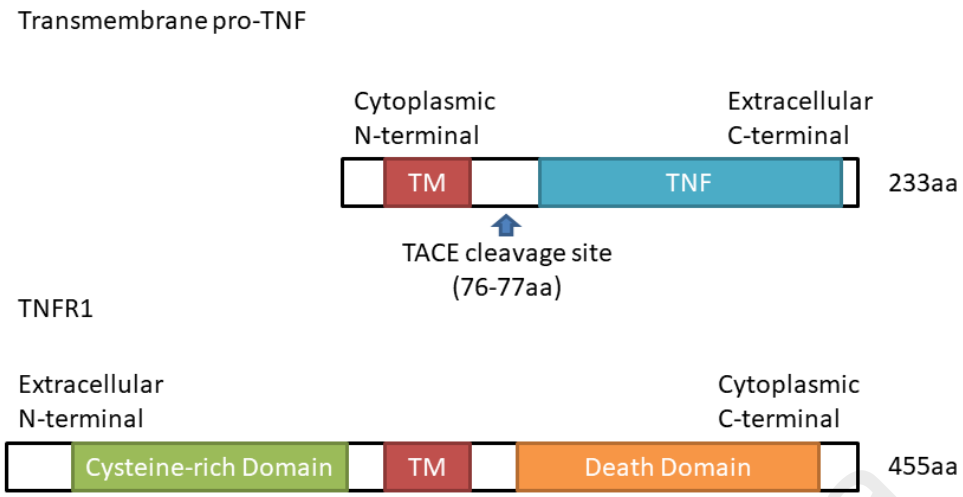


Figure 2.5: The structural domains of transmembrane pro-TNF and TNFR1.
The top diagram shows the transmembrane pro-TNF. It is further processed or cleaved by a metalloprotease to become a soluble form of TNF with a molecular mass of approximately 17 kDa. The bottom diagram shows TNFR1 with an extracellular cysteine-rich domain for TNFα interaction, transmembrane domain (TM) and C-terminal death domain (DD).

TNFR1 contains two important domains, namely an extracellular cysteine-rich domain for soluble TNF binding and a cytoplasmic death domain (DD) that is crucial for the recruitment of adaptor proteins. (Lavrik et al., 2005; Tartaglia et al., 1993) (Figure 2.5). Upon ligation of TNF to the extracellular region of TNFR1, TNFR1 trimerizes and triggers biochemical signaling leading to the activation of NF-κB, as well as MAP kinases, apoptosis or necroptosis (Aggarwal, 2003; Berghe et al., 2014; Chan et al., 2000; MacEwan, 2002) (Figure 2.6). The DD is 80 amino acids long with evolutionarily conserved sequences. The DD of TNFR1 is essential for NF-κB activation (Lavrik et al., 2005). The silencer of death domains (SODD) was found to prevent TNFR1 self-association. Upon the binding of TNF to TNFR1, SODD will dissociate from the TNFR1-DD and promote NF-κB signaling (Jiang et al., 1999). The trimerization of TNFR1 attracts or encourages the binding of a DD-containing adaptor protein, TNFR1 associated death domain protein (TRADD).

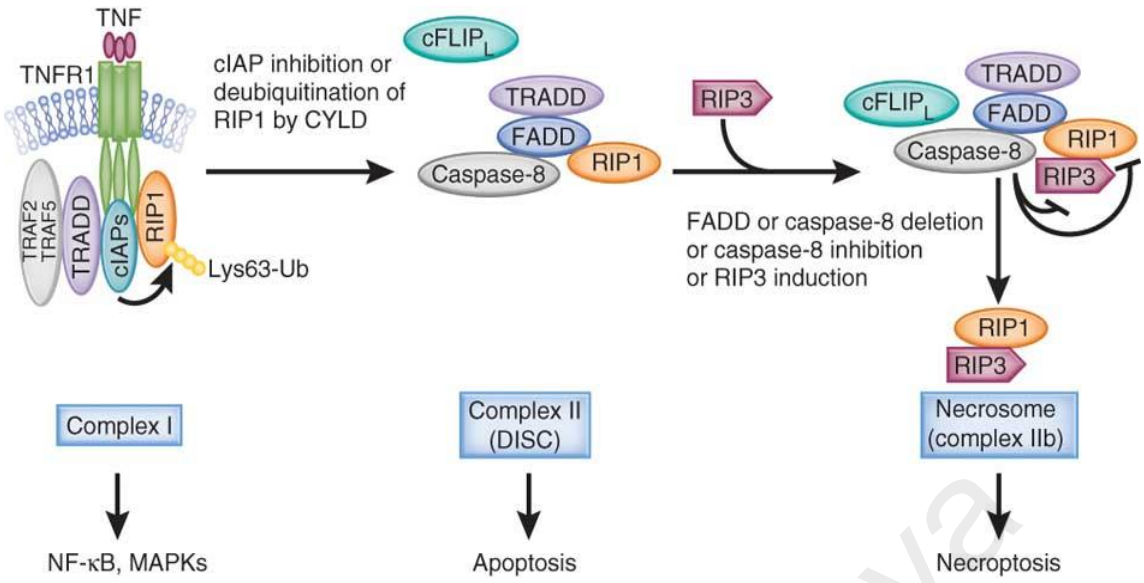


Figure 2.6: TNF-induced NF-κB, apoptotic and necroptotic signaling.
Upon ligation of TNF to the extracellular region of TNFR1, TNFR1 trimerizes and triggers biochemical signaling that leads to the activation of NF-κB, as well as MAPK, apoptosis or necrosis. Cytoplasmic portion of TNFR1 recruits multiple protein adapters to trigger the pro-survival TNFR1 complex I. Deubiquitination and cIAP inhibition promote the formation of pro-apoptotic complex II, also known as cytosolic death-inducing signaling complex (DISC). In the cells with defective FADD or caspase-8 as well as higher level of RIP3, TNF-treatment will lead to the initiation of necroptosis. (Adapted by permission from Han et al. (2011)).

2.2.2 TRADD

TRADD is a 34 kDa protein that binds to the C-terminal DD of TNFR1 (Figure 2.7). The binding of TRADD is crucial for TNFR1 induced NF-κB signaling and apoptosis. TRADD is also essential for germline center formation, Death Receptor 3 (DR3)-mediated costimulation of T cells and TNFα-mediated inflammatory responses *in vivo*. Furthermore, TRADD has novel roles in TLR3 and TLR4 signaling pathways (Chen et al., 2008). Overexpression of TRADD is capable of activating two major events, namely TNF-induced NF-κB and apoptosis. The 118 amino acids from the C-terminal domain of TRADD are essential for the activation of both pathways (Hsu et al., 1995). Moreover, TNFR1 receptor complex formation is reduced in TRADD knockout mice (Pobezinskaya et al., 2008) and human B cells (Schneider et al., 2008). Interestingly, TRADD is essential for TNFR1 complex formation in mouse embryonic fibroblast but not in macrophage due to higher expression levels of another TNFR1 adaptor, receptor-interacting serine/threonine-protein kinase 1 (RIPK1/RIP1) which permits limited signal

transmission in the absence of TRADD (Pobezinskaya et al., 2008). The recruitment of TRADD to TNFR1 can lead to the recruitment of the second adaptor protein RIP1 and TNFR-associated factor 2 (TRAF2) (Hsu et al., 1996a, 1996b; Liu et al., 1996).

Figure 2.7: The structural domains of TRADD.
TRADD contains a TRADD N-terminal domain for TRAF2 interaction and C-terminal death domain (DD) for TNFR1 DD interaction.

2.2.3 RIP1

RIP1 is a 74 kDa protein that comprises of an N-terminal kinase domain, a α-helical intermediate domain and a C-terminal death-domain (Stanger et al., 1995) (Figure 2.8). The N-terminal kinase domain is essential for RIP1-associated necroptosis together with RIP3 (another receptor-interacting serine/threonine-protein kinase) and RIP1 autophosphorylation (Hsu et al., 1996a; Ting et al., 1996). However, the kinase domain is dispensable for TNF-induced NF-κB, since kinase-defective-RIP1 is able to restore the TNF-induced NF-κB activation in RIP1 deficient mice (Ting et al., 1996). A mouse with RIP1 deletion leads to premature lethality and immunity abnormalities, suffering severe cell death in the lymphoid and adipose cells (Kelliher et al., 1998). RIP1 is highly expressed in lymphoid tissue and lymphocyte population especially in immature B cells in bone marrow and peripheral T and B cells (Zhang et al., 2011). RIP1 deficiency induced cell death is partially associated with the failure of NF-κB activation (Cusson et al., 2002). $RIP1^{-/-}$ cells are sensitized to TNF-induced apoptosis due to the reduction of NF-κB stimulation (Kelliher et al., 1998). In addition, a RIP1 kinase-dead ($RIP1^{K45A}$) mouse shows viable and healthy phenotypes, suggesting that pro-survival role of RIP1 does not require its kinase activity (Berger et al., 2014; Kaiser et al., 2014).

The intermediate domain and DD of RIP1 are involved in mediating NF-κB activation. The intermediate domain contains a RIP homotypic interaction motif (RHIM) which is necessary for the interaction with RHIM from RIP3 for necroptosis activation (Festjens et al., 2007). On the other hand, the DD of RIP1 mediates the interaction of RIP1 to Fas or DD-containing protein such as ligand-bound TNFR1 (Zheng et al., 2006). Although RIP1 can bind directly to DD-containing TNFR1, the interaction is more efficient in the presence of TRADD (Hsu et al., 1996a, 1996b). Interestingly, RIP1 contains TRAF binding motif that is believed to contribute to the recruitment of TRAF2 to the receptor complex under certain situations (Pobezinskaya et al., 2008) and may not be solely dependent on TRADD. Upon the formation of TNFR1 complex 1, RIP1 is polyubiquitinated with multiple forms of ubiquitin chain such as K63-, K48-, K11-, and linear (M1)-linked polyubiquitin chains that are essential for activating downstream signaling (Ea et al., 2006; Kanayama et al., 2004; Legler et al., 2003; Li et al., 2006; Zhang et al., 2000).

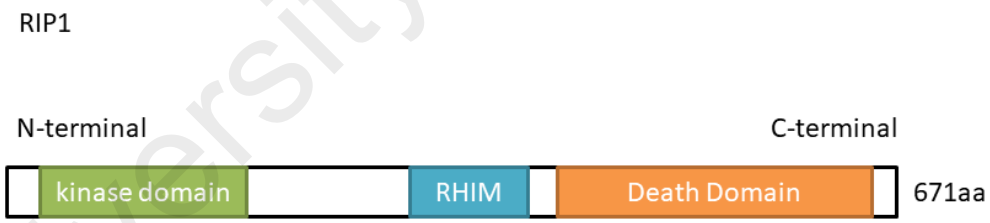


Figure 2.8: The structural domains of RIP1.
RIP1 contains an N-terminal kinase domain, an intermediate domain which contains a RIP homotypic interaction motif (RHIM), and a C-terminal death domain.

2.2.4 TRAF2 and TRAF5

TRAF2 is a 54 kDa protein that was discovered along with TRAF1 through yeast two hybrid study using a C-terminal domain of TNFR2 as bait (Rothe et al., 1994). Shortly after, TRAF5 was discovered via interaction with a C-terminal domain of CD40 in another yeast two hybrid screen (Ishida et al., 1996). Similar to other TRAF family members, TRAF2 and TRAF5 contain an N-terminal really interesting new gene (RING)

finger and five zinc finger motifs, a conserved C-terminal TRAF homology domain which can be divided into an N-terminal coil-coiled region (TRAF-N) and a β-sandwich (TRAF-C) (Wu et al., 1999) (Figure 2.9). The TRAF domain is capable of mediating protein-protein interaction with other adaptor proteins in a signaling cascade as well as mediating TRAF family protein oligomerization (Ha et al., 2009; Wajant et al., 2001). The N-terminal RING finger of TRAF is commonly found in E3 ubiquitin ligases, suggesting that TRAF2 and TRAF5 also play a role in ubiquitination as an E3 ubiquitin ligase (Xie, 2013). However, TRAF2 has an unusual RING structure and some have claimed that TRAF2 does not and cannot have E3 ligase activity (Yin et al., 2009).

TRAF2 and TRAF5 are structurally and functionally similar but have different expression patterns. TRAF2 is expressed ubiquitously whereas TRAF5 is detected only in limited tissues with significant levels in lung, thymus, spleen, and kidney and at a lower level in brain and liver tissues (Ishida et al., 1996; Rothe et al., 1994; Yeh et al., 1997). This phenomenon has been shown through the severity of TRAF2 deletion mice that exhibit perinatal lethality whereas TRAF5 knockout only leads to a certain defect in CD40 and CD27 mediated lymphocyte development. However, both TRAF2 and TRAF5 double knockdown exhibit some functional redundancy and defects in NF-κB activation (Nakano et al., 1999; Tada et al., 2001; Yeh et al., 1997).

In contrast to TNFR2 mediated NF-κB, TNFR1, which does not have any TRAF interacting motif, recruits TRAF2 via TRADD (Hsu et al., 1995; Rothe et al., 1995; Tartaglia et al., 1993). Upon stimulation with TNFα, TRAF2 is recruited to the receptor complex by TRADD-TRAF binding domain. Moreover, the structural study of the interaction between TRADD and TRAF2 suggests that interaction between TRAF2 and TRADD is stronger than the typical interaction between TRAF2 and other TNFR family member with TRAF interacting domain such as TNFR2. In contrast to TRAF2, TRADD has a much lower binding affinity to TRAF5 (Park et al., 2000). Therefore, in TNF-

induced TNFR1 in wild type cells, TRAF2 is more likely to be recruited to the receptor complex than TRAF5. In addition, in TRAF2 deficient mice, TRAF5 may compensate TRAF2 to activate NF-κB weakly but not for AP-1 activation. In contrast to TRAF2, TRAF5 deletion exhibits normal NF-κB and AP-1 activation. The massive defects in NF-κB and AP-1 activation are only achievable in TRAF2/5 double deletion mice (Nakano et al., 1999; Tada et al., 2001; Yeh et al., 1997). Upon the recruitment of TRAF2 to TRADD, TRAF2 is able to recruit another key player c-IAP1 and c-IAP2 to the TNFR1 complex (Park et al., 2000; Uren et al., 1996).

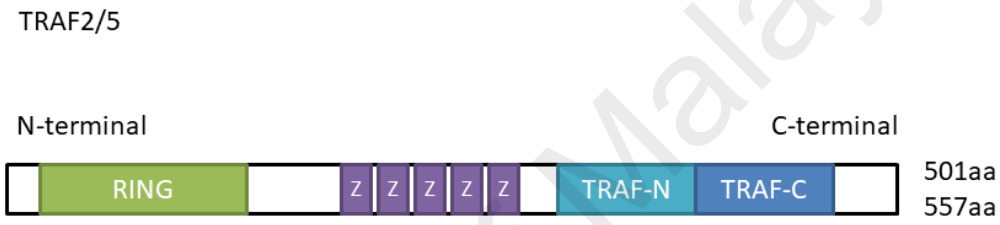


Figure 2.9: The structural domains of TRAF2/5.
TRAF2 (501 amino acids) and TRAF5 (557 amino acids) contain an N-terminal RING finger and five zinc finger motifs (Z), a conserved C-terminal TRAF homology domain which can be divided into an N-terminal coil-coiled region (TRAF-N) and a β-sandwich (TRAF-C).

2.2.5 cIAPs

Inhibitor of Apoptosis (IAP) was discovered in 1993 as a baculovirus protein that maintains cell survival for efficient viral replication in insect host cells (Crook et al., 1993). Afterward, scientists revealed eight IAP proteins that were encoded by human genomes, such as Baculoviral IAP repeat-containing protein 1 (BIRC1)/NAIP, BIRC2/cIAP1, BIRC3/cIAP2, BIRC4/XIAP, BIRC5/Survivin, BIRC6/BRUCE, BIRC7/ML-IAP and BIRC8/ILP2 (Estornes & Bertrand, 2015; Salvesen & Duckett, 2002). In the early years, IAPs were thought to be a specific inhibitor of cellular apoptosis. However, it was later proven to be involved in a wider spectrum of cellular processes. Recently, researchers have also reported the involvement of IAPs in the regulation of inflammation and innate immunity (Estornes & Bertrand, 2015; Gyrd-Hansen & Meier, 2010).

IAPs are characterised by the presence of one or more baculovirus IAP repeat (BIR) domains (Birnbaum et al., 1994), which contain approximately 70 amino acid residues that comprise of Zinc binding motif that facilitates protein-protein interaction. Human IAP proteins contain between one to three copies of BIR domain. For example, cIAP1 and cIAP2 contain three copies of BIR domain at the N-terminus (Figure 2.10). The first BIR domain (BIR1) of cIAP1 and cIAP2 is associated with TRAF2 for their recruitment to receptor complex (Samuel et al., 2006). Besides, they possess an ubiquitin-associated domain (UBA) that gives them poly-ubiquitin chain binding ability. In addition, cIAP1 and cIAP2 have a RING domain in their C-terminal portion that provides them with an E3 ligase capability. To date, cIAPs have been shown to mediate the conjugation of K48-, K63- and K11-linked polyubiquitin chains to substrate protein (Silke & Meier, 2013). Interestingly, cIAP1 and cIAP2 have an extra, evolutionary conserved, caspase recruitment domain (CARD) located between UBA and RING domain, which is a suppressor for its E3 ligase activity (Dueber et al., 2011; Lopez et al., 2011).

The association of cIAP and TNFR1 was first reported by Shu et al. (1996). It was further established by the studies of *Drosophila* IAP (DIAP2) that showed the involvement of DIAP2 in TAK-TAB2 or TAB3 mediated NF-κB activation via immune deficiency signaling cascade (*Imd*) in response to gram-negative bacterial infection. Surprisingly, the *Imd* signaling cascade resembles the human TNFR1 signaling (Gesellchen et al., 2005; Huh et al., 2007; Kleino et al., 2005; Lemaitre & Hoffmann, 2007; Leulier et al., 2006). In *DIAP2* deletion *Drosophila*, it failed to activate NF-κB when triggered by bacterial infection (Huh et al., 2007; Leulier et al., 2006). Besides, the E3 ligase activity of DIAP2 has been shown to be essential for NF-κB activation (Huh et al., 2007; Leulier et al., 2006; Meinander et al., 2012; Paquette et al., 2010). Similarly, in humans, cIAP1 and cIAP2 are essential for canonical NF-κB and MAP kinases activation through TNFR1 (Mahoney et al., 2008; Varfolomeev et al., 2008). Deletion of individual

cIAPs in mice shows normal development with limited cell-death phenotypes. However, deletion of both cIAP1 and cIAP2 causes embryonic lethality. This phenomenon indicates the existence of functional redundancy among cIAP proteins. Intriguingly, this embryonic lethality phenotype can be reversed with TNFR1 deletion which strengthens the association of cIAPs and TNF signaling pathway (Heard et al., 2015; Moulin et al., 2012, 2015).

Upon stimulation, the formation of TNFR1 complex I that consists of trimeric TNF-TNFR1, TRADD, TRAF2, cIAPs and RIP1, is critical for the canonical NF-κB and MAPK activation (Micheau & Tschopp, 2003). With E3 ligase activity of cIAPs, ubiquitins are conjugated to RIP1. Although TRAF2 possesses a RING domain initially thought to be responsible for RIP1 ubiquitination, previous evidence suggests that cIAP1 and cIAP2 are responsible for RIP1 ubiquitination (Bertrand et al., 2008; Varfolomeev et al., 2008; Xu et al., 2009; Yin et al., 2009). It is more likely that TRAF2 is crucial for the recruitment of cIAP1 and cIAP2 rather than directly catalyzing the ubiquitination of RIP1 (Hayden & Ghosh, 2014; Silke, 2011; Yin et al., 2009). Subsequently, polyubiquitination chain acts as a platform for the recruitment of linear ubiquitin chain assembly complex [LUBAC, which consists of Heme-oxidized IRP2 ubiquitin ligase 1 (HOIL1), HOIL-1-interacting protein (HOIP), and SHANK associated RH domain interactor (SHARPIN)], TAK kinase complexes (TAK1, TAB2 and TAB3) and IKK kinase complexes (Hayden & Ghosh, 2014; Silke, 2011). Collectively, cIAP1 and cIAP2 are regulators of apoptosis and inflammation (Estornes & Bertrand, 2015; Gyrd-Hansen & Meier, 2010; Silke & Meier, 2013).

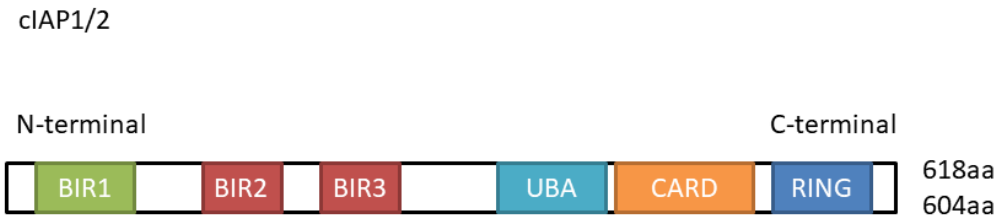


Figure 2.10: The structural domains of cIAP1/2.
Both cIAP1 and cIAP2 contain an N-terminal BIR1 domain, followed by BIR2 and BIR3, an ubiquitin-associated domain (UBA), a RING domain in their C-terminal portion and a caspase recruitment domain (CARD) between its UBA and RING domains.

2.2.6 TAK1

Transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1), also known as mitogenic-activated protein (MAP) kinase kinases 7 (MAP3K7), belongs to MAP3K family. In 1995, TAK1 was discovered in a MAP kinase study in yeast. TAK1 can be activated by TGF-β and bone morphogenetic protein (BMP) (Yamaguchi et al., 1995). Since then, it was found to be associated with other proinflammatory cytokines and other stimuli such as TNFα, Interleukin-1β (IL-1β), TLR, T-cell receptor (TCR) and B-cell receptor (BCR) (Ajibade et al., 2013; Dai et al., 2012). TAK1 forms a complex with TAK1-binding proteins (TAB1, TAB2 or TAB3) and the formations of either TAK1-TAB1-TAB2 or TAK1-TAB1-TAB3 complexes are crucial for TAK1 autophosphorylation (Besse et al., 2006; Cheung et al., 2004; Ishitani et al., 2003; Shibuya et al., 1996; Takaesu et al., 2000). TAK1 is essential for the regulation of the transcriptional activities of NF-κB and AP-1 in particularly for inflammatory signaling pathway, which in turn leads to diverse cellular responses, such as cell proliferation, survival, innate and acquired immunity (Hayden & Ghosh, 2014). Deletion of TAK1 in mice results in an embryonic lethality. Moreover, NF-κB and AP-1 activation are severely impaired in TAK1 deficient MEF cells but not in TAB1 or TAB2 deficiency cells (Shim et al., 2005).

TAK1 contains an N-terminal kinase domain, adjacent TAB1 binding domain and C-terminal TAB2/3 binding domain (Besse et al., 2006; Ono et al., 2003; Yamaguchi et al.,

1995) (Figure 2.11). TAK1 with a deletion of N-terminal 22 amino acid is constitutively active which suggests that the N-terminal region of TAK1 exhibit an autoinhibitory effect on its kinase activity (Shibuya et al., 1996; Yamaguchi et al., 1995). TAB1 is different from TAB2 and TAB3. TAB1 contains an N-terminal pseudo phosphatase domain and a C-terminal TAK1-binding domain. On the other hand, TAB2 and TAB3 possess an N-terminal coupling of ubiquitin conjugation to endoplasmic reticulum degradation (CUE) domain and a C-terminal TAK1 binding domain and a Npl4 zinc finger (NZF) ubiquitin binding domain (Besse et al., 2006; Hirata et al., 2017; Ishitani et al., 2003; Sakurai et al., 2000) (Figure 2.11).

After the ubiquitination of RIP1, TAK1, TAB1, and TAB2 or TAB3 complexes will be recruited to the K63-linked ubiquitin chain of RIP1 through the TAB2 or TAB3's NZF domain. In turn, conformation change of TAK1 complexes promotes the autophosphorylation of TAK1 that is critical for TAK1 activation (Kanayama et al., 2004; Lee et al., 2004; Wang et al., 2001). The interaction of NZF domain of TAB2 and TAB3 towards K63-linked ubiquitin chain has been proven in crystal structure analysis (Kulathu et al., 2009; Sato et al., 2009). In addition, the K63-linked ubiquitin chain of RIP1 acts as a scaffold for TAK1 substrates, such as IKK complexes and MAP kinase kinases (MKKs), assembly to close proximity to TAK1 for phosphorylation-dependent activation (Adhikari et al., 2007). The assembly of these complexes leads to IKKβ phosphorylation and MAPK activation which subsequently result in the activation of transcriptional activity of NF-κB and AP-1 (Hayden & Ghosh, 2014; Symons et al., 2006).

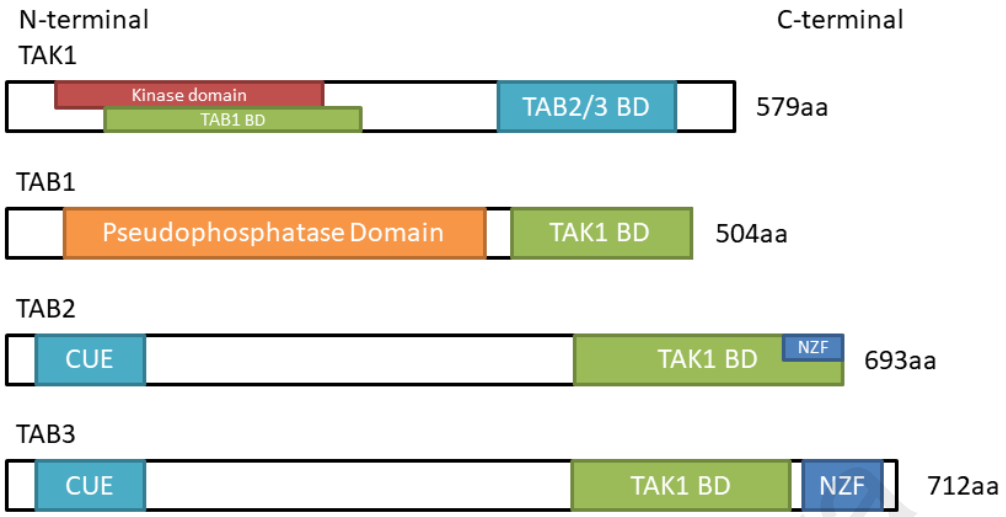


Figure 2.11: The structural domains of TAK1, TAB1, TAB2, and TAB3. TAK1 contains an N-terminal kinase domain, TAB1 binding domain, and C-terminal TAB2/3 binding domain. TAB1 contains an N-terminal pseudophosphatase domain and a C-terminal TAK1-binding domain. TAB2 and TAB3 contain an N-terminal coupling of ubiquitin conjugation to the endoplasmic reticulum degradation (CUE) domain and a C-terminal TAK1 binding domain and a Npl4 zinc finger (NZF) ubiquitin binding domain.

2.2.7 IKK complex

The IKK complex was discovered by Maniatis and colleagues in 1996 as a 700 kDa macromolecular complex with the ability to phosphorylate IκBα *in vitro* (Chen et al., 1996). Since then, multiple groups of scientists have sought to identify the components of this macromolecule kinase. In 1997, three separate research groups identified the key element proteins of IKK complexes such as IKKα and IKKβ that were involved in IκBα phosphorylation and provided the evidence for the missing link of NF-κB activation (DiDonato et al., 1997; Mercurio et al., 1997; Régnier et al., 1997; Zandi et al., 1997). In the following year, another key protein NEMO was identified by two other groups. Alain Isreal's group in Paris recovered a full-length cDNA encoding a 48 kDa protein, NEMO using the genetic complementation approach (Yamaoka et al., 1998). At the same time, Karin's group also discovered the NEMO protein through immunoprecipitation of IKK complex with IKKα antibody (Rothwarf et al., 1998). In the canonical pathway, IKK complexes exist in a ratio of 1:1:2 whereby one IKKα and IKKβ heterodimer is bound to the dimer of NEMO (Häcker & Karin, 2006; Hayden & Ghosh, 2008; Scheidereit, 2006; Tegethoff et al., 2003).

The two kinases of IKK complex, IKKα and IKKβ, have a size of 85kDa and 87kDa, respectively. Both IKKα and IKKβ share 51% sequence homology (Mercurio et al., 1997). Both of them have an N-terminal kinase domain with two key serine residues (S176 and S180 for IKKα, S177, and S181 for IKKβ) that are essential for phosphorylation and IKK kinase activation. Activation of IKK relies on the phosphorylations of these serine residues that causes conformation changes and kinase activation (Delhase et al., 1999; DiDonato et al., 1997; Johnson et al., 1996; Ling et al., 1998; Mercurio et al., 1997). Besides a kinase domain, IKKα and IKKβ possess a leucine zipper (LZ) domain for homo- and heterodimerization, a helix-loop-helix (HLH) domain that is essential in regulating IKK kinase activity and a C-terminal NEMO-binding domain (NBD) for its interaction with NEMO (Delhase et al., 1999; Israël, 2010; Liu et al., 2012) (Figure 2.12). Interestingly, IKKβ has an additional ubiquitin-like domain (ULD) and IKKβ lacking the ULD is catalytically inactive and unable to induce NF-κB. IKKβ with site mutagenesis of L353 on its ULD is catalytically active but unable to dissociate from p65. These phenomena suggest that ULD is necessary for the dissociation of IKK complex from NF-κB after phosphorylation of its target IκBα (May et al., 2004).

NEMO comprises two coil-coiled domains (CC1 and CC2) for protein-protein interaction (Figure 2.12). The N-terminus and CC1 of NEMO are responsible for dimerization and interacting with the NBD of IKKα and IKKβ (Marienfeld et al., 2006). Moreover, CC2 of NEMO with the adjacent LZ domain possesses an ubiquitin binding ability which is known as UBAN (ubiquitin-binding in ABIN and NEMO) domain (Rahighi et al., 2009). Studies have reported that NEMO binds M1-linked polyubiquitin chains with IKK activation and NF-κB responses, significantly reduced with site-mutagenesis that abolishes its polyubiquitin binding ability (Ea et al., 2006; Tang et al., 2003; Wu et al., 2006). Besides, NEMO contains a C-terminal zinc finger (ZNF) domain for IκBα binding and is essential for substrate recognition (Schröfelbauer et al., 2012).

Similar to p65, NEMO and IKKβ knockout mice exhibit embryonic lethality at day E13 with severe liver apoptosis (Li et al., 1999a, 1999b). The lethality phenotype can be rescued with crossing the IKKβ deletion mice with TNFR1 deletion mice (Li et al., 1999a). Thus, IKK complexes are central coordinators of TNF-induced NF-κB. Upon stimulation by TNFα, activated IKK complexes phosphorylate IκBα at S32 and S36 rapidly that leads to K48-linked polyubiquitination and proteasome-dependent degradation of IκBα (Chen, 2012; Chen et al., 1995b, 1996).

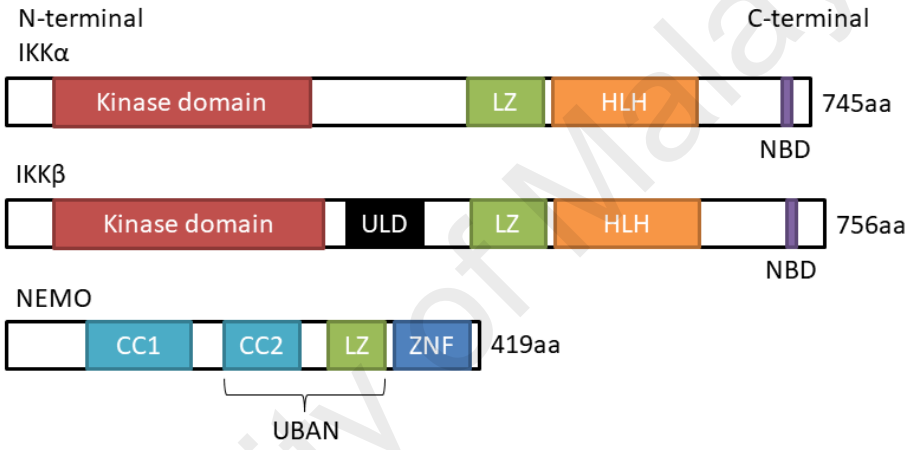


Figure 2.12: The structural domains of IKK complex.
Both IKKα and IKKβ possess an N-terminal kinase domain, a leucine zipper (LZ) domain, a helix-loop-helix (HLH) domain and a C-terminal NEMO-binding domain (NBD) whereas IKKβ only contains an extra ubiquitin-like domain (ULD). NEMO contains two coil-coiled domains (CC1 and CC2), a leucine zipper (LZ) domain, UBAN (ubiquitin-binding in ABIN and NEMO) domain and C-terminal zinc finger (ZNF) domain.

2.2.8 Regulation of NF-κB pathway by post-translational modification

The general framework of NF-κB activation by TNF stimulation has been described previously in section 2.2.1 to 2.2.7. However, emerging evidence implies that NF-κB activation is not solely dependent on protein adaptor recruitment, protein-protein interaction, IKK activation, IκBα degradation, or NF-κB nuclear translocation. Besides the standard framework of activation, there are many exceptions and alternative ways of regulating and controlling NF-κB. Current advances suggest that NF-κB signaling shall be governed via numerous post-translational modifications on core components of the

pathway, such as RIP1, IKK kinases, IκB or NF-κB itself. Moreover, NF-κB is regulated either positively or negatively by different types of post-translational modifications to fine-tune the transcriptional activities of NF-κB. The various post-translational modifications include ubiquitination, phosphorylation, acetylation, sumoylation, nitrosylation, and methylation, which vary in response to different stimuli and lead to different outcome and specificity of NF-κB activities (Huang et al., 2010; Perkins, 2006).

2.2.8.1 Roles of ubiquitination

Ubiquitin is a highly conserved and ubiquitously expressed protein in eukaryotes from yeast to human. Ubiquitin contains 74 amino acids with a flexible C-terminal glycine that is capable of forming an isopeptide bond with the epsilon amine group of lysine or the N-terminal methionine of another ubiquitin through a stepwise enzymatic reaction. These processes are facilitated by three unique proteins known as ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3) (Figure 2.13). The C-terminal of ubiquitin is able to form a homotypic linkage with multiple lysine residues of another ubiquitin, such as K6, K11, K27, K29, K33, K48, and K63, to form a polyubiquitin chain or with the N-terminal methionine to form a linear polyubiquitin chain (M1-polyubiquitin chain). Each type of modification will decide the fate of the substrate protein. For example, K48-linked ubiquitination is linked to proteasome-dependent degradation, while K63-linked ubiquitination is involved in non-proteolytic protein activation (Chen & Chen, 2013; Chen & Sun, 2009; Liu & Chen, 2011).

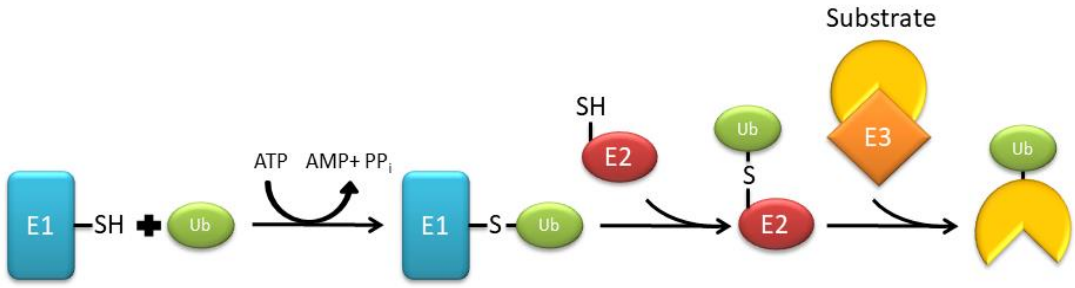


Figure 2.13 The mechanism and machinery of Ubiquitination.
The ubiquitination cascade requires three enzymatic steps. Firstly, ubiquitin activating enzyme (E1) activates an ubiquitin via ATP hydrolysis. Next, the activated ubiquitin is conjugated with ubiquitin conjugating enzyme (E2). Finally, an ubiquitin ligase (E3) facilitates the transfer of the ubiquitin from E2 to the substrate. Ub, ubiquitin; E1, ubiquitin activating enzyme; E2, ubiquitin conjugating enzyme; E3, ubiquitin ligase; PP_i, pyrophosphate.

Besides K48-linked polyubiquitination mediated degradation of IκBα as discussed earlier, TNF pathway has been shown to be regulated by other non-degradable polyubiquitin chains, including K11-linked, K63-linked and M1-linked polyubiquitin chains. There is a general agreement that non-degradable polyubiquitination chain acts as a scaffold for IKK activation in TNF-induced NF-κB (Adhikari et al., 2007). It is worth noting that, LUBAC has been shown to facilitate M1-linked polyubiquitin chains on key component proteins of TNF signaling pathway such as RIP1 and NEMO which further emphasize the role of linear ubiquitination (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011). However, the physiological targets of polyubiquitination remain a conundrum. With the rapid improvement in scientific tools, multiple modifications on component proteins of TNF-induced NF-κB was identified, including TNFR1, TRADD, TRAF2, RIP1, cIAPs, TAB2, TAK1, NEMO and even p65. All of them play certain roles in fine-tuning TNF-induced NF-κB.

For example, Draber et al. (2015) demonstrated that TNFR1, TRADD, and RIP1 undergo M1-polyubiquitination that regulates the stability of TNFR1 complex I (Draber et al., 2015). Moreover, K63-linked polyubiquitination on TNFR1 mediated by RNF8 acts as an early molecular checkpoint for the cell fate decision between cell survival and death (Fritsch et al., 2014). In addition, RIP1 is modified by K11-linked, K48-linked,

K63-linked and M1-linked polyubiquitin chains mediated by cIAP1 and UbcH5 to activate TAK1 and IKK complexes (Bertrand et al., 2008; Blackwell et al., 2013; Dynek et al., 2010; Varfolomeev et al., 2008; Witt & Vucic, 2017). Like RIP1, TRAF2 becomes polyubiquitinated with K48- and K63-linked polyubiquitin chains upon TNFα stimulation (Habelhah et al., 2004; Li et al., 2009; Shi & Kehrl, 2003; Wu et al., 2005). Moreover, TAK1 and TABs also exhibit K48- and K63-linked ubiquitination on multiple lysine residues (Hirata et al., 2017). It is also worth noting that NEMO undergoes multiple types of ubiquitination that affect its function. For instance, LUBAC linearly polyubiquitinates NEMO and regulates NF-κB activation (Gerlach et al., 2011; Tokunaga et al., 2011) while cIAP1 mediated K-6 linked polyubiquitination chain on NEMO ZNF region is necessary for activation of IKK by TNF-α (Tang et al., 2003).

Since the central consequence of TNF-induced NF-κB relies on the transcriptional activity of NF-κB itself, therefore, it must be well monitored and controlled to prevent a catastrophic outcome. P65 is monoubiquitinated on multiple lysine residues that decrease NF-κB transcriptional activity in a non-proteolytical manner (Hochrainer et al., 2012). Moreover, HERC3, an E3 ligase facilitates K48-linked polyubiquitination on K195 and K315 of p65 independently of its catalytic domain that leads to the attenuation of p65 activity and promotes proteasomal degradation of p65 (Hochrainer et al., 2015). Substitution of K195R abolishes its polyubiquitination and degradation as well as promotes stronger NF-κB activation (Fan et al., 2009). Moreover, p65 is also regulated by PDZ and LIM domain 2 (PDLIM2), a nuclear E3 ligase that promotes p65 polyubiquitination and its subsequent degradation (Tanaka et al., 2007). Similarly, phosphorylation of p65 facilitates the interaction of COMM domain-containing 1 (COMMD1) and an E3 ligase cullin 2 which is capable of mediating p65 ubiquitination and proteasomal degradation (Geng et al., 2009). All of these modifications give rise to another layer of complexity onto TNF-induced NF-κB signaling.

Ubiquitination is a reversible post-translational modification. The removal of polyubiquitin is carried out by deubiquitinases (DUBs), proteases that cleave or remove ubiquitin. There are about 100 DUBs encoded by the human genome (Skaug et al., 2009). These DUBs can be classified into 5 different classes based on their specific domains, such as C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTUs), Josephins, and JAB1/MPN/MOV34 metalloenzymes (JAMMs) (Nijman et al., 2005). There are multiple DUBs involve in TNF-induced NF-κB signaling. A20 (also known as Tumor necrosis factor alpha-induced protein 3 or TNFAIP3) is an NF-κB regulated OTU-type of DUBs that negatively regulate NF-κB activation (Ma & Malynn, 2012). Mice with A20 deficiency are hypersensitive to TNFα, exhibit severe inflammation and cachexia as well as suffer premature death, indicating that A20 is an essential negative regulator for NF-κB (Lee et al., 2000). A20 is capable of cleaving K63-linked polyubiquitin chain on RIP1 and TNFR1 through its N-terminal OTU domains. Interestingly, although A20 is a DUB, it possesses E3 ligase ability that facilitates K48-linked polyubiquitination on RIP1 which targets RIP1 for proteasomal degradation (Wertz et al., 2004, 2015). Cylindromatosis (CYLD) is another well-known DUB that regulates NF-κB negatively. CYLD is a tumor suppressor with UCH domain. It disassembles linear and K63-linked polyubiquitin chain which in turn inhibits IKK activation (Draber et al., 2015; Kovalenko & Chable-bessia, 2003). Cells with CYLD deficiency are more susceptible to tumor development whereas cells with overexpressed CYLD prevent ubiquitination on RIP1, TRAF2 and NEMO (Brummelkamp et al., 2003; Chen & Chen, 2013; Kovalenko & Chable-bessia, 2003; Trompouki et al., 2003). In brief, all non-typical ubiquitination can be summarized in Table 2.1.

Table 2.1: List of non-typical ubiquitination that regulates TNF-induced NF-κB.

Protein	Modification	Enzyme	Effect	References
TNFR1, TRADD, RIP1	M1-polyubiquitination	LUBAC	Stability of TNFR1 complex I	Draber et al., 2015
TNFR1	K63-linked polyubiquitination	RNF8	Checkpoint for cell fate decision	Fritsch et al., 2014
RIP1	K11-linked, K48-linked, K63-linked and M1-linked polyubiquitination	cIAP1 and UbcH5	To activate TAK1 and IKK complexes	Bertrand et al., 2008; Blackwell et al., 2013; Dynek et al., 2010; Varfolomeev et al., 2008; Witt & Vucic, 2017
TRAF2	K48- and K63-linked polyubiquitination	TRAF2 and Ubc13	Activation of JNK	Habelhah et al., 2004; Li et al., 2009; Shi & Kehrl, 2003; Wu et al., 2005
NEMO	K-6 linked polyubiquitination	cIAP1	Activation of IKK	Tang et al., 2003
p65	Monoubiquitination	Unknown	Decrease NF-κB transcriptional activity	Hochrainer et al., 2012
p65 (K195 & K315)	K-48 linked polyubiquitination	HERC3	Attenuation of p65 activity and degradation	Hochrainer et al., 2015
p65	K-48 linked polyubiquitination	PDLIM2/ E3 ligase cullin 2	Degradation	Tanaka et al., 2007; Geng et al., 2009

2.2.8.2 Roles of phosphorylation

Phosphorylation refers to the process of adding or transferring a gamma-phosphate of ATP to a serine (S), threonine (T) or tyrosine (Y) residue of substrate proteins by protein kinases. Phosphate is a negatively charged molecule that can change protein conformation and regulate protein function positively or negatively when added to the particular target amino acid residue (Johnson, 2009; Johnson et al., 1996). Phosphorylation plays a major role in TNF-induced NF-κB signaling. Besides the typical phosphorylation, such as TAK1 autophosphorylation-directed activation, IKK kinase phosphorylation-directed activation and IκBα phosphorylation-directed proteasomal degradation, multiple key players of TNF-induced NF-κB signaling undergo phosphorylation on numerous serine, threonine or tyrosine residues that fine-tune and manipulate the outcome of NF-κB activities.

While typical autophosphorylation site of TAK1 at T187 and S192 within its activation loop was thought to be the essential post-translational modification for TAK1 activation (Kishimoto et al., 2000; Sakurai et al., 2000, 2002; Singhirunnusorn et al., 2005), yet another phosphorylation site for example S412 has also been shown to play a critical role in regulating TNF-induced responses (Kobayashi et al., 2005; Ouyang et al., 2014).

In addition to TAK1-mediated IKKβ phosphorylation within its activation loop at S177 and S181, c-Src has been shown to phosphorylate IKKβ at Y188 and Y199 following TNF stimulation in a Protein kinase C (PKC)-dependent manner, which in turn modulates the expression of Intercellular adhesion molecule 1 (ICAM1) in A549 and Cytochrome c oxidase subunit 2 (COX2) in NCI-H292 human alveolar epithelial cells, respectively (Huang et al., 2003b, 2003c). Multiple phosphorylations at serine residues within the C-terminal of IKKβ serine rich region also show significant feedback regulation of IKK kinase activity (Delhase et al., 1999). These phosphorylations are associated with G4-1, also referred to as G5PR, which recruits Protein Phosphatase 5 (PP5) to terminate IKKβ activation (Chiang et al., 2011). Moreover, Polo-like kinase 1 (Plk1) is able to phosphorylate IKKβ at S733, S740, and S750 within its NEMO-binding domain *in vitro*, which negatively regulates TNF-induced IKK activation and cyclin D1 expression level (Higashimoto et al., 2008). Similarly, T23 of IKKα has been shown to be phosphorylated by Akt, which regulates TNF-induced NF-κB positively (Ozes et al., 1999).

Moreover, IκBα is phosphorylated at Y42 by c-Src following TNF treatment in murine bone marrow macrophages (BMMs), which modulates transcriptional activation of IL-6 secretion (Abu-Amer et al., 1998). Interestingly, another kinase, cellular-Abelson murine leukemia tyrosine kinase (c-Abl) phosphorylates IκBα at Y305 and regulates the stability and nuclear accumulation of IκBα that promotes TNF-induced apoptosis instead of NF-κB activation (Kawai et al., 2002).

On the other hand, p65 exhibits dynamic phosphorylations on S42 and S45 in responses to TNF stimulation which modulates its DNA binding and transcriptional activity (Lanucara et al., 2016). In addition, phosphorylation of p65 at T254 recruits Pin1 that inhibits p65 and IκBα interaction and thus promotes NF-κB nuclear translocation and stability upon cytokine stimulation (Ryo et al., 2003). Inhibition of p65 phosphorylation at S205, S276, S281, and S311 results in impaired TNF stimulation of NF-κB (Dong et al., 2008; Duran et al., 2003; Hochrainer et al., 2007, 2013). Besides, p65 is also phosphorylated at T435 within its TAD that disrupts its interaction with Histone deacetylases 1 (HDAC1) in response to TNFα stimulation (Yeh et al., 2004). S468 is another inducible phosphorylation site on p65 by IKKβ or IKKε upon TNF or IL1β stimulation, which suppresses NF-κB activity by promoting NF-κB degradation through interacting with COMMD1-containing E3 ligase complex (Geng et al., 2009; Schwabe & Sakurai, 2005). Additionally, S529, which modulates NF-κB transcriptional potential, is phosphorylated by Casein kinase II (CKII) when stimulated with TNF (Wang et al., 2000; Xing et al., 2011). S536, which controls transcriptional activation of NF-κB, is another well-known p65 phosphorylation site catalyzed by IKK (Sakurai et al., 1999, 2003). Collectively, protein phosphorylation in TNF-induced NF-κB signaling pathway provides another layer of regulation in fine-tuning and controlling NF-κB activity. In brief, all non-typical phosphorylation can be summarized in Table 2.2.

Table 2.2: List of non-typical phosphorylation that regulates TNF-induced NF-κB.

Protein	Modification	Enzyme	Effect	References
TAK1 (S412)	Phosphorylation	PKA	Regulates TNF-induced responses positively	Kobayashi et al., 2005; Ouyang et al., 2014
IKKβ (Y188 & Y199)	Phosphorylation	c-Src	Modulates the expression of ICAM1 and COX2	Huang et al., 2003b, 2003c.
Multiple serine at C-terminal of IKKβ	Phosphorylation	IKKβ	Regulates IKK kinase activity	Delhase et al., 1999
IKKβ (S733, S740 & S750)	Phosphorylation	Plk1	Negatively regulates TNF-induced IKK activation and cyclin D1 expression level	Higashimoto et al., 2008
IKKα (T23)	Phosphorylation	Akt	Regulates TNF-induced NF-κB positively	Ozes et al., 1999
IκBα (Y42)	Phosphorylation	c-Src	Modulates transcriptional activation of IL-6 secretion	Abu-Amer et al., 1998
IκBα (Y305)	Phosphorylation	c-Abl	Regulates the stability and nuclear accumulation of IκBα	Kawai et al., 2002
p65 (S42 & S45)	Phosphorylation	Unknown	Modulates its DNA binding and transcriptional activity	Lanucara et al., 2016
p65 (T254)	Phosphorylation	Unknown	Recruits Pin1 that inhibits p65 and IκBα interaction	Ryo et al., 2003
p65 (S205, S276, S281 & S311)	Phosphorylation	Unknown	TNF stimulation of NF-κB	Dong et al., 2008; Duran et al., 2003; Hochrainer et al., 2007, 2013
p65 (T435)	Phosphorylation	Unknown	Disrupts its interaction with HDAC1	Yeh et al., 2004
p65 (S468)	Phosphorylation	IKKβ or IKKε	Suppresses NF-κB activity	Geng et al., 2009; Schwabe & Sakurai, 2005
P65 (S529)	Phosphorylation	Casein kinase II (CKII)	Modulates NF-κB transcriptional potential	Wang et al., 2000; Xing et al., 2011
P65 (S536)	Phosphorylation	IKK	Controls transcriptional activation of NF-κB	Sakurai et al., 1999, 2003

2.2.8.3 Roles of methylation

Protein methylation plays a vital role in cell biology and modulates diverse cellular processes. Protein methylation can occur on lysine (K) and arginine (R) residues of a target protein. The ε-amine group of lysine residue can be modified with mono-, di- or trimethylation by a specific lysine methyltransferase (KMT) using a common co-substrate, S-adenosylmethionine (SAM and a.k.a. adoMet), as a methyl donor (Lake & Bedford, 2007; Paik et al., 2007; Smith & Denu, 2009). On the other hand, arginine residues of a target protein can be modified with mono- or dimethylation by protein arginine methyltransferase (PRMT). Moreover, dimethylated arginine can either be symmetric or asymmetric in which two methyl groups are conjugated to both its guanidine nitrogen side chains or single guanidine nitrogen side chain respectively (Bedford & Richard, 2005). Methylation is a reversible process as methylated-protein can be demethylated by lysine or arginine demethylases that remove a methyl group from the lysine or arginine side chain (Biggar & Li, 2015) (Figure 2.14).

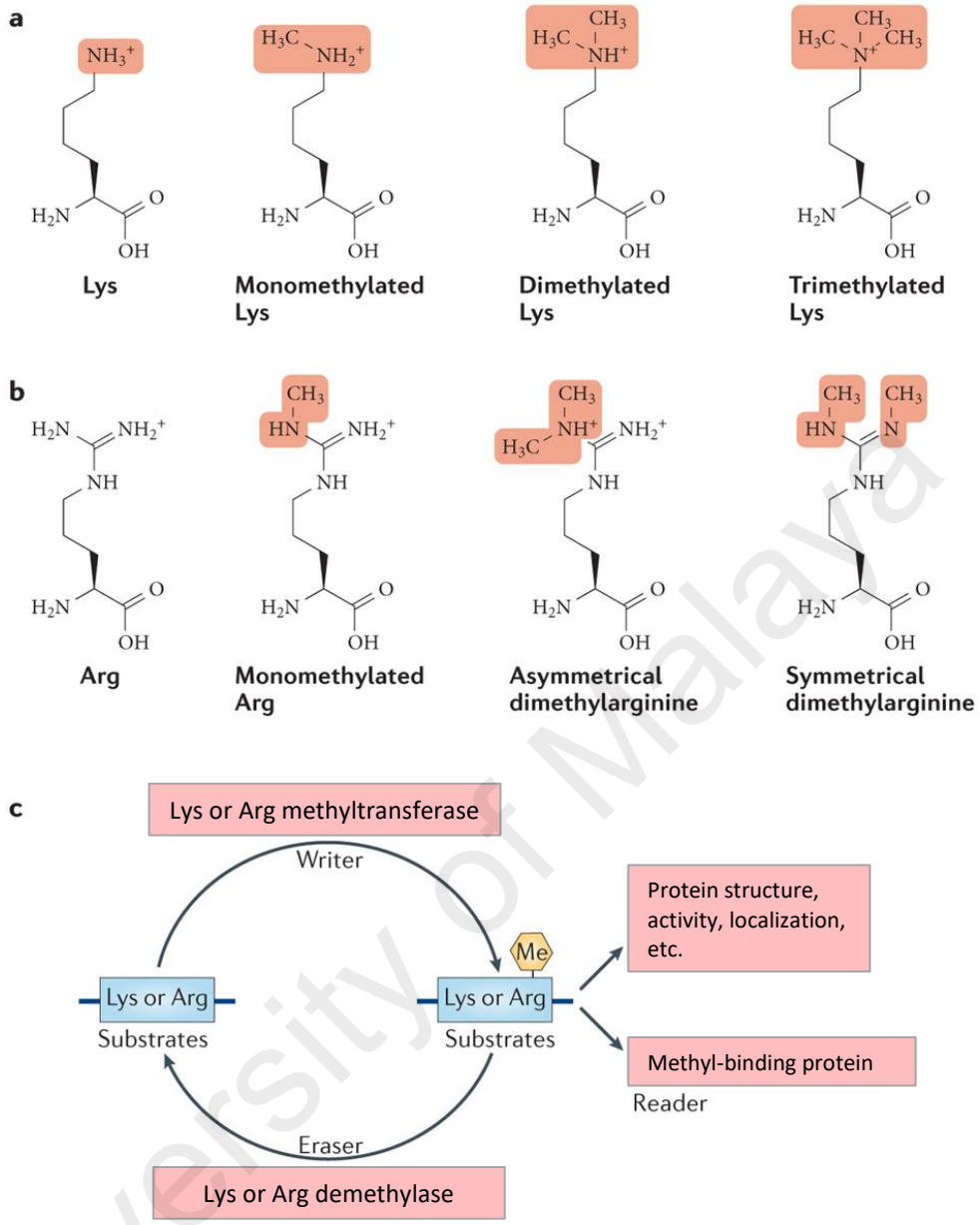


Figure 2.14: Schematic diagram of protein methylation and demethylation.
a. A lysine can be modified by mono-, di- or trimethylation at the ε-amine group side chain. b. An arginine can be modified as monomethylated at either one of its guanidine nitrogen side chains, or dimethylated at both guanidine nitrogen side chain (symmetrical) or single guanidine nitrogen side chain (asymmetrical). c. Protein methylation is reversible with methyltransferase as writer and demethylase as an eraser. (Adapted with modification by permission from Biggar and Li (2015)).

Proteomic studies have shown that some proteins of the TNF-induced signaling pathway are modified by methylation. The modifications include mono-methylated R477 and R487 of RIP1(Geoghegan et al., 2015); mono-methylated R173 (Geoghegan et al., 2015; Guo et al., 2014; Larsen et al., 2016), di-methylated R173 (Guo et al., 2014), mono-methylated R441 of TAB2 (Larsen et al., 2016) and mono-methylated R187 of p65

(Larsen et al., 2016). However, the biological roles of these modifications remain unknown.

P65 is dimethylated at R30, R35, and R174 in a signal-dependent manner by PRMT5. PRMT5 is a methyltransferase that facilitates symmetrical dimethylarginine formation in mammalian cells (Harris et al., 2014, 2016; Krause et al., 2007; Wang et al., 2014). Dimethylated R30 will enhance DNA binding capability and transactivation activity of NF-κB. Consistently, overexpression of PRMT5 enhances NF-κB transcriptional activity, while repressed NF-κB transcriptional activity was observed after PRMT5 depletion.

In addition, K218 and K221 of p65 are methylated by nuclear receptor-binding SET domain-containing protein (NSD1) and demethylated by F-box and leucine-rich repeat protein 11 (FBXL11), which negatively regulates NF-κB (Lu et al., 2009, 2010, 2013). Structural analysis shows that p65 with methylated K218 and K221 interacts with DNA with higher affinity (Lu et al., 2013). Reconstitution of p65 deficient cells with K218/221Q double mutant or K37Q alone p65 shows that K218/221Q mutation down-regulates 48% of NF-κB target genes and K37Q mutation affects 28% of NF-κB regulated genes (Lu et al., 2013).

Additionally, p65 is mono-methylated by SET domain containing 6 (SETD6) at K310 in resting cells and p65 with methylated K310 (p65K310me1) associates with chromatin in the nucleus (Chang et al., 2011; Levy et al., 2011; Lu et al., 2013). The p65K310me1 is recognized by AnkR of G9a-like protein (GLP), which subsequently generates a repressive histone mark via promoting H3K9me2/1 methylation that modulates basal NF-κB genes in unstimulated cells (Chang et al., 2011; Levy et al., 2011). Upon stimulation with TNFα, the p65K310me1 level at chromatin is reduced compared to unstimulated cells (Levy et al., 2011).

P65 is also methylated at K37 by SET9 methyltransferase following TNFα or IL1β induction (Ea & Baltimore, 2009; Lu et al., 2013). Interestingly, SET9-mediated

methylation of p65 at K37 is required for DNA binding and promoter recruitment of NF-κB (Ea & Baltimore, 2009). However, other studies show that p65 is negatively regulated by SET9 mediated mono-methylation at K314 and K315 in response to TNF stimulation (Lu et al., 2013; Yang et al., 2009, 2010) which promotes degradation of DNA bound p65. Intriguingly, reconstitution of p65 with K314/315R mutation abolishes SET9 induced degradation and enhances TNF-induced IL8 and IL6 expression compared to control cells, suggesting that SET9 is a negative regulator for TNF-induced NF-κB (Yang et al., 2009). At the time of writing, it remains unclear about the antagonistic relationship between SET9 and p65. In brief, the non-typical methylation or demethylation can be summarized in Table 2.3.

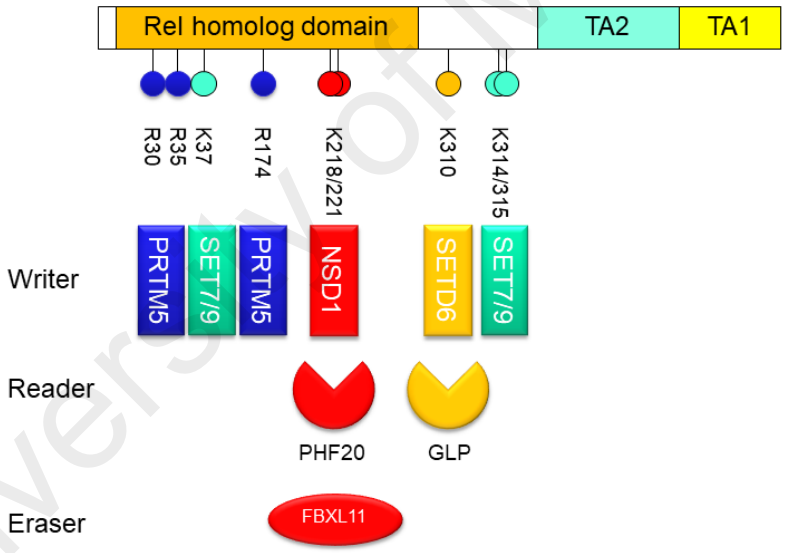


Figure 2.15: Schematic diagram of p65 methylation.
Representation of p65 with indicated domain and site of arginine and lysine methylation together with their corresponding writer, reader, and eraser. TA1, TA2, transcriptional activation domain 1 and 2. (Adapted by permission from Carr et al. (2015)).

Table 2.3: List of non-typical methylation that regulates TNF-induced NF-κB.

Protein	Modification	Enzyme	Effect	References
RIP1 (R477 & R487)	Mono-methylation	Unknown	Unknown	Geoghegan et al., 2015
TAB2 (R173 & R441)	Mono/di-methylation	Unknown	Unknown	Geoghegan et al., 2015; Guo et al., 2014; Larsen et al., 2016
p65 (R187)	Mono-methylation	Unknown	Unknown	Larsen et al., 2016
p65 (R30, R35 & R174)	Di-methylation	PRMT5	Enhances DNA binding capability and transactivation activity of NF-κB	Harris et al., 2014, 2016; Krause et al., 2007; Wang et al., 2014
p65 (K37)	Mono-methylation	SET9	Regulates transcriptional activity of NF-κB	Ea & Baltimore, 2009; Lu et al., 2013
p65 (K218 & K221)	Methylation/ demethylation	NSD1/ FBXL11	Regulates NF-κB	Lu et al., 2009, 2010, 2013
p65 (K310)	Mono-methylation	SETD6	Associates with chromatin in nucleus	Chang et al., 2011; Levy et al., 2011; Lu et al., 2013
p65 (K314 & K315)	Mono-methylation	SET9	Regulates NF-κB negatively	Lu et al., 2013; Yang et al., 2009, 2010

2.2.8.4 Other modifications

Acetylation of targeted protein is another post-translational modification that regulates NF-κB pathway. Acetylation is governed by histone acetyltransferase and histone deacetylase that facilitates the addition and removal of the acetyl group at lysine residues of a protein respectively (Huang et al., 2010). For example, p50 undergoes acetylation at K431, K440 and K441 by p300 that enhances DNA binding of p50 (Deng et al., 2003; Furia et al., 2002). Likewise, multiple lysine residues of p65 have been shown to be acetylated by p300, including K122, K123, K218, K221, K310, K314 and K315 (Huang et al., 2010). In addition to p300, PCAF acetylates K122 and K123 of p65 subunit as well (Kiernan et al., 2003). Acetylation of p65 at these lysine residues changes the functions of NF-κB. For instance, acetylation of K122 and K123 of p65 by p300 or PCAF negatively modulates the DNA binding of p65, and the effects are reversed by histone deacetylase 3 (HDAC3) (Kiernan et al., 2003). Moreover, acetylation of K221 of p65 enhances the DNA binding of p65 and weakens IκBα association. Besides, acetylated

K310 of p65 is required for the full transcriptional activity of NF-κB (Chen et al., 2002) and is reversed by HDAC3, which enhance the IκBα assembly and promotes nuclear export of NF-κB-IκBα complexes (Chen et al., 2001). In addition to HDAC3, HDAC1 is capable of deacetylating K310 and reduces NF-κB transcriptional activity at chromatin level in cooperation with breast cancer metastasis suppressor 1 (BRMS1) (Liu et al., 2006). Acetylated-K310 is also recognized by Sirtuin 1 (SIRT1) deacetylase, which inhibits NF-κB transactivation activity and sensitizes the cells to TNF-α-induced apoptosis (Yeung et al., 2004).

Small ubiquitin-related modifier (SUMO) is an ubiquitin-like protein, which can be attached to lysine residues of a protein by a specific enzyme in an ATP-dependent manner that regulates cellular processes comparable to the classical ubiquitination reactions (Flotho & Melchior, 2013). SUMOylation has been detected in proteins of TNF signaling pathway. For instance, IKKγ/NEMO undergoes SUMOylation at K277 and K309, which modulates NEMO nuclear transition in NF-κB genotoxic stress pathway and is also detectable upon TNFα stimulation (Huang et al., 2003a). Moreover, SUMO1-linked SUMOylation of IκBα at K21 inhibits TNF-induced proteolysis of IκBα by antagonizing the ubiquitination-dependent degradation of IκBα. (Desterro et al., 1998). In contrast to SUMO1, SUMO2/3 forms a heterologous chain with ubiquitin on IκBα that optimises TNF-mediated proteolysis of IκBα (Aillet et al., 2012).

Next, *O*-GlcNAcylation is another post-translational modification that plays critical roles in regulating NF-κB targeted gene expression through direct modification of NF-κB. *O*-GlcNAcylation is a process of adding an *O*-linked β-N-acetylglucosamine (*O*-GlcNAc) carbohydrate moiety to the hydroxyl group of serine or threonine residues of a protein covalently by a β-N-acetylglucosaminyltransferase (OGT) enzyme (Hart et al., 2011). P65 has been shown to be modified by *O*-GlcNAcylation at multiple sites (Yang et al., 2008, 2015) and *O*-GlcNAc tunes and regulates NF-κB activity through interplay

with phosphorylation and acetylation (Ma et al., 2017). For example, the attachment of *O*-GlcNAc moiety to p65 is essential for K310 acetylation, which is required for the full transcriptional activity of NF-κB when stimulated with TNFα (Allison et al., 2012).

Taken together, these complex post-translational modifications of proteins of TNF signaling pathway bring another layer of complexity to NF-κB functions that not only fine-tunes the signaling cascades but also contributes to the induction of the expression of specific NF-κB target gene programs. In brief, the non-typical post-translational modification can be summarized in Table 2.4.

Table 2.4: List of non-typical post-translational modification that regulates TNF-induced NF-κB.

Protein	Modification	Enzyme	Effect	References
p50 (K431, K440 & K441)	Acetylation	p300	Enhances DNA binding of p50	Deng et al., 2003; Furia et al., 2002
p65 (K122, K123, K218, K221, K310, K314 & K315)	Acetylation/ deacetylation	p300, CBP PCAF, HDAC3	Regulates NF-κB activity positively or negatively	Huang et al., 2010; Kiernan et al., 2003; Chen et al., 2001, 2002
p65 (K310)	Deacetylation	HDAC1	Reduces NF-κB transcriptional activity at chromatin	Liu et al., 2006
p65 (K310)	Deacetylation	SIRT1	Inhibits NF-κB transactivation activity	Yeung et al., 2004
NEMO (K277 & K309)	SUMOylation	Unknown	Modulates NEMO nuclear transition	Huang et al., 2003a
IκBα (K21)	SUMOylation (SUMO1)	Unknown	Inhibits TNF-induced proteolysis of IκBα	Desterro et al., 1998
IκBα	SUMOylation (SUMO2/3)	Unknown	Optimizes TNF-mediated proteolysis of IκBα	Aillet et al., 2012
p65 (K310)	O-GlcNAcylation	OGT	Required for the full transcriptional activity of NF-κB	Aillet et al., 2012; Yang et al., 2008, 2015

2.3 JmjC domain-containing protein

The JmjC domain-containing proteins are a class of redox enzymes that catalyze protein hydroxylation or demethylation (Accari & Fisher, 2015; Tsukada et al., 2006). The JmjC domain was first classified by Takeuchi et al. in 1995 (Takeuchi et al., 1995). Jumonji, which means cruciform in Japanese, was named after the observation of abnormal cross-like neural groove formation in *Jumonji* mutant embryos. Soon after the

discovery of this JmjC domain, the JmjC domain-containing superfamily has been expanded with more than 10,000 proteins in the databases, such as UniProt, Pfam, SMART and Interpro (Hahn et al., 2008). Initially, it was thought that a JmjC domain usually co-exists with a Jumonji N terminal (JmjN) domain in a non-adjacent manner in the same protein. However, a JmjC domain was later found to exist without a JmjN domain in various proteins that are conserved from bacteria to human (Balciunas & Ronne, 2000; Clissold & Ponting, 2001).

The JmjC domain is characterized by a double-stranded β helix (DSBH) fold that consists of eight antiparallel β sheets that fold into a barrel-like structure. This β barrel shape topology of JmjC domain belongs to a cupin metalloenzyme superfamily, which was named after a Latin word "cupa" that means a small barrel (Dunwell et al., 2001). The Jumonji domain-containing proteins can be further classified into a 2-oxoglutarate (a.k.a. α-ketoglutarate) oxygenase subclass of cupin proteins that utilizes its β barrel topology as a binding groove to accommodate iron (II) (Fe^{2+}) and α-ketoglutarate molecules (Clifton et al., 2006) (Figure 2.16).

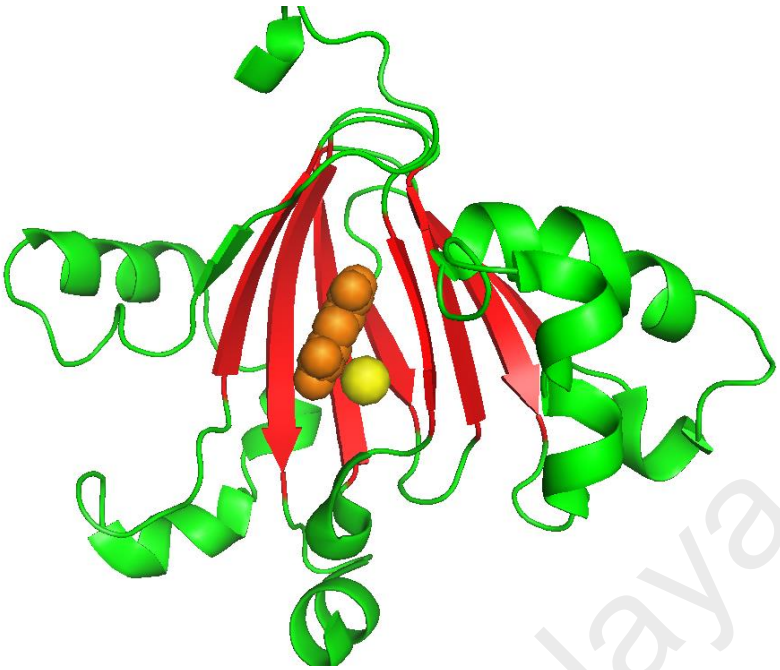


Figure 2.16: Example of a tertiary structure of DSBH.
The JMJD6 structure from Protein Data Bank (PDB: 3LDB) was highlighted at 120-300 amino acids. The eight antiparallel β sheets that fold into a barrel-like structure were highlighted in red. The iron (Fe^{2+}) and α-ketoglutarate molecules are highlighted in yellow and orange colors respectively (Figure generated by Pymol).

The JmjC domain-containing protein superfamily can be divided into two groups, which are histone demethylases that remove a methyl group from methylated lysine or arginine residues on the N-terminal tail of histones (KDM2-KDM7) and the JmjC domain-only proteins with ill-defined functions (Accari & Fisher, 2015). The majority of the histone demethylases contain additional protein domains that facilitate their interaction with histone. For example, Jmjd2C/GASC/KDM4C, a H3K9 specific demethylase (Accari & Fisher, 2015; Cloos et al., 2006), contains a conserved TUDOR and a plant homeodomain (PHD) that bind methylated lysines or arginines.

More importantly, recent studies have demonstrated that JmjC domain-containing proteins regulate numerous signaling pathways involved in cellular development, differentiation as well as proliferation, and perturbations of JmjC domain-containing protein expression are associated with several human malignancies (Berry & Janknecht, 2013; Labbé et al., 2014; Shi & Whetstine, 2007; Soini et al., 2015; Takeuchi et al., 1995, 2006; Vieira et al., 2013).

2.3.1 JmjC domain-only protein

The JmjC domain-only proteins are a diverse group of enzymes with distinct functions such as factor inhibiting HIF (FIH), or those with an unknown function such as JMJD8. This group of proteins contains only a Jumonji C domain and lack any chromatin binding domains. Recent studies have shown that some of these jmjC domain-only proteins play a role in histone modification. Moreover, this group of protein has shown to be involved in hydroxylation of asparagine or histidine residues of non-histone proteins (Johansson et al., 2014). Recent reviews have summarized some characteristics and putative functions of this JmjC domain-only subgroup (Accari & Fisher, 2015; Johansson et al., 2014).

Jumonji domain-containing protein 6 (JMJD6) is the first member of the JmjC domain-only subgroup that shown to function as a H3R4me2 (symmetrical)-specific histone demethylase (Accari & Fisher, 2015; Chang et al., 2007) and as a histone lysine hydroxylase (Hong et al., 2010; Unoki et al., 2013; Webby et al., 2009). Interestingly, a non-histone protein has also been identified to be associated with JMJD6. For instance, TRAF6 is demethylated by JMJD6 when stimulated with TLR ligands and this demethylation is essential for TLR-induced NF-κB signaling (Tikhanovich et al., 2015).

Jumonji domain-containing protein 5 (JMJD5) (also known as KDM8) is the second member of JmjC domain-only subgroup with a histone demethylase activity (Hsia et al., 2010; Ishimura et al., 2012). Besides its histone demethylase activity, JMJD5 has been shown to hydroxylate non-histone proteins, such as NFATc1, whereby JMJD5 negatively regulated Nuclear factor of activated T-cells 1 (NFATc1) protein level with its hydroxylase activity (Youn et al., 2012). Moreover, JMJD5 negatively regulates p53 transcriptional activity via interacting with p53 independent of its demethylase activity (Huang et al., 2015), suggesting that JmjC domain-only protein may regulate other proteins even without its enzymatic activity. Interestingly, Huang et al. (2015) have shown that JMJD5 interacts with NF-κB (p105/p50) (Youn et al., 2012), suggesting

another possibility of an association between a member of JmjC domain-only subgroup and NF-κB.

Besides, factor inhibiting HIF (FIH) is a well-known member of JmjC domain-only subgroup. FIH, an asparaginyl hydroxylase, hydroxylates the TAD of HIFα protein at Asp-803 that inhibits the recruitment of CBP/p300 transcriptional coactivators (Lando et al., 2002; Mahon et al., 2001). Interestingly, FIH is capable of altering asparagine residues at AnkR of NF-κB precursor (p105) and IκBα (Cockman et al., 2006), but their biological roles remain unknown. All these examples give rise to the possibility of JmjC domain-only protein in NF-κB signaling. In brief, the list of JmjC domain-only proteins and its responding targets are summarized in Table 2.1.

Table 2.5: List of JmjC domain-only demethylases and/or hydroxylases and its substrates.

Name	Histone substrate	Non-histone substrate	References
JMJD4	Unknown	Unknown	
JMJD5 (KDM8)	H3K36me2/me1	NFATc1 hydroxylation	(Hsia et al., 2010; Ishimura et al., 2012; Youn et al., 2012)
JMJD6	H3R2me2, H4R3me2/me1, H2A/H2B, H3, H4	U2AF2/U2AF65, LUC7L2	(Chang et al., 2007; Mantri et al., 2011; Unoki et al., 2013; Webby et al., 2009)
JMJD7	Unknown	Unknown	
JMJD8	Unknown	Unknown	
NO66	H3K4me1/me2/me3, H3K36me2/me3	Rpl8	(Ge et al., 2012; Sinha et al., 2010)
MINA53	H3K9me3	Rpl27a	(Ge et al., 2012; Lu, Chang, et al., 2009)
FIH/ HIF1AN	Unknown	HIFα, IκBα, p105	(Cockman et al., 2006; Lando et al., 2002; Mahon et al., 2001)
HSPBAP1	Unknown	Unknown	
TYW5	Unknown	$tRNA^{phe}$	(Noma et al., 2010)
Hairless	H3K9me1/me2	Unknown	(Liu et al., 2014)

2.3.1.1 Jumonji domain-containing protein 8

JMJD8 is a member of a JmjC-domain-only subgroup with unknown function. It was first identified as C16orf20 in 2001 (Daniels et al., 2001). JMJD8 was later shown to be related to cancer progression and cellular proliferation as more than 80% inhibition of SCC23/MET cell invasiveness was observed when the expression of JMJD8 was knocked down by siRNA (Ding et al., 2013; Zhu et al., 2016). A recent study has shown that JMJD8 is involved in angiogenesis and cellular metabolism via interacting with pyruvate kinase M2 (Boeckel et al., 2016). In particular, JMJD8 is upregulated in endothelial cells (EC) and ECs with JMJD8 deficiency show impaired angiogenesis sprouting. Moreover, Boeckel et al. (2016) revealed that JMJD8 is localized to the extranuclear compartment, excluding its possibility as a histone-modification enzyme. Also, metabolism rate was significantly reduced when the JMJD8 expression was suppressed with siRNA, while the opposite effect is observed when JMJD8 is ectopically overexpressed in ECs (Boeckel et al., 2016). Although the biological role of JMJD8 in angiogenesis is slowly being established, the physical and biochemical properties of JMJD8 as well as its role in other signaling pathways remain unknown.

CHAPTER 3: METHODOLOGY

3.1 Cell culture

Human embryonic kidney (HEK) 293T cells and HeLa S3 (Homo sapiens cervix adenocarcinoma) cells were obtained from ATCC ®, USA (CRL-11268^{TM} and CCL-2.2™, respectively), HONE1 (Nasopharyngeal carcinoma cells) was obtained from Associate Professor Dr. Ng Ching Ching as a gift, HaCat (Immortalized human keratinocytes) and U2OS (Osteosarcoma cells) were obtained from Professor Dr. David Baltimore. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% (v/v) Fetal bovine serum (FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco).

3.2 Reagents and antibodies

Human TNFα was purchased from Gold Biotechnology (St. Louis, MO). Antibodies against TNFR1 (H-271), NEMO (FL-419), IKKβ (C-20), IκBα (C-21), p65 (C-20 and F-6), Heat shock protein 90α or HSP90α (C-20), α-Tubulin (TU-02), Poly [ADP-ribose] polymerase 1 or PARP1 (F-2), Caspase 8 (C-20), Caspase 3 (E-8), JNK (D-6 and N-18), ERK1 (K-23), ERK2 (C-14), p38 (H-174), c-Myc (9E10), Hemagglutinin (HA)-probe (HA.C5), and Sucrose nonfermenting protein 2 homolog or SNF2H (H300) were acquired from Santa Cruz Biotechnology; whereas, cleaved-caspase 3 (5A1E), RIP1 (D94C12), Early endosome antigen 1 or EEA1 (C45B10), Receptor-binding cancer antigen expressed on SiSo cells or RCAS1 (D2B6N), Apoptosis inducing factor or AIF (D39D2), Kinectin 1 (D5F7J) and Lyric/Metadherin (MTDH) (2F11C3) and phosphorylated form of JNK, ERK, p38 (D3F9), IKKα/β (16A6), FLAG (9A3) and IκBα were bought from Cell Signaling Technology. Anti-JMJD8 and Protein disulfide isomerase Family A member 3 (PDIA3) were purchased from Abnova. Anti-calnexin antibody was purchased

from Abcam. LysoTracker® Red DND-99 and ER-Tracker™ Red dye were purchased from Invitrogen.

3.3 Mammalian and bacterial expression vectors

Transcript of human JMJD8 was amplified by polymerase chain reaction (PCR) from human complementary DNA (cDNA) from HEK293T cells and cloned into a pcDNA3 vector (Invitrogen) to generate pcDNA3-hJMJD8 with 2×FLAG and 2×HA or eCFP at the C-terminus. To remove the siRNA targeting site, a site directed mutagenesis was conducted to generate silent mutations at five nucleotides from "CTG GTG AGG TGC TGT ACT T" to "CTG GTG AAG TCT TAT ATT T" (pcDNA3-hJMJD8-siJMJD8*). To generate signal peptide deletion (Δ45-JMJD8) and transmembrane domain deletion (ΔTM-JMJD8) mutants, full-length JMJD8 was used as a template for PCR with specific deletion primers for each construct. These plasmids were subsequently verified by Sanger sequencing. Recombinant GST-TNF and GST-IκBα were expressed in Top10 cells and purified with Glutathione beads according to the manufacturer's recommendation.

3.4 siRNA

The siRNAs for hJMJD8 were purchased from Sigma [(siJMJD8a) SASI_Hs02_00305057, (siJMJD8b) SASI_Hs01_00228274 and (siJMJD8c) SASI_Hs01_00228276). The control siRNAs (D-001810-10-50) were purchased from Dharmacon. The siRNAs were transfected into HEK293T cells via calcium phosphate precipitation. Briefly, one volume of siRNA with 250 mM $CaCl_2$ was mixed with one volume of 2× HBS (50 mM HEPES, pH 7.05, 1.5 mM Na_2HPO_4, 140 mM NaCl, 10 mM KCl and 12 mM Dextrose) and added drop-wise into the culture media. The amount of transfection mixture was 10% volume of the total culture media used. The siRNA was transfected at a final concentration of 20 nM on the first and second day to enhance

knockdown efficiency. Media was changed on the third day and incubated overnight before treating the cells with and without TNFα for the indicated time points.

3.5 RNA isolation and qPCR

Total RNA was prepared with the Thermo Scientific GeneJET RNA Purification Kit according to the manufacturer's protocol. Briefly, cells were lysed in 600 µl lysis buffer supplemented with β-mercaptoethanol and mixed with vortex. Then, 360 µl of absolute ethanol was added to the lysate and mixed. The mixture was then transferred to GeneJET RNA Purification Column and centrifuged at 11000 rpm for 1 minute. The flow-through was discarded followed by washing with 700 µl of Wash Buffer 1 and twice with 600 µl and 250 µl of Wash Buffer 2. The washing tubes were centrifuged at 11000 rpm for 1 minute except the second wash of Wash Buffer 2 at 13000 rpm for 2 minutes and the flow-through was discarded after each step. Finally, the column was placed in a new tube and the RNA was eluted from the membrane with 50 µl of RNase-free water. The concentrations of the RNA were quantified using Nanodrop 2000c spectrophotometer (Thermoscientific, USA). The cDNA was synthesized from 0.5-1 µg of RNA (DNase I-treated, Thermoscientific) using random hexamer (Invitrogen), dNTPs (Thermoscientific), RNase inhibitor and Moloney Murine Leukemia Virus (M-MuLV/MMLV) Reverse Transcriptase (NEB) according to the manufacturer's recommendation. Generated cDNA was used for subsequent quantitative PCR (qPCR) assays. The qPCR was carried out with indicated primers and KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems) according to the manufacturer's protocol. All data were then normalized to Succinate dehydrogenase complex flavoprotein subunit A (SDHA). The primer sequences are listed in Table 3.1.

Table 3.1: List of primers used in qPCR assays.

Genes	Primers Sequence 5' – 3'
IL8 Forward	AGCTCTGTCTGGACCCCAAG
IL8 Reverse	GAATTCTCAGCCCTCTTCAAAAAC
TNFα Forward	GCCCAGGCAGTCAGATCATCT
TNFα Reverse	TTGAGGGTTTGCTACAACATGG
IκBα Forward	CTCCGAGACTTTCGAGGAAATAC
IκBα Reverse	GCCATTGTAGTTGGTAGCCTTCA
CCL5 Forward	CCCAGCAGTCGTCTTTGTCA
CCL5 Reverse	TCCCGAACCCATTTCTTCTCT
IFNβ Forward	ACTGCCTCAAGGACAGGATG
IFNβ Reverse	AGCCAGGAGGTTCTCAACAA
JMJD8 Forward	GACAGGTTGCTGGCTTCGTT
JMJD8 Reverse	AGGGCAAGTCCACTTTGTGGTA
SDHA Forward	TGGGAACAAGAGGGCATCTG
SDHA Reverse	CCACCACTGCATCAAATTCATG

3.6 Subcellular fractionation

To examine the nuclear translocation of NF-κB, the cells were fractionated into cytoplasmic and nuclear fractions. Briefly, HEK293T cells treated with or without TNFα were washed 3 times with 1x PBS and lysed with a hypotonic lysis buffer (10 mM Tris, pH 7.5; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5 mM DTT; 0.5 mM PMSF; 1× Protease Inhibitor, 0.05% NP40). The nuclear fraction was isolated by centrifugation at 500 g, 4℃ for 5 minutes and resuspended in a nuclear lysis buffer (25 mM Tris, pH7.5; 420 mM NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 25% Glycerol; 0.5 mM DTT; 0.5 mM PMSF; 1× Protease Inhibitor). Debris from both cytoplasmic and nuclear fractions was cleared by centrifugation at max speed at 4℃ for 5 minutes.

3.7 IKK kinase Assays

To study the IKK kinase activity, the IKK complex was immunoprecipitated from control and JMJD8 knockdown HEK293T cells which were treated with TNFα at the indicated time points. Briefly, the cells were lysed in an IPKA lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 25 mM β-glycerol-phosphate, 1 mM orthovanadate,

1 mM DTT, 1 mM PMSF, 1% Triton X100) and total protein level was quantitated using Bradford assay. Next, 500 µg of total proteins were immunoprecipitated with an anti-IKKγ (sc-8330) antibody and 15 µl of 50% slurry protein A/G beads for 1 hour at 4℃, then washed with IPKA lysis buffer twice and 1× kinase buffer (20 mM HEPES pH 7.5, 50 mM NaCl, 20 mM β-glycerol-phosphate, 10 mM $MgCl_2$, 1 mM orthovanadate, 200 µM ATP) without ATP for the third wash. The beads were incubated with 200 ng of glutathione S-transferase (GST)-IκBα in 1× kinase buffer for 30 minutes at 30℃. After the incubation, the products were analyzed by immunoblotting using an anti-p-IκBα antibody.

3.8 TNFR1 recruitment assays

The control and JMJD8-knockdown cells were induced with 1 µg/ml of GST-TNFα for the indicated time points, washed with ice-cold 1x PBS for three times and lysed in IPKA lysis buffer. Next, the lysates were preclear with protein A/G beads on a rotator for 1 hour at 4℃. TNFR1 complexes were pulled down with Glutathione beads and bound proteins were analyzed by immunoblotting using the indicated antibodies.

3.9 Immunofluorescence assays

To examine the protein localization, cells were fixed with 4% formaldehyde for 15 minutes and then permeabilized and blocked with 1x PBS supplemented with 5% fetal bovine serum and 0.3% Triton X-100 for 30 minutes. Then, cells were incubated overnight with the primary antibodies (p65) according to manufacturer's recommended dilution about 1:500. Next, cells were washed 3 times with 1x PBS followed by 1-hour incubation with specific AlexaFluor-conjugated secondary antibodies (AlexaFluor 488 or AlexaFluor 555) in recommended dilution about 1:1000 (Cell Signaling Technology). Images were acquired with an Olympus IX71 fluorescent microscope with a 40x

objective. Images were analyzed using the cellSens standard and FV10-ASW viewer software (Olympus).

3.10 Immunoprecipitation

To examine the interaction between RIP1 and JMJD8, HEK293T cells were transfected with myc-RIP1 and hJMJD8 with 2×FLAG and 2×HA-expressing constructs. The cells were lysed in the IPKA lysis buffer and quantified as previously described. Five hundred microgram of total proteins were immunoprecipitated with an anti-c-Myc (sc-40), FLAG (8146S) or mouse IgG antibody (as negative control), and 10 µl of 50% slurry protein A/G beads (Pierce) for overnight at 4℃, then washed with the IPKA lysis buffer for 4 times. Bound proteins were analyzed by immunoblotting using the indicated antibodies. Thirty micrograms of total cell lysate was included as positive control.

To examine the oligomerization of JMJD8, HEK293T cells were co-transfected with different JMJD8-expressing (hJMJD8 with 2×FLAG and 2×HA, JMJD8-CFP, Δ45-JMJD8 and ΔTM-JMJD8) constructs. The cells were lysed in an IPKA lysis buffer, and the total protein concentration was quantified as previously described. Five hundred microgram of total proteins were immunoprecipitated with an anti-HA or mouse IgG antibodies, and 10 µl of 50% slurry protein A/G beads (Pierce) overnight at 4℃, then the beads were washed 4 times with the IPKA lysis buffer. The efficiency of pull-down assay was verified by immunoblotting using the indicated antibodies. Thirty micrograms of total cell lysate were included as a positive control.

3.11 Luciferase assays

Stable HEK293T cells carrying a luciferase reporter (generous gift from Professor Dr. David Baltimore) driven by NF-κB enhancer found in the immunoglobulin kappa light chain gene (293T-luc cells) were transfected with siRNA for knockdown study or JMJD8-expressing vector for overexpression study before treating the cells with and without TNFα (10 ng/ml) for an additional 12 hours to accumulate sufficient amount of luciferase protein for detection. Cells were lysed in luciferase lysis buffer (100 mM Sodium Phosphate buffer pH7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100 and 15% glycerol) and the luciferase activities were measured using a TECAN M200 plate reader according to the manufacturer's instructions.

3.12 Flow cytometry of TNFR1

Two and half million of HEK293T cells were seeded in 10 cm plate. The cells were subjected to siRNA transfection (siControl, siJMJD8a and siJMJD8b). After transfection, the cells were harvested prior to immunostaining. The cells were trypsinized and washed twice with 1× PBS, then collected in 15ml tube. Next, the cells were fixed in 4% formaldehyde at 4°C for 15 minutes and then collected by centrifugation. The cells were washed three times with 1× PBS and incubated for 5 minutes on ice. The cells were then blocked with 5% FBS in 1× PBS for 30 minutes 4°C for 30 minutes. The cells were immunoblotted with anti-TNFR1 antibody (1:100) on a rotator for 1 hour at 4°C. Then, the cells were washed three times with 1× PBS on a rotator for 5 minutes at 4°C followed by a secondary antibody AlexaFluor 488 (1:500) on a rotator for 1 hour at 4°C. Before subjected to flow cytometry, the cells were washed for another three times with 1× PBS on a rotator for 5 minutes at 4°C. The cell cells were analyzed using MACSQuant Analyzer (Miltenyi Biotec, Germany) to quantify AlexaFluor 488-positive cells. The histograms were further analyzed and plotted with FlowJo software.

3.13 JMJD8 localization assay

To examine the localization of JMJD8, HEK293T cells stably express JMJD8-eCFP were fixed with 4% formaldehyde for 15 minutes and then permeabilized and blocked with 1× PBS supplemented with 5% fetal bovine serum and 0.3% Triton X-100 for 30 minutes. The cells were then incubated overnight with primary antibodies (AIF or EEA1) according to manufacturer's recommended dilution about 1:500. Next, cells were washed 3 times with 1× PBS followed by 1-hour incubation with specific AlexaFluor-conjugated secondary antibodies (Cell Signaling Technology). For detection of lysosome and endoplasmic reticulum, the cells were treated with either 25 nM LysoTracker® or 0.25 μM ER-Tracker™ for 15 minutes. Images were acquired with an Olympus FV1000 confocal microscope using a 100× objective lens. Images were analyzed using the cellSens standard and FV10-ASW viewer software (Olympus).

3.14 Protease Protection assay

To examine the topology of JMJD8, five million overnight culture of HEK293T cells stably expressing C-terminal FLAG-HA-tagged JMJD8 were washed two times with KHM buffer (110 mM Potassium acetate, 7.5 mM Magnesium Chloride, and 20 mM HEPES pH7.2) and permeabilized with 50 μg/ml of Digitonin for 5 minutes. Next, cells were washed three times with ice-cold KHM buffer and scraped in ice-cold KHM buffer. The cells were pelleted and split into three tubes and treated with or without 10 μg/ml of proteinase K at 37℃ for 5 minutes. NP40 was added to one of the tubes to solubilize organelle proteins and treated with proteinase K as a positive control of protein digestion. Protease activities were terminated by the addition of 5 mM phenylmethylsulfonyl fluoride (PMSF). The samples were treated with SDS-sample buffer, boiled for 10 minutes, and subjected to immunoblotting analysis by anti-Kinectin, anti-MTDH, anti-PDIA3 and anti-FLAG antibodies.

3.15 Bioinformatic analysis and phylogenetic tree generation

The protein sequence of JMJD8 was obtained from NCBI (NM_001005920.2) and subjected to signal peptide (Signal P 4.1) (Petersen et al., 2011), transmembrane motif analysis (TopPred2) (Claros & Heijne, 1994) and GlycoMine (Li et al., 2015). To determine if JMJD8 clusters specifically with a particular group of JmjC domain-containing proteins, the sequences of JmjC protein were retrieved from UniProtKB (Bateman et al., 2015) with the following accession numbers and amino acid regions: JHDM1A (Q9Y2K7; 148-316), JHDM1B (Q8NHM5; 178-346), JMJD1A (Q9Y4C1; 1058-1281), JMJD1B (Q7LBC6; 1498-1721), JMJD1C (Q15652; 2274-2498), JMJD2A (O75164; 142-308), JMJD2B (O94953; 146-309), JMJD2C (Q9H3R0; 144-310), JMJD2D (Q6B0I6; 146-312), JARID1A (P29375; 437-603), JARID1B (Q9UGL1; 453-619), JARID1C (P41229; 468-634), JARID1D (Q9BY66; 458-624), UTX (O15550; 1095-1258), JMJD3 (O15054; 1339-1502), PHF8 (Q9UPP1; 231-387), JHDM1D (Q6ZMT4; 230-386), TYW5 (A2RUC4; 102-267), HIF1AN (Q9NWT6; 142-312), PHF2 (O75151; 197-353), UTY (O14607; 1042-1205), JMJD4 (Q9H9V9; 188-347), JMJD5 (Q8N371; 271-416), JMJD6 (Q6NYC1; 141-305), JMJD7 (P0C870; 128-307), JMJD8 (Q96S16; 201-334), JARID2 (Q92833; 884-1048), KDM4E (B2RXH2; 143-309), HSPBAP1 (Q96EW2; 124-288), Hairless (O43593; 946-1157), MINA53 (Q8IUF8; 139-271), and NO66 (Q9H6W3; 294-439). The phylogenetic tree was generated according to the recommendation by Hall et al. (Hall, 2013) using the Maximum Likelihood method based on the Le and Gascuel model (Le & Gascuel, 2008). The phylogenetic tree was drawn using MEGA6 with 32 amino acid sequences (Tamura et al., 2013).

3.16 Gel filtration assays

About 175 µg of total cell lysate in the lysis buffer of 20 mM Tris, pH7.5, 150 mM NaCl and 10% glycerol from HeLa S3 cells stably expressing a JMJD8-FLAG-HA (HeLa-JMJD8-FLAG-HA) was subjected to gel filtration (Superdex 200) analysis with a constant flow rate of 0.5 ml/min. The fractions were collected in 1 ml per tube. The collected fractions were immunoblotted with anti-FLAG antibody. The gel filtration high molecular weight (HMW) kit was used as a protein size reference. The molecular weights corresponded to each fraction were calculated by comparing its $K_{AV} = (V_e - V_0) / (V_c - V_0)$ to the K_{AV} of standard from HMW kit, where K_{AV} is partition coefficient, V_e is the elution volume (fraction number), V_0 is the void volume and V_c is the column volume.

3.17 Sample preparation for mass spectrometry

To examine the interaction partners of JMJD8, ten million cells were first fractionated to obtain nuclear wash (Nw) fraction. Briefly, HEK293T-JMJD8-FLAG-HA cells were washed three times with 1× PBS and lysed with a hypotonic lysis buffer (10 mM Tris, pH 7.5; 1.5 mM $MgCl_2$; 10 mM KCl; 0.5 mM DTT; 0.5 mM PMSF and 1× Protease Inhibitor). The nuclear pellet was isolated by centrifugation at 500 g, 4℃ for 5 minutes. The pellet was washed once with hypotonic lysis buffer, resuspended in a Nw buffer (50 mM Tris pH 7.5; 10 mM $MgCl_2$; 250 mM Sucrose; 0.2% NP40; 0.5 mM DTT; 0.5 mM PMSF and 1× Protease Inhibitor), and rotated at 4℃ for 1 hour. The Nw fraction was obtained via centrifugation at 500 g, 4℃ for 5 minutes. The protein concentration of Nw fraction was quantified with the Bradford assay. Three milligrams of Nw fraction was precleared with 60 µl of A/G beads (Pierce, USA) and subjected to immunoprecipitation as previously described with FLAG-agarose beads (Pierce, USA). The beads were washed 3 times with TBS containing 0.5% NP40 and 2 more times with TBS only. On-bead digestion protocol adapted from Turriziani, B. et al. (Turriziani et al., 2014) was

used to denature and digest the immunoprecipitated proteins. Briefly, 60 µl of buffer 1 (50 mM Tris pH 7.5; 2 M Urea; 5 ng/ul Trypsin) was added to the washed beads and incubated for 30 minutes at 27℃ with shaking at 800 rpm. The supernatant was collected via centrifugation at 1000 g for 1 minute. Next, the beads were washed twice with 25 µl buffer 2 (50 mM Tris, pH 7.5; 2 M Urea; 1 mM DTT) and the supernatant was pooled together with buffer 1. Then, the sample was kept at room temperature for overnight digestion. After digestion, 20 µl of 5 mg/ml Iodoacetamide (IAA) was added to the mixture and incubated at room temperature in the dark for 30 minutes. The reaction was stopped by addition of 1 µl of 100% trifluoroacetic acid (TFA). The reaction mix was desalted with a C18 spin column as per manufacturer's recommendation. Briefly, the peptides were subjected to an activated C18 spin column and spun for 1500 g for 1 minute. The column was washed twice with 200 ul of 0.5% TFA in 5% Acetonitrile (ACN) and the peptides were eluted with 50 µl 70% ACN twice. The eluates were speedvac-concentrated and resuspended with 35 µl of 0.1% formic acid and analyzed with mass spectrometry.

3.18 Mass Spectrometry

All samples were analyzed with a Thermo Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific, San Jose, CA) equipped with a Thermo EASY-nanoLC system (Thermo Scientific, San Jose, CA) and a nanoelectrospray source. Five microliters of sample were injected into an Acclaim™ PepMap™ 100 C18 LC Column (Thermo Scientific, San Jose, CA) and separated with the Thermo EASY-nanoLC system loaded with a Thermo Scientific™ EASY-Spray™ C18 LC Column (Thermo Scientific, San Jose, CA) (2 µm, 75 µm × 50 cm). The samples were separated at a flow rate of 250 nl/min over 80 minutes with a gradient from 5 % to 95 % buffer B (99.9 % Acetonitrile / 0.1 % formic acid). The raw data were collected continuously with a mass spectrometer

in a data-dependent manner. A survey scan was recorded in the Orbitrap analyzer with a 60,000 resolution over a mass range between m/z 400-1600 Da and an automatic gain control (AGC) target at $4.0e^5$. Then, it was followed by the second stage of higher-energy collisional dissociation (HCD) MS/MS scans to the top 20 most intense ions from the survey scan with an AGC target of $1e^3$, a signal threshold of 1,000, auto scan range mode and 28 % of HCD collision energy. Charge state was assigned to focus on ions that have a charge state of +2 and +3. Dynamic exclusion was enabled for 30 seconds repeat duration and 20 seconds exclusion duration to with a repeat count of 3.

3.19 Analysis of mass spectrometry data

The raw files generated from the mass spectrometer were analyzed with a MaxQuant software package (Cox & Mann, 2008). The raw files were scanned against human-reviewed protein database (UniProtKB: Taxonomy number: 9606) with a Maximum error tolerance (ppm) of 20 ppm for the first search and a 6 ppm for the main search with a false discovery rate (FDR) of 0.01. Every raw file was considered as an independent experiment, and the replicates were group together for subsequent statistical analysis. Carbamidomethylation for cysteine residue was set as fixed modification whereas methionine oxidation and acetylation of N-termini of proteins were defined as variable modification. The analyzed data were subjected to statistical analysis using the Perseus (Tyanova et al., 2016). A volcano plot was generated from student t-test with a FDR of 0.01 and S0 of 1.5. The positive hits of JMJD8 interacting partners were further analyzed with the PANTHER classification system Version 11.1 (Mi et al., 2016).

3.20 Statistical analysis

Data were analyzed with Microsoft Excel and presented as mean ± SD. Data are representative of two or more independent experiments. Statistical significance was assessed using two-tailed unpaired Student's t-test (*$p < 0.05$, **$p < 0.01$).

CHAPTER 4: RESULTS

4.1 JMJD8 is required for TNF-induced NF-κB-dependent gene expression.

A previous finding from our group showing that methylation of p65 protein regulates its transcriptional activity (Ea & Baltimore, 2009) prompted me to evaluate whether demethylases are also involved in TNF-induced NF-κB signaling. A preliminary RNAi screening of a group of Jumonji domain-containing protein founds that the JMJD8, a JmjC domain-only protein may participate in regulating TNF-induced NF-κB signaling. To verify this observation, the TNF-induced transcription kinetics of a few well-known NF-κB-dependent genes were compared between control and JMJD8 knockdown HEK293T cells. As shown in Figure 4.1a, the TNF-induced NF-κB transcriptional activity was almost completely abrogated in JMJD8 knockdown cells compared to the control cells. The effect of JMJD8 knockdown on TNF-induced NF-κB signaling was further supported by a NF-κB luciferase reporter assay (Figure 4.2a). The 293T-Luc cells were treated with 10 ng/ml of TNFα for 12 hours, which was different from the previous assay (Figure 4.1a), to prolong the expression of luciferase genes that optimized the signal detection. JMJD8 knockdown efficiency was verified by immunoblotting with a JMJD8 specific antibody. Although a band that correspond to the size of JMJD8 were detected and reduced in JMJD8 knockdown cells, multiple bands were detected at the same time (Figure 4.2a), this may be due to the non-specific interaction with proteins from the total cell lysate used.

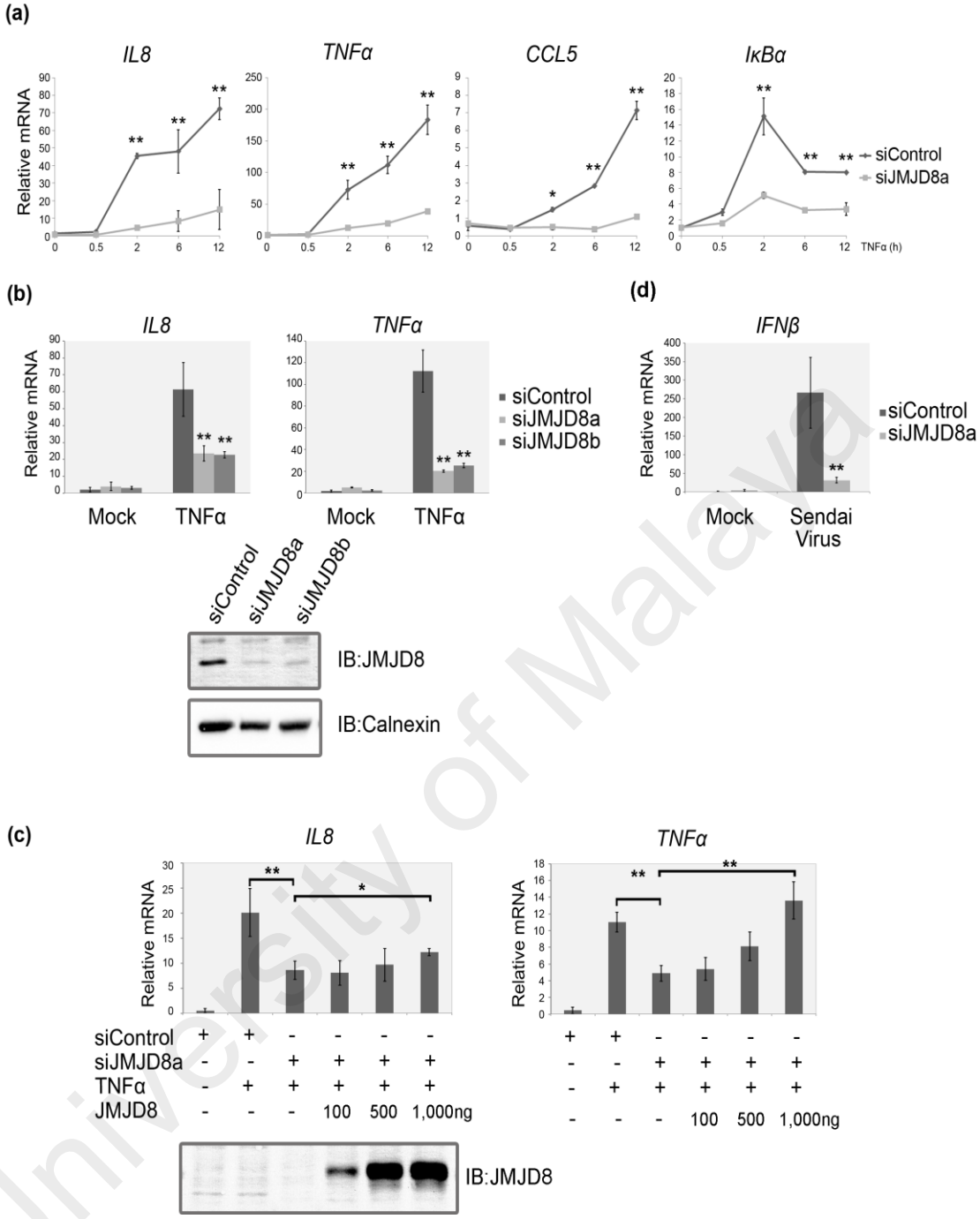


Figure 4.1: JMJD8 positively regulates NF-κB.
(a) HEK293T cells transfected with control and JMJD8 targeting siRNA oligos were treated with and without 10 ng/ml of TNFα for 0, 0.5, 2, 6 and 12 hours. The expression of TNFα, IL8, CCL5 and IκBα were measured by RT-qPCR (n = 4). (b) HEK293T cells transfected with control, JMJD8a and JMJD8b siRNA oligos were treated with and without 10 ng/ml of TNFα for 2 hours, the expression of TNFα and IL8 were measured by RT-qPCR. The knockdown expression of JMJD8 by each siRNA oligo was verified by immunoblotting with a JMJD8 specific antibody (n = 4). (c) JMJD8 knockdown HEK293T cells reconstituted with JMJD8 were treated with TNFα for 2 hours and the expression of TNFα and IL8 were measured by RT-qPCR. The transient expression of ectopic JMJD8 was verified by immunoblotting with a JMJD8 specific antibody (n = 4). (d) Control and JMJD8 knockdown HEK293T cells were infected with Sendai virus (150 HAU/ml) and the levels of IFNβ were measured by RT-qPCR (n = 4). Data represent the means ± SD. (*p < 0.05, **p < 0.01). The "n" represents the number of repeats. Full-length blots are presented in Appendix A.

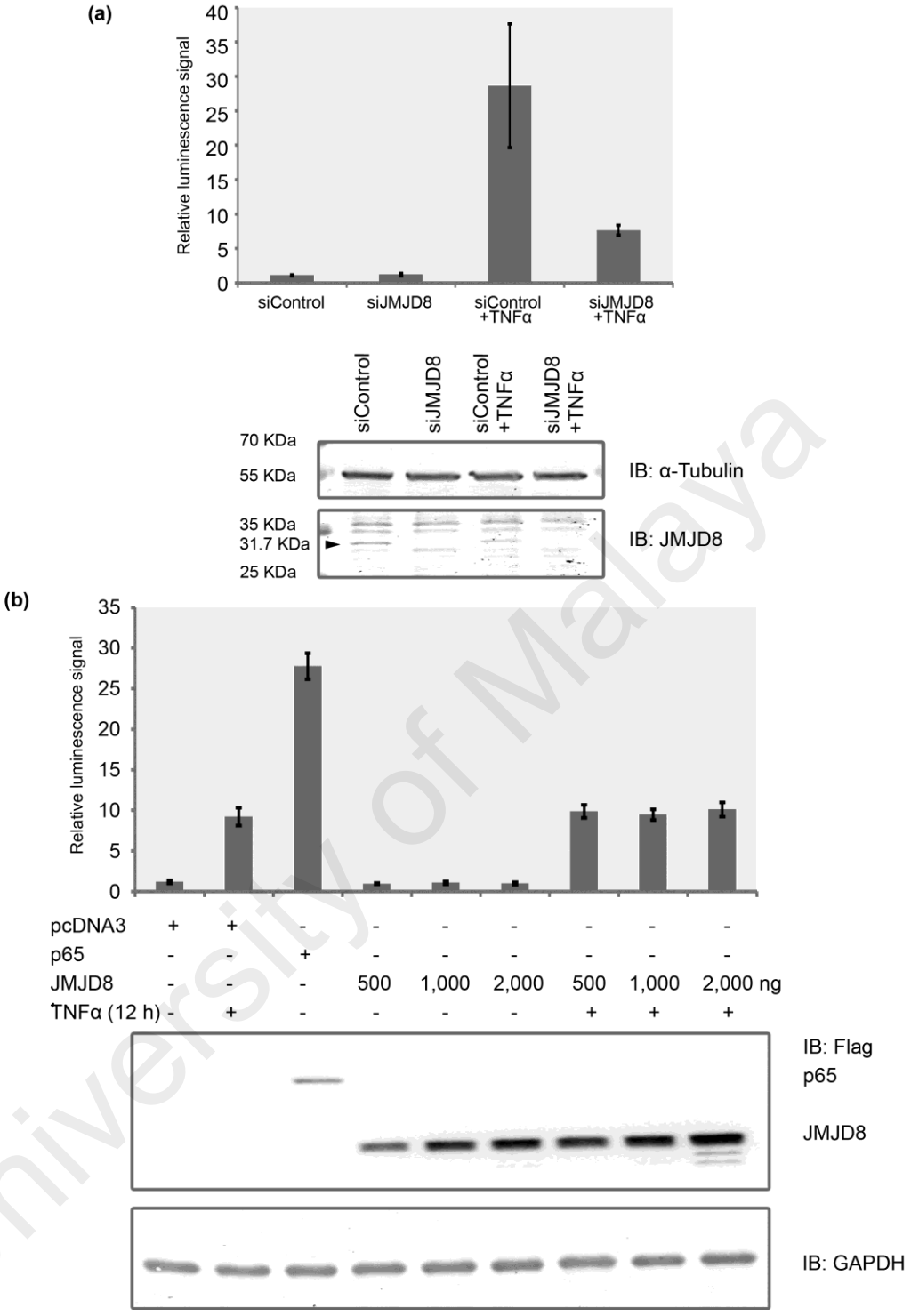


Figure 4.2: The effect of knock down and overexpression of JMJD8 in TNF-induced NF-κB activity.
(a) Control and JMJD8 knockdown (siJMJD8a) 293T-Luc cells were treated with 10 ng/ml of TNFα for 12 hours. Total cell lysates were prepared and luciferase activity was quantified with TECAN M200 microplate reader. (b) 293T-luc cells were transiently transfected with different construct as indicated and were treated with TNFα for 12 hours. Total cell lysates were prepared and luciferase activity was quantified with TECAN M200 microplate reader. The transient expression of ectopic JMJD8 was verified by immunoblotting with the indicated antibodies. Data represent means ± SD. (n = 4). The "n" represents the number of repeats.

To ensure that the NF-κB activation defect observed in the JMJD8 knockdown cells is not due to an off-target effect, a second siRNA oligo that targets an alternative site of the JMJD8 transcript was tested. The knockdown of JMJD8 by each siRNA oligo was verified by immunoblotting with a JMJD8 specific antibody (Figure 4.1b, lower panel). In line with the previous observation, transfection of both siRNA oligos specific for JMJD8 into HEK293T cells not only resulted in a decrease of JMJD8 protein level but also led to a significant reduction of TNF-induced expression of *IL8* and *TNFα* transcripts (Figure 4.1b, upper panel). The same effect was observed with JMJD8 knockdown in HONE1 (Nasopharyngeal carcinoma cells), HaCat (Immortalized human keratinocytes) and U2OS (Osteosarcoma cells) cell lines indicating that the observed defects in NF-κB activation caused by JMJD8 knockdown are not cell-type specific (Figure 4.3). In addition, a rescue assay of the defective TNF-induced NF-κB activation was carried out by reconstituting the JMJD8 knockdown HEK293T cells with a siRNA-resistant JMJD8 transcript. Transient over-expression of the siRNA-resistant JMJD8 in JMJD8 knockdown HEK293T cells leads to significant recovery of both *IL8* and *TNFα* expression in a dose-dependent manner albeit less pronounced in *IL8* suggesting that JMJD8 is indeed a positive regulator of TNF-induced NF-κB signaling (Figure 4.1c). Surprisingly, no augmentation of NF-κB activation was detected when wild-type JMJD8 was transiently overexpressed in 293T-luc cells, with or without TNFα stimulation (Figure 4.2b). This suggests that JMJD8 may affect NF-κB activation indirectly and a secondary factor is required for its positive regulatory function.

To determine whether the observed defect in NF-κB activation caused by JMJD8 knockdown is specific to TNF-induced NF-κB signaling, the control and JMJD8 knockdown HEK293T cells were infected with and without Sendai virus. Surprisingly, JMJD8 knockdown markedly suppressed IFNβ induction by Sendai virus infection

(Figure 4.1d). This observation suggests that JMJD8 may be involved in other pathways and may not be specifically restricted to TNF-induced NF-κB signaling.

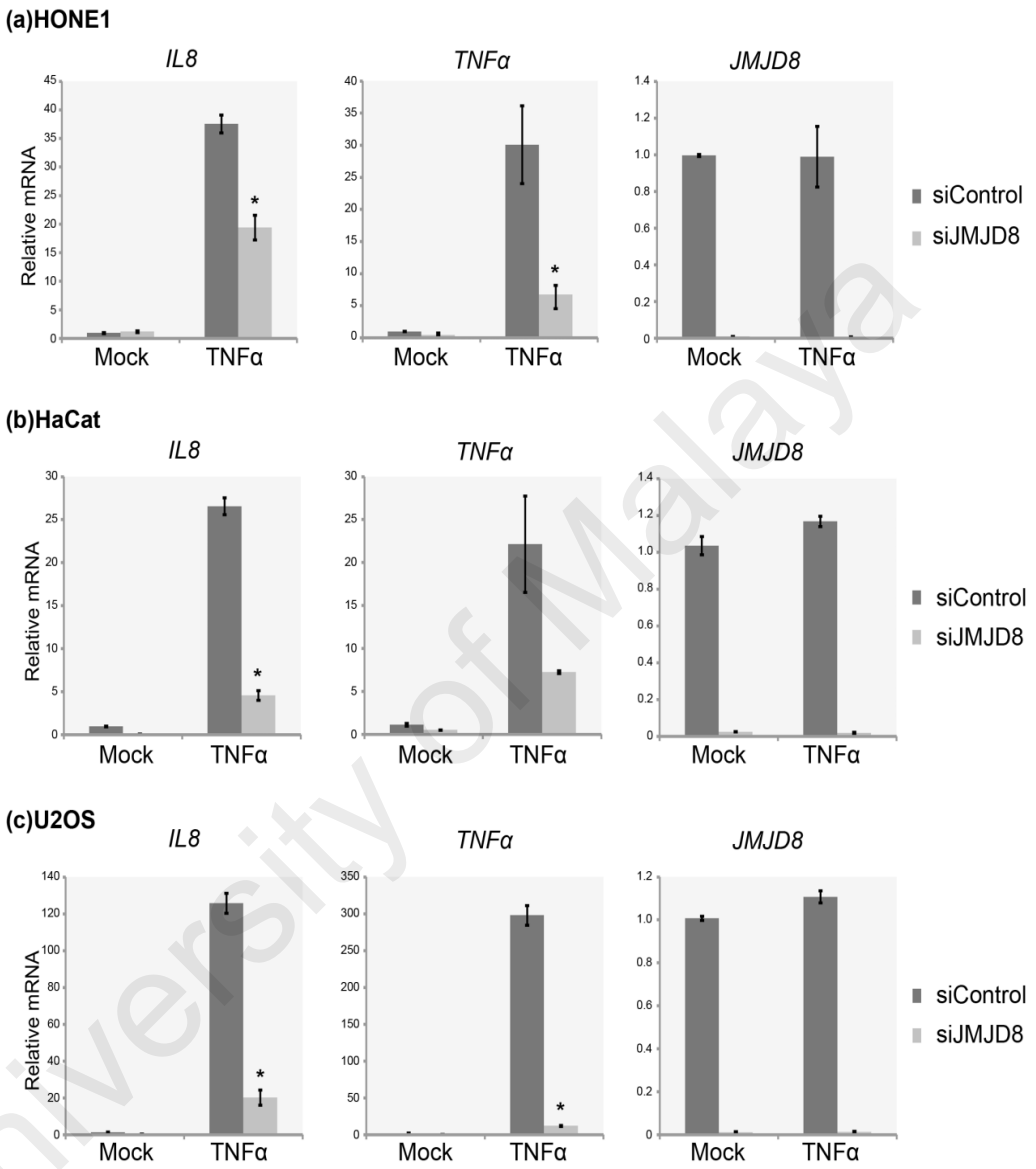


Figure 4.3: JMJD8 regulation of TNF-induced NF-κB signaling is not cell-type specific. (a) HONE1, (b) HaCat and (c) U2OS cells transfected with control and JMJD8 siRNA (siJMJD8a) oligos were treated with or without 10 ng/ml of TNFα for 2 hours, the expression of *TNFα* and *IL8* were measured by RT-qPCR. (n = 4). Data represent means ± SD. (*p < 0.05). The "n" represents the number of repeats.

4.2 JMJD8 deficiency reduces TNF-induced IκBα degradation and p65 translocation.

To dissect the role of JMJD8 in the TNF pathway, the degradation of IκBα and the nuclear translocation of NF-κB were first investigated, which are the two biochemical hallmarks of NF-κB activation. HEK293T cells were transfected with control or JMJD8-targeting siRNA oligos and treated with TNFα at the indicated time points. The cells were harvested and fractionated into cytoplasmic and nuclear fractions. TNF-induced degradation of IκBα peaked at 30 minutes followed by a resynthesis of IκBα at 90 minutes in the control cells (Figure 4.4). IκBα degradation was reduced or delayed in the JMJD8-deficient cells and no resynthesis of IκBα was observed. However, the level of IκBα at 0-minute time point of JMJD8 knockdown cells was lower than 4-45 minutes time points, this may be due to unequal loading since the HSP90α was lower at 0-minute time point than 4-45 minutes time points as well. Consistent with impaired IκBα degradation, a significant reduction of TNF-induced p65 nuclear translocation was observed in JMJD8-deficient cells (Figure 4.4). To further confirm this observation, immunofluorescence assay was performed to visualize the p65 subcellular localization in control and JMJD8 knockdown HEK293T cells with and without TNFα stimulation. Consistently, a complete blockage of p65 translocation into the nucleus of JMJD8 knockdown cells was observed (Figure 4.5), indicating that JMJD8 is required for both IκBα degradation and the release of NF-κB into the nucleus.

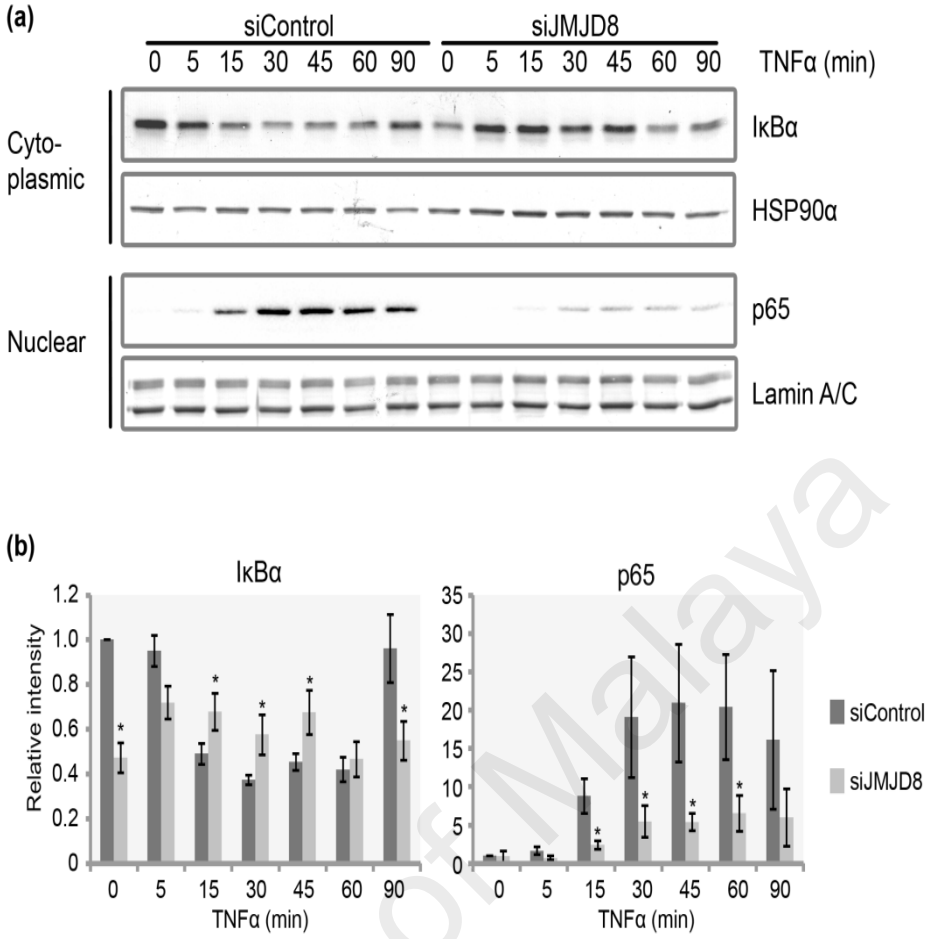


Figure 4.4: JMJD8 deficiency reduces TNF-induced IκBα degradation and p65 translocation. (a) Control and JMJD8 (siJMJD8a) knockdown HEK293T cells were treated with 10 ng/ml of TNFα for 0, 5, 15, 30, 45, 60 and 90 minutes. Cytoplasmic and nuclear fractions were prepared, and immunoblotted for IκBα and p65. HSP90α and Lamin A/C were used as cytoplasmic and nuclear loading controls respectively. (b) The relative intensity of bands was quantified using the Image Lab (BioRad)/ImageJ software, were normalized to HSP90α or Lamin A/C, and shown in relative to 0 minute of siControl (n = 3). Data represent means ± SD. (*$p < 0.05$). The "n" represents the number of repeats. Full-length blots are presented in Appendix B.

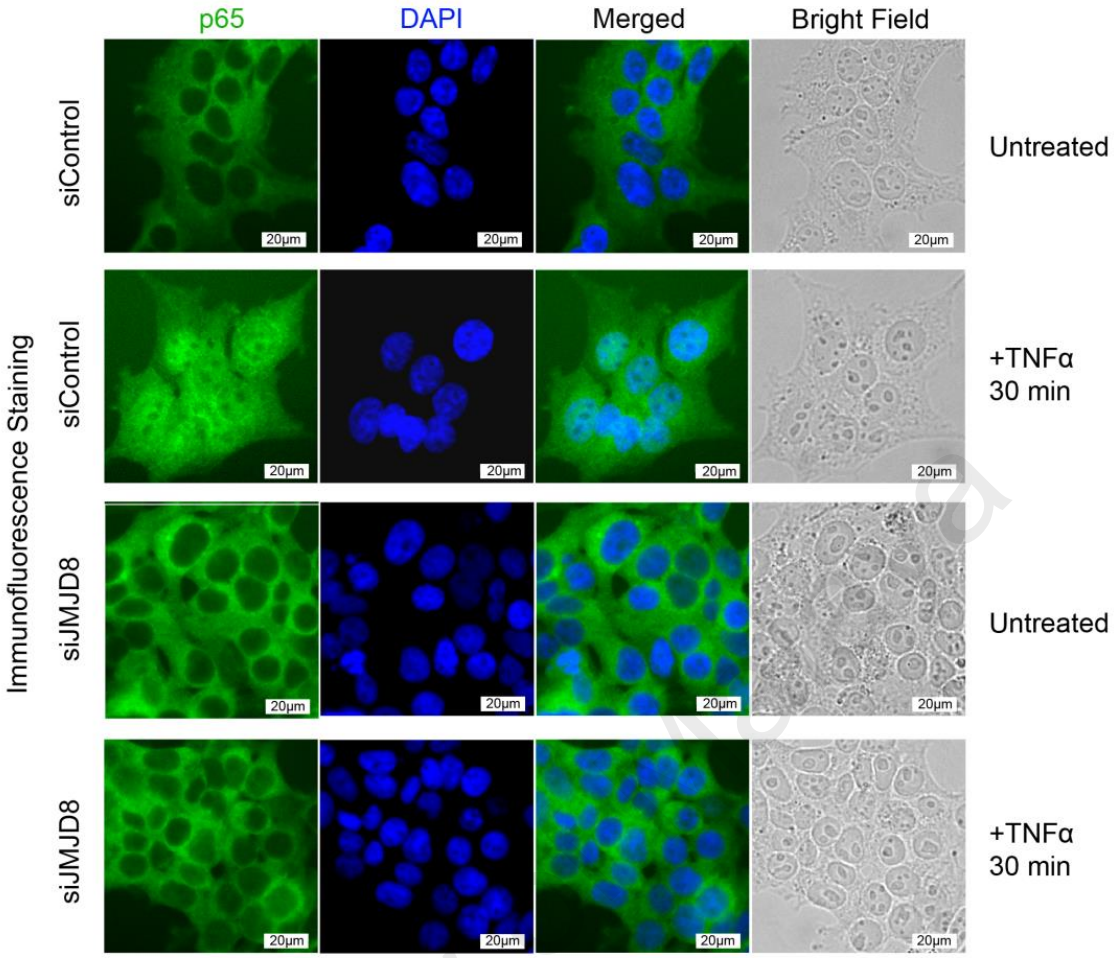


Figure 4.5: Complete blockage of p65 translocation into the nucleus of JMJD8 knockdown cells. Control and JMJD8 (siJMJD8a) knockdown HEK293T cells were treated with 10 ng/ml of TNFα for 30 minutes. P65 localization was visualized with an immunofluorescence assay. Images were acquired with an Olympus IX71 fluorescence microscope. Scale bar: 20 µm. (n = 3). The "n" represents the number of repeats.

4.3 JMJD8 is essential for IKK kinase activation.

The observed defect in IκBα degradation in JMJD8 knockdown cells suggests that JMJD8 may regulate the upstream signal transduction of TNF pathway. IκBα phosphorylation by IKK complexes is a prerequisite step for IκBα degradation (Hayden & Ghosh, 2014). Therefore, the activation of IKK in the control and JMJD8 knockdown HEK293T cells was investigated. The control and JMJD8 knockdown HEK293T cells were treated with TNFα at the indicated time points and the IKK kinase activity was measured with an *in vitro* IKK kinase assay. IKK kinase activity was detected as early as 5 minutes post-TNF stimulation and peaked at 10 minutes (Figure 4.6). With the

knockdown of JMJD8, TNF-induced IKK activation was significantly reduced as measured by the *in vitro* IKK kinase assay as well as the immunoblotting of p-IκBα in the total cell extracts. This result suggests that JMJD8 is required for TNF-induced activation of IKK.

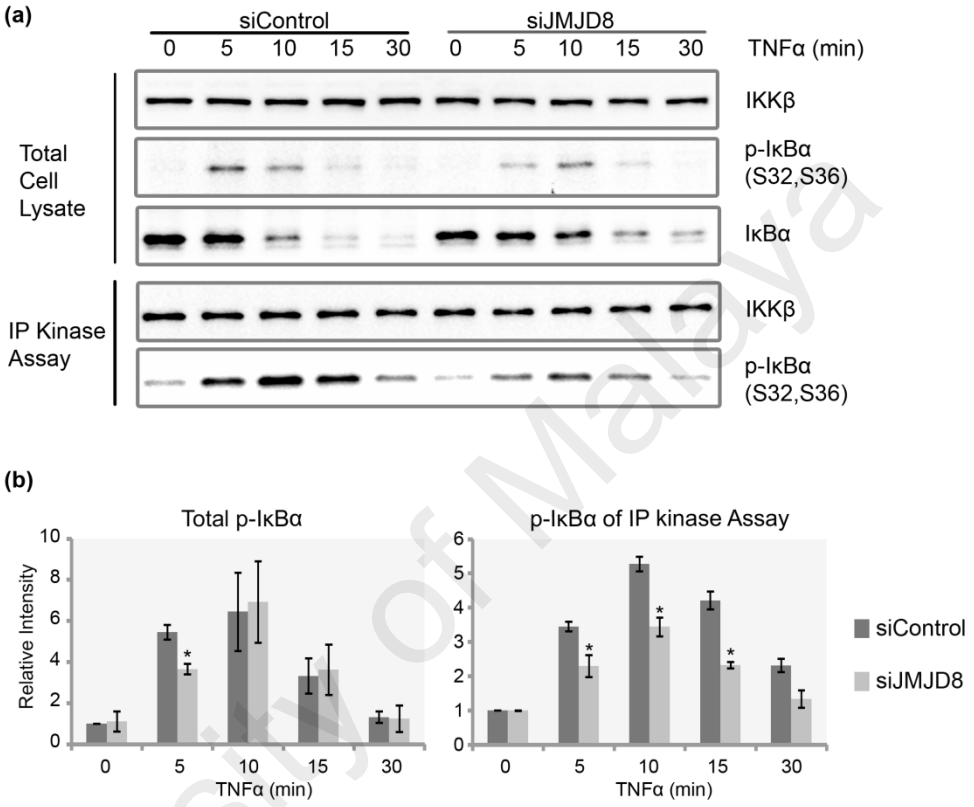


Figure 4.6: JMJD8 is required for TNF-induced IKK kinase activity.
(a) Control and JMJD8 (siJMJD8a) knockdown HEK293T cells were induced with 10 ng/ml of TNFα for 0, 5, 10, 15 and 30 minutes. IKK kinase activity was measured with an *in vitro* kinase assay followed by immunoblotting using the anti-p-IκBα and anti-IKKβ antibodies. (b) The relative intensity of bands was quantified using the Image Lab (BioRad)/ImageJ, were normalized to IKKβ, and shown in relative to 0 minute of siControl (n = 2). Data represent means ± SD. (*p < 0.05). The "n" represents the number of repeats. Full-length blots are presented in Appendix C.

In parallel to the activation of NF-κB, TNF also activates the MAPK pathways, including ERK, JNK and p38 pathways (Karin & Gallagher, 2009). To examine whether TNF-induced MAPK pathways are affected in JMJD8 knockdown cells, the activation status of MAPKs in response to TNF stimulation was examined. Unexpectedly, activation of MAP kinases was also reduced in JMJD8 knockdown cells (Figure 4.7). There was a significant reduction of the phosphorylation of JNK1/2 (p-JNK1/2), ERK1/2 (p-ERK1/2) and p38 (p-p38) in JMJD8 knockdown cells compared to the control cells. These results

suggest that JMJD8 is required for the activation of MAPKs in response to TNF stimulation.

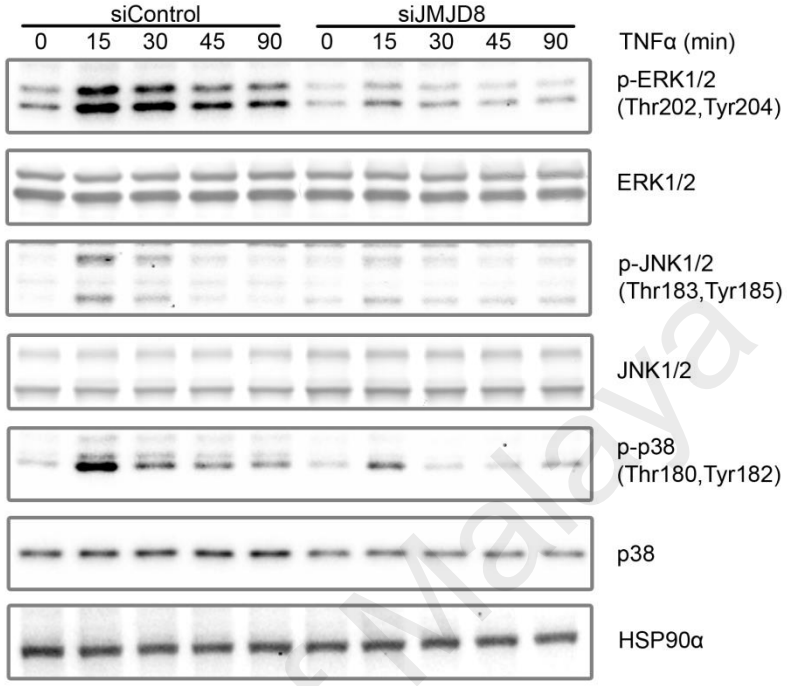


Figure 4.7: JMJD8 is required for TNF-induced MAP kinase.
Control and JMJD8 (siJMJD8a) knockdown HEK293T cells were induced with 10 ng/ml of TNFα for 0, 15, 30, 45 and 90 minutes. Total cell lysates were prepared and immunoblotted with the indicated antibodies (n = 2). The "n" represents the number of repeats. Full-length blots are presented in Appendix D, E and F, respectively.

4.4 JMJD8 is required for IKK phosphorylation and RIP1 ubiquitination.

Phosphorylation of IKK at S177 and S181 in the activation loop of IKKβ (S176 and S180 in IKKα) is required for its kinase activity (Delhase et al., 1999). To investigate the phosphorylation status of IKK, the control and JMJD8-deficient cells were treated with TNFα at indicated time points and measured the amount of p-IKK. Consistent with the IKK kinase assay, the amount of p-IKK was significantly lower in JMJD8 knockdown cells (Figure 4.8).

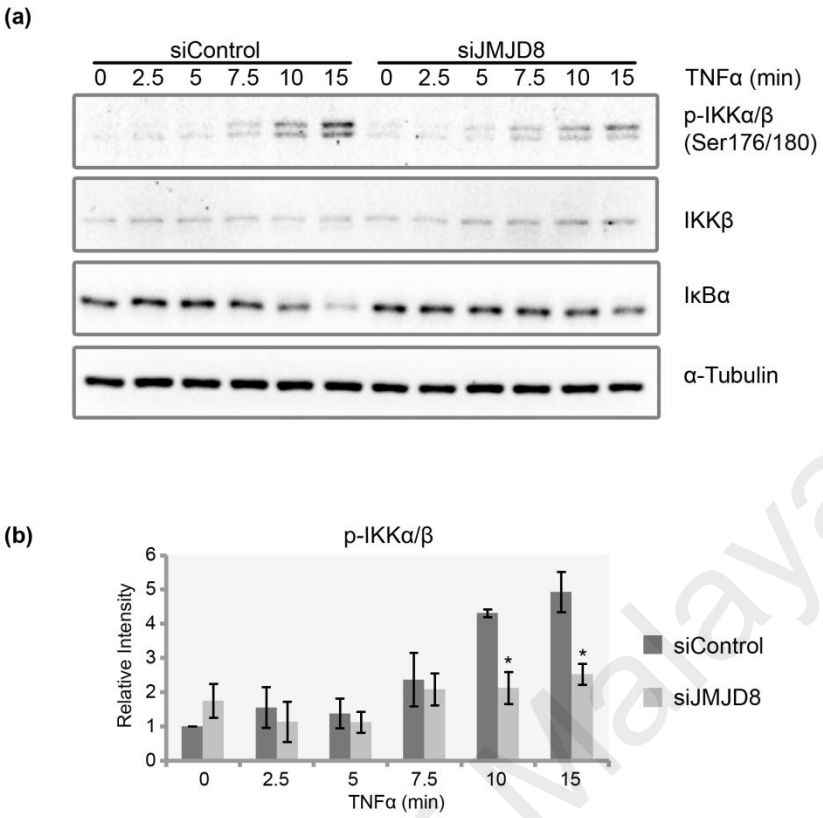


Figure 4.8: JMJD8 is required for IKK phosphorylation.
(a) Control and JMJD8 (siJMJD8a) knockdown HEK293T cells were induced with 10 ng/ml of TNFα for 0, 2.5, 5, 7.5, 10 and 15 minutes. Total cell lysates were prepared and immunoblotted with the indicated antibodies. (b) The relative intensity of bands was quantified using the Image Lab (BioRad)/ImageJ, were normalized to IKK or α-Tubulin, and shown in relative to 0 minute of siControl (n = 2). Data represent means ± SD. (*$p < 0.05$). The "n" represents the number of repeats. Full-length blots are presented in Appendix G.

RIP1 ubiquitination is another key event that is essential for TNF-induced NF-κB signaling (Ea et al., 2006; Li et al., 2006). To examine whether RIP1 ubiquitination is affected in JMJD8-deficient cells, the TNFR1 receptor complex was pulled down from control and JMJD8 knockdown HEK293T cells that were treated with and without GST-TNFα and examined the RIP1 ubiquitination by immunoblotting with a specific antibody against RIP1. Interestingly, a significant reduction of RIP1 ubiquitination in JMJD8 knockdown cells was detected (Figure 4.9), suggesting that JMJD8 may regulate the upstream components of TNF-induced NF-κB signaling. However, no interaction between RIP1 and JMJD8 was detected with a co-immunoprecipitation assay (Figure 4.10). Besides, it is possible that the loss of JMJD8 interferes with the presentation or

expression of TNFR1. However, no change in the levels of TNFR1 was detected with flow cytometry in JMJD8 knockdown cells compared to the control cells (Figure 4.11).

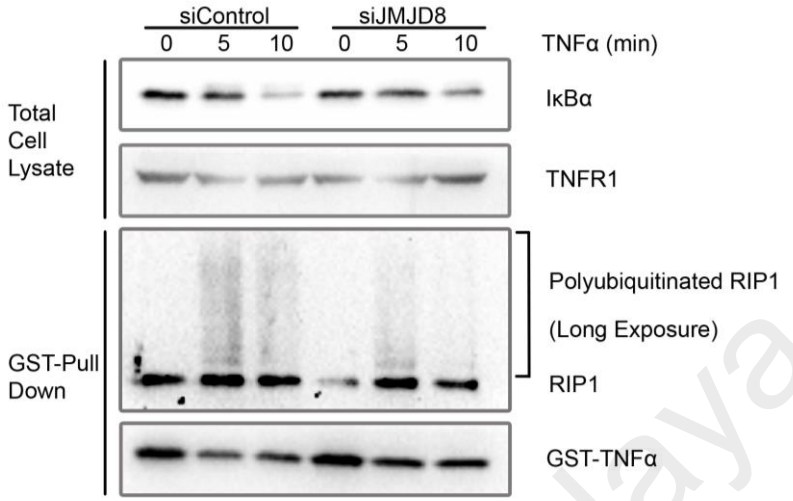


Figure 4.9: JMJD8 is important for RIP1 ubiquitination.
Control and JMJD8 (siJMJD8a) knockdown HEK293T cells were induced with 1 μg/ml of GST-TNFα for 0, 5 and 10 minutes. TNFR1 complexes were pulled down with Glutathione beads and immunoblot for RIP1 and TNFα (n = 10). The "n" represents the number of repeats. Full-length blots are presented in Appendix H.

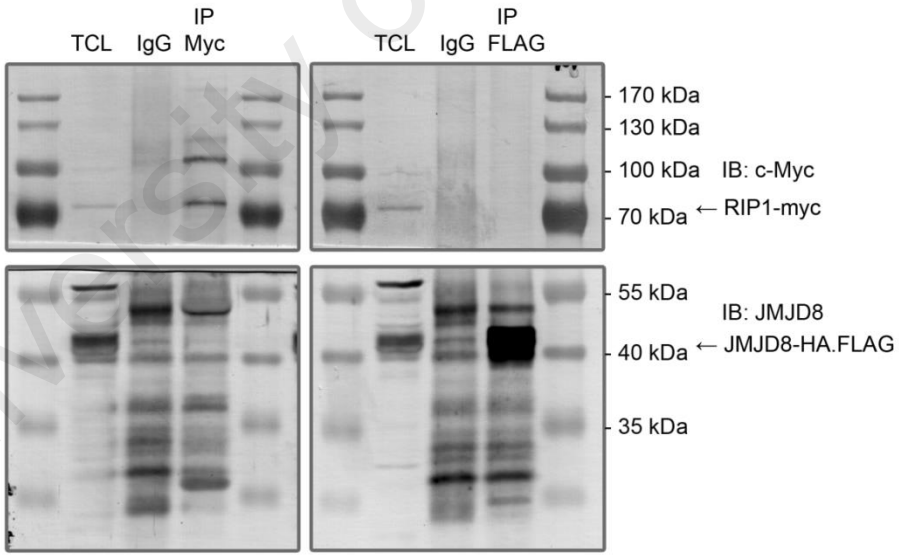


Figure 4.10: JMJD8 and RIPK1 do not interact when over expressed in 293T cells.
HEK293T cells were transfected with RIP1-myc and JMJD8-HA-FLAG-expressing constructs. Total cell lysates were prepared 24 hours post-transfection and immunoprecipitated with the indicated antibodies. The bound proteins were analyzed by immunoblotting with the indicated antibodies. TCL=Total cell lysate. (n = 2). The "n" represents the number of repeats.

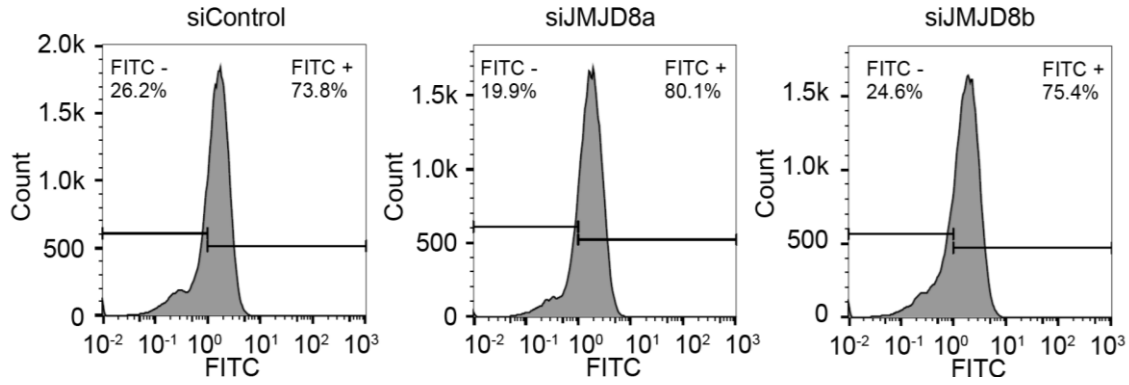


Figure 4.11: Loss of JMJD8 does not change the level of TNFR1.
Control and JMJD8 knockdown HEK293T cells were immunostained with TNFR1 antibody followed by FITC-labelled secondary antibody. TNFR1 levels were measured with flow-cytometry via detection of the level of FITC signal. (n = 3). The "n" represents the number of repeats.

4.5 JMJD8 deficiency favors cells towards TNF-induced apoptosis.

TNFα is a pleiotropic cytokine which can lead to two distinct cell fates which are the pro-survival path, mainly through the activation of pro-survival genes by NF-κB, or pro-apoptotic path through the signaling cascade of caspases activation (Brenner et al., 2015). Thus, the defective in the pro-survival path may favor the cells towards pro-apoptotic pathway. To investigate this speculation, the control and JMJD8 knockdown HEK293T cells were treated with and without TNFα and apoptosis was examined by immunoblotting total cell lysates with specific antibodies against caspase 3, cleaved-caspase 3, caspase 8 and PARP-1. TNF-only treatment induced a moderate level of apoptosis in control cells. Apoptosis was further enhanced in the presence of both TNF and cycloheximide (CHX) as evidenced by the presence of cleaved PARP-1, reduced level of pro-caspase 3 and 8, an increased level of cleaved-caspase 3 (Figure 4.12). In the knockdown of JMJD8, TNF-only treatment induced high level of apoptosis that was comparable to the control cells treated with both TNF and CHX. Collectively, these results indicate that JMJD8 is required for the pro-survival pathway of TNF-induced NF-κB signaling.

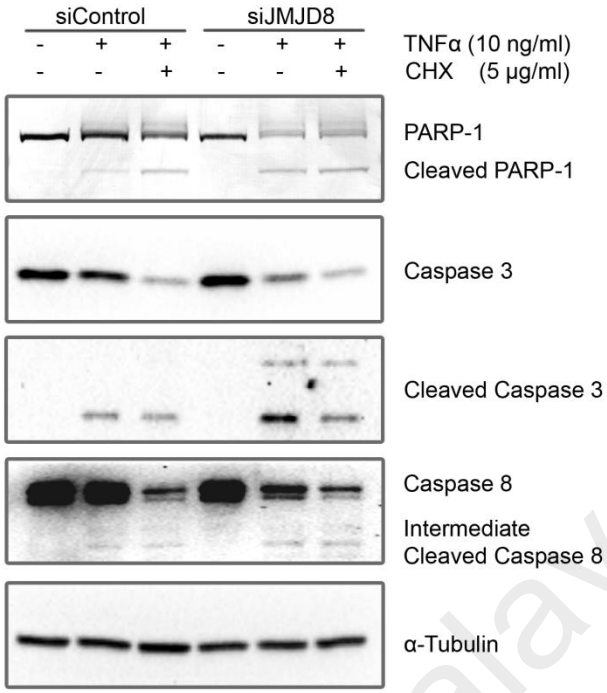


Figure 4.12: JMJD8 deficiency sensitizes HEK293T cells to TNF-induced apoptosis. Control and JMJD8 (siJMJD8a) knockdown HEK293T cells were treated with either 10 ng/ml of TNFα alone or together with 5 μg/ml of Cycloheximide (CHX) for 12 hours. Total cell lysates were prepared and immunoblotted with the indicated antibodies (n = 3). The "n" represents the number of repeats. Full-length blots are presented in Appendix I.

4.6 JMJD8 contains a signal peptide that is essential for its endoplasmic reticulum (ER) localization.

To better understand the biochemical properties of JMJD8, its protein sequence was retrieved from NCBI (NM_001005920.2) and a sequence analysis was performed (Figure 4.13a). Using TopPred II analysis software (Claros & Heijne, 1994), a putative transmembrane domain was identified located between 175 – 196 amino acid residues of JMJD8. According to SignalP 4.1 (Petersen et al., 2011), a discrimination score (D-score) of 0.590 was obtained at the position of 1-44 amino acid residues, which was above the default cut-off point of 0.450, suggesting that there is a signal peptide at the N-terminus of JMJD8 (Figure 4.13b).

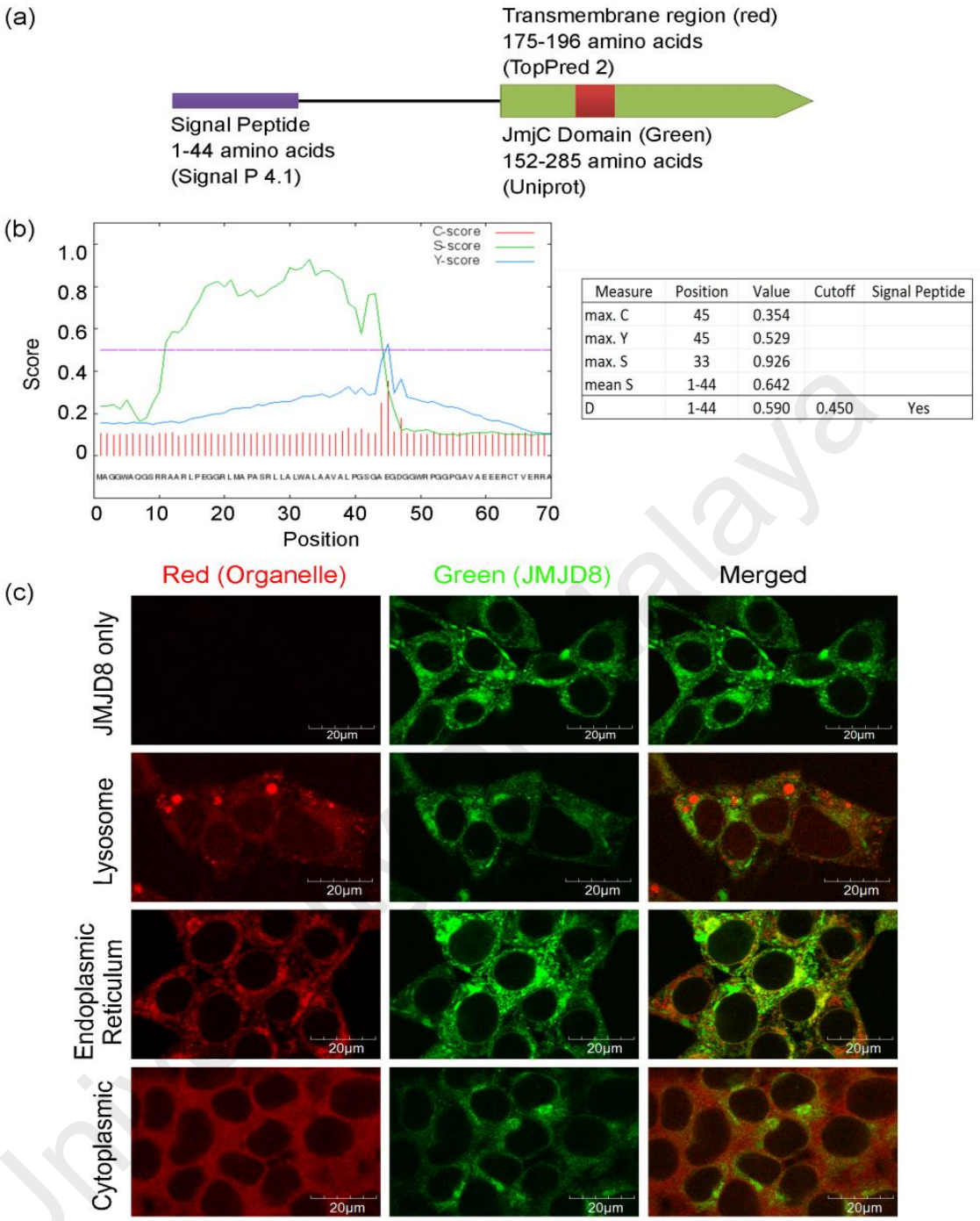


Figure 4.13: JMJD8 is an ER protein.
(a) Schematic structure of JMJD8 predicted using indicated bioinformatics tools. (b) The protein sequence of JMJD8 obtained from NCBI was subjected to SignalP 4.1 analysis and three different scores were measured. The raw cleavage site score (C-score) is the output of the cleavage site prediction network, which is trained to distinguish signal peptide cleavage site; signal peptide score (S-score) is the output of signal peptide prediction network, which is trained to locate the signal peptides of a protein; and Y-score is a combination of C-score and the slope of S-score. In addition, mean S represented the average S-score of the possible signal peptide, whereas discrimination score (D-score) represented the mean S and maximum Y scores, which is used to discriminate signal peptide from non-signal peptide. (c) HEK293T-JMJD8-eCFP stable cells were stained with LysoTracker (Lysosome), ER-tracker (Endoplasmic reticulum) or an anti-p65 antibody (Cytoplasmic). The yellow staining in the overlay image indicates colocalization of JMJD8 with ER. Images were acquired with an Olympus FV1000 confocal microscope. Scale bar: 20 μm. (n = 3). The "n" represents the number of repeats.

JmjC domain-containing proteins that function as histone demethylases are mainly localized to nucleus (Accari & Fisher, 2015). In contrast, the presence of a putative transmembrane domain and a signal peptide imply that JMJD8 may be a membrane bound protein localized to the cell membrane or other organelles. To determine the subcellular localization of JMJD8, confocal microscopy was employed to image HEK293T cells stably expressing JMJD8 fused with an eCFP at the C-terminus (Figure 4.13c). JMJD8 showed a distinct cytoplasmic staining that partially overlapped with the staining pattern of ER-Tracker™ Red dye, a fluorescent marker that specifically stains ER. JMJD8 did not colocalize with p65, a cytoplasmic protein, as well as other organelles, including lysosome, nucleus, endosome, Golgi and mitochondria (Figure 4.13c and Figure 4.14). Interestingly, JMJD8 lacking the signal peptide (Δ45-JMJD8-eCFP) lost its ER localization and was distributed both in nucleus and cytoplasm, whereas the transmembrane domain deleted JMJD8 (ΔTM-JMJD8-eCFP) still localized to ER (Figure 4.15).

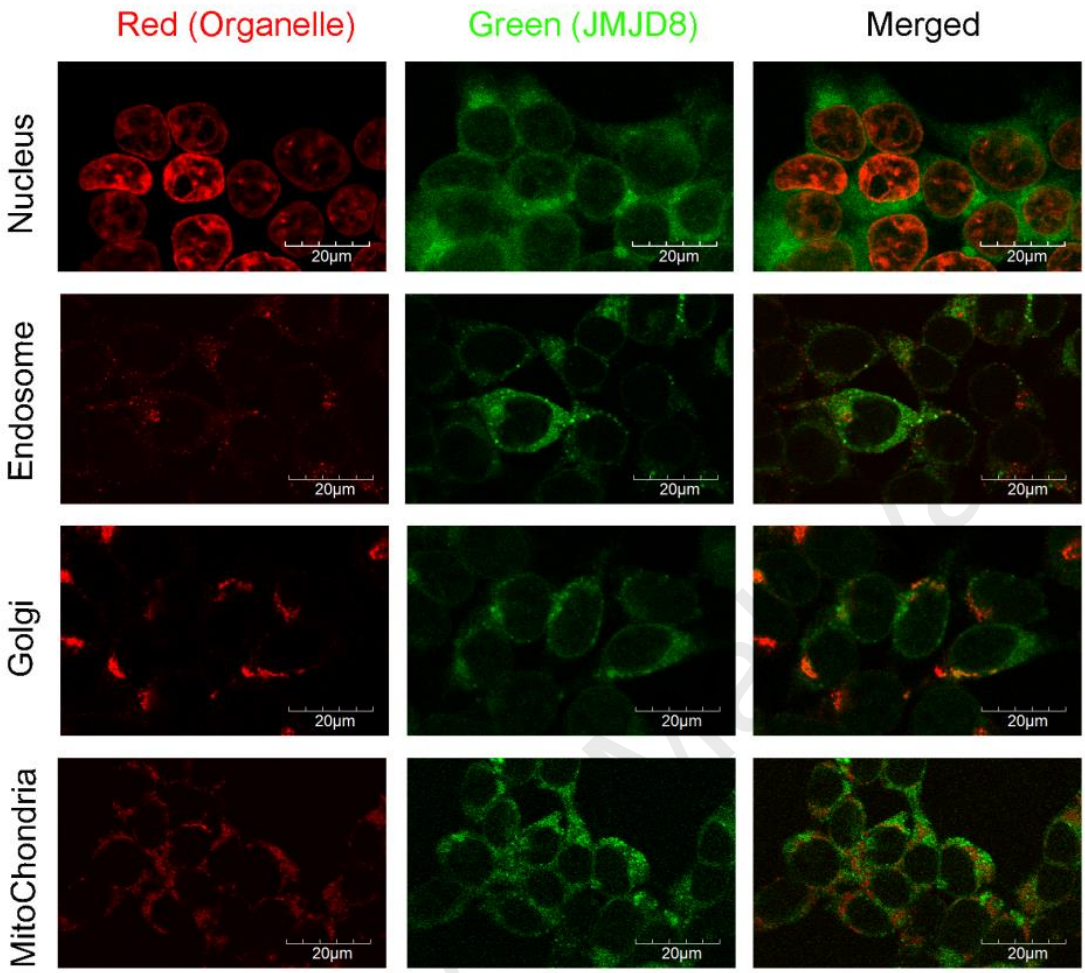


Figure 4.14: Localization of JMJD8.
Localization of JMJD8 in HEK293T-JMJD8-eCFP stable cells were visualized by immunofluorescence microscopy co-stained with organelle specific antibodies such as EEA1 (C45B10) (Cell Signaling Technology, USA) for endosomes, RCAS1 (D2B6N) (Cell Signaling Technology, USA) for Golgi, and AIF (D39D2) (Cell Signaling Technology, USA) for mitochondria. Images were acquired with an Olympus FV1000 confocal microscope. Scale bar: 20 μm. (n = 2). The "n" represents the number of repeats.

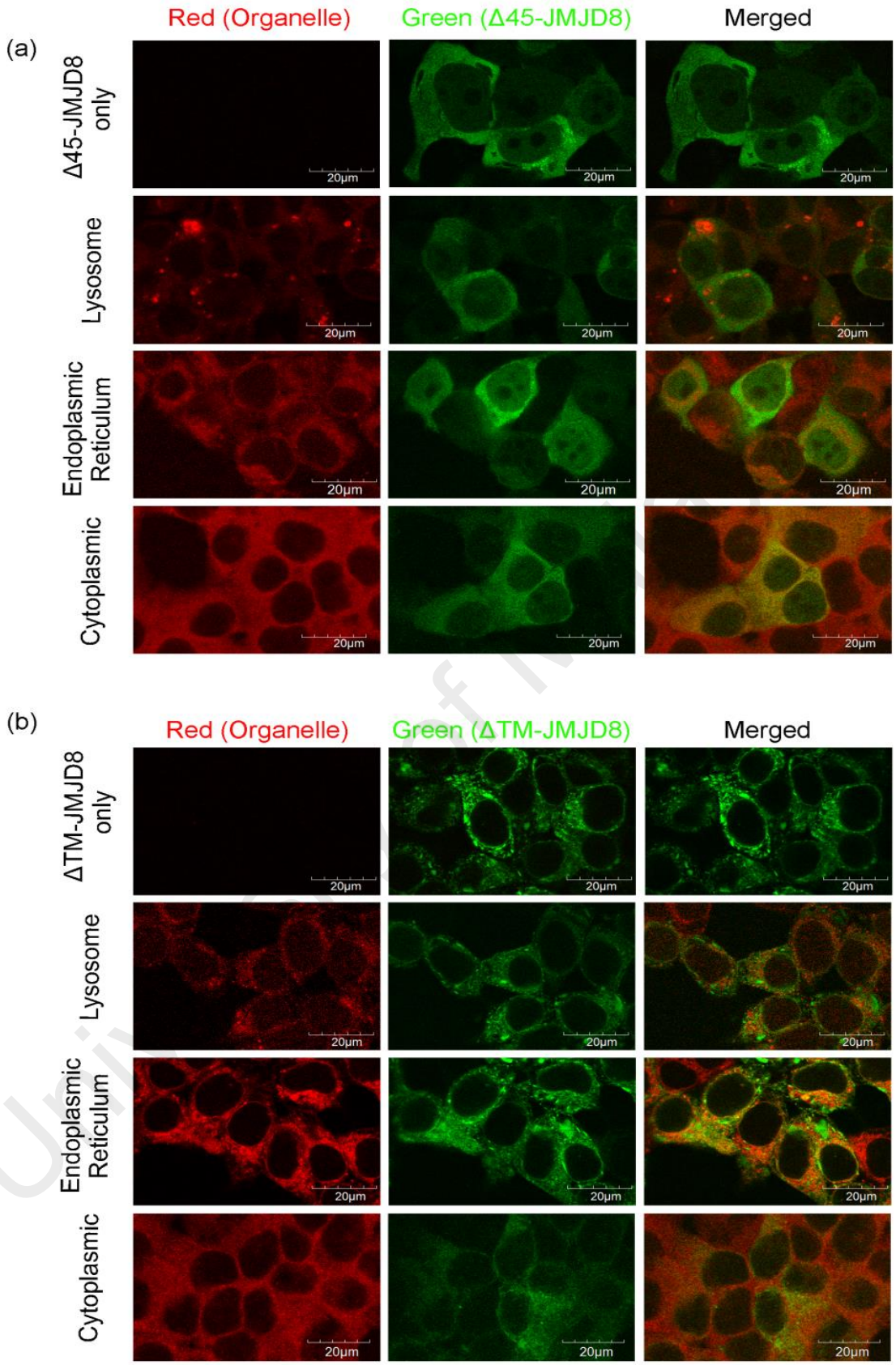


Figure 4.15: Localization of JMJD8 mutants.
(a) Localization of Δ45-JMJD8 and (b) ΔTM-JMJD8 in HEK293T stable cells were visualized and compared with LysoTracker- (Lysosome), ER-tracker-(Endoplasmic reticulum) and p65-(Cytoplasmic) immunofluorescence staining. Images were acquired with an Olympus FV1000 confocal microscope. Scale bar: 20 μm. (n = 3). The "n" represents the number of repeats.

To determine the subcellular localization of endogenous JMJD8, a crude subcellular fractionation experiment was carried out. Firstly, the HEK293T cells were transfected with 3 different siRNA oligos that target JMJD8 to facilitate the identification of JMJD8 by immunoblotting. Although these siRNAs are able to knockdown *JMJD8* transcript, but only siJMJD8a and siJMJD8b are able to reduce the endogenous JMJD8 protein, this may be due to a possible off-target effect that prolongs the JMJD8 protein stability by siJMJD8c or siJMJD8c transfection promotes the expression of a non-specific protein with its molecular weight about the size of JMJD8. The cells were lysed in a hypotonic buffer and isolated the nuclear, cytoplasmic and heavy membrane (HM) fractions. HM fraction is enriched with lysosomes, ER and mitochondria (Alexia et al., 2013). However, calnexin, an ER protein, was present in both HM and nuclear fractions. Some ER, especially rough ER, is connected to the outer nuclear membrane and thus may co-purify with nucleus (Huber et al., 2003; Wilkie & Schirmer, 2008). To extract ER proteins from the nuclear fraction, the nuclear pellets were washed with a nuclear wash buffer. With the additional step, the majority of calnexin was able to be extracted from the nuclear fraction without breaking the nucleus as SNF2H, a nuclear protein, which was not extracted (Figure 4.16). Intriguingly, endogenous JMJD8 co-purified with calnexin in the nuclear wash fraction (Nw) but not in the HM fraction, this may be due to the sensitivity of the antibody. In line with the endogenous JMJD8, ΔTM-JMJD8-eCFP is also enriched in the Nw fraction (Figure 4.17).

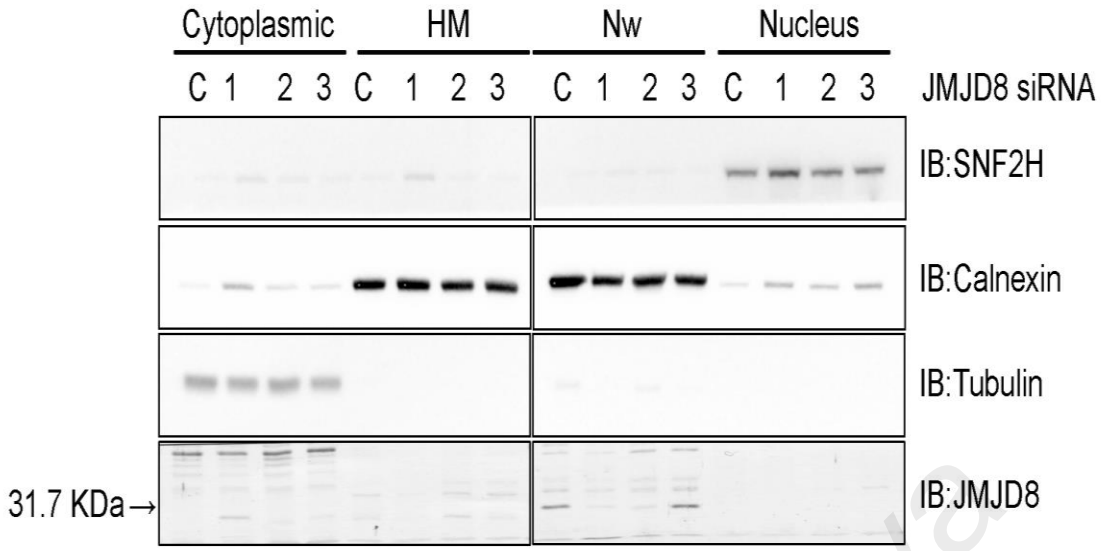


Figure 4.16: JMJD8 is enriched in Nw fraction.
HEK293T cells were transfected with 10 nM control (C) and siRNA targeting JMJD8 (1= siJMJD8b, 2=siJMJD8a and 3=siJMJD8c). Three siRNA oligos were tested to facilitate the identification of endogenous JMJD8 by immunoblotting. Cell lysates were prepared and fractionated into cytoplasmic, heavy membrane (HM-rich in lysosomes, ER, and mitochondria), nuclear wash (Nw-rich in ER) and nuclear fractions. The organelle specific proteins and JMJD8 were analyzed by immunoblotting with the indicated antibodies (Nuclear with SNF2h, ER with calnexin and cytoplasmic with tubulin). (n = 3). The "n" represents the number of repeats.

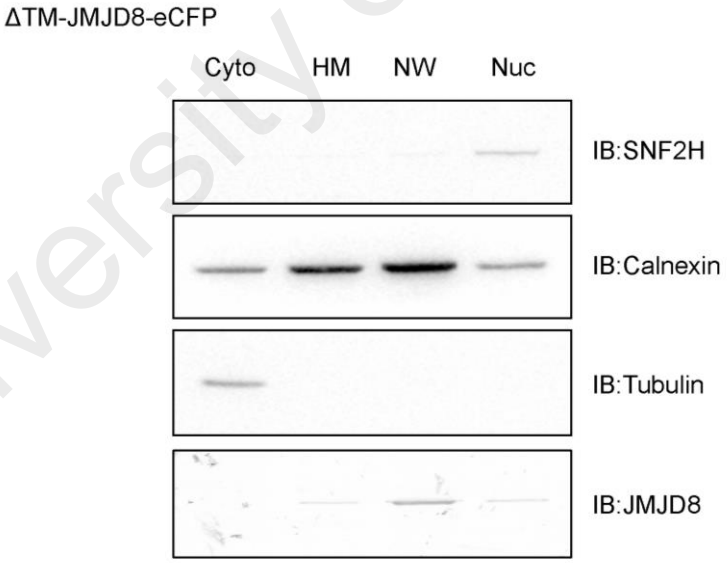


Figure 4.17: Subcellular fractionation of ΔTM-JMJD8-eCFP mutants.
Cell lysates from ΔTM-JMJD8-eCFP expressing HEK293T stable cells were prepared and fractionated into cytoplasmic, heavy membrane (HM-rich in lysosomes, ER, and mitochondria), nuclear wash (Nw-rich in ER) and nuclear fractions. The organelle specific proteins and JMJD8 were analyzed by immunoblotting with the indicated antibodies (Nuclear with SNF2h, ER with calnexin and cytoplasmic with tubulin). (n = 3). The "n" represents the number of repeats.

To further determine if JMJD8 exist as an ER membrane or lumen protein, a limited permeabilization and protease protection assay was employed to determine the orientation of JMJD8 in the ER. ER integrity and protease efficiency were monitored by immunoblotting with ER membrane and lumen proteins. Interestingly, similar to luminal ER protein (PDIA3), JMJD8-FLAG-HA is resistant to proteinase K treatment in the protease protection assay compared to ER membrane proteins (Kinectin and MTDH), suggesting that it is a luminal ER protein (Figure 4.18a). In addition, we also identified three potential N-glycosylation sites located at asparagine residues 151, 161 and 230 using GlycoMine (Li et al., 2015) (Table 4.1) and shown that JMJD8 is sensitive to endoglycosidase H (endoH) digestion which suggest that JMJD8 is N-glycosylated (Figure 4.18b). Together, these results demonstrate that JMJD8 is a luminal ER protein and the signal peptide is required for its ER localization.

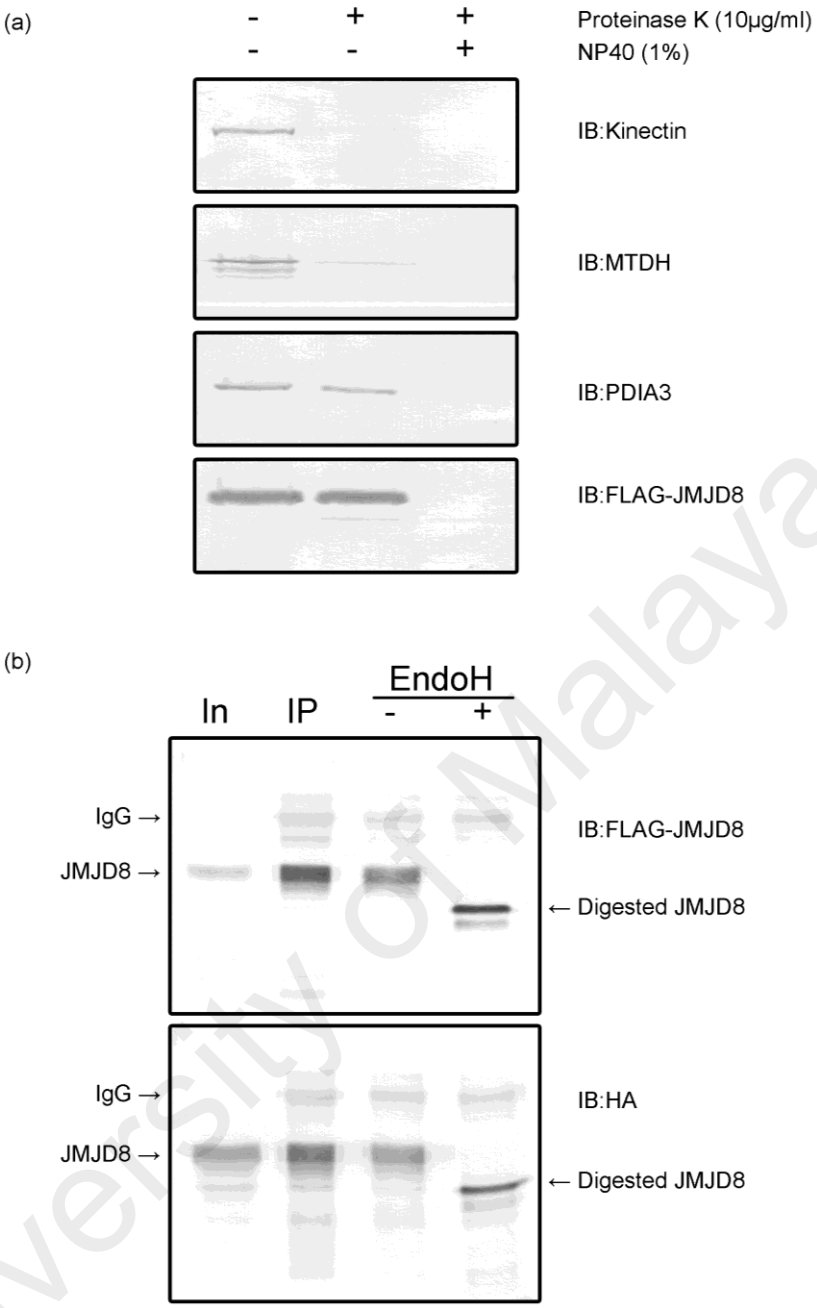


Figure 4.18: Protease protection assay and EndoH sensitivity assay.
(a) HEK293T cells stably expressing JMJD8-FLAG-HA were permeabilized with 50 μg/ml Digitonin for 5 minutes and treated with or without 10 μg/ml of proteinase K. Cells were solubilized with 1% of NP40 as a positive control for proteinase K digestion. ER membrane proteins and JMJD8 were analyzed by immunoblotting with the indicated antibodies (Kinectin and MTDH are ER membrane proteins, while PDIA3 is a luminal ER protein). (n = 2). (b) Immunoprecipitated JMJD8-FLAG-HA was subjected to EndoH digestion at 37°C for 1 hour. JMJD8 were analyzed by immunoblotting with the indicated antibodies. (n = 2). The "n" represents the number of repeats.

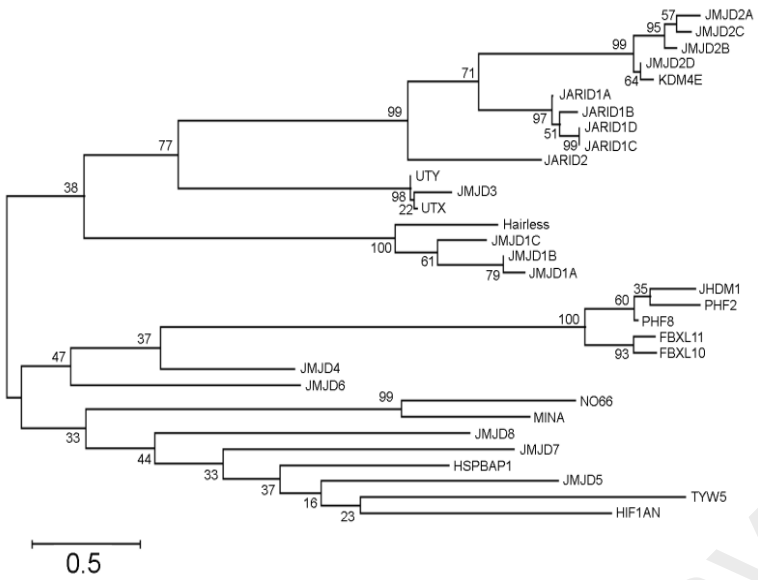
Table 4.1: N-glycosylation prediction with GlycoMine for JMJD8.

Protein	Position	Adjacent residues	Probability
JMJD8	151	QDPTSLGNDTLYFFG	0.823
JMJD8	161	LYFFGDNNFTEWASL	0.823
JMJD8	230	KTPEFHPNKTTLAWL	0.823
JMJD8	122	VVRLSTANTYSYHKV	0.086
JMJD8	160	TLYFFGDNNFTEWAS	0.074
JMJD8	273	RWWHATLNLDTSVFI	0.043
JMJD8	95	ILQGLTDNSRFRALC	0.018

4.7 Amino acid sequence comparison between JMJD8 and other JmjC domain-containing proteins.

JMJD8, like JMJD6, is unique that it contains only a JmjC domain without any other functional domain. To determine if JMJD8 clusters specifically with a particular group of JmjC domain-containing proteins, a phylogenetic analysis was employed to compare JMJD8 with 31 JmjC domain-containing proteins retrieved from UniProtKB. Interestingly, JMJD8 segregates into a cluster containing JMJD7, HSPBAP1, JMJD5, TYW5 and HIF1AN (Figure 4.19a). Moreover, sequence alignment of JmjC domains showed that JMJD8 has a histidine (H), which is conserved among JMJD8 from different species, instead of an aspartic acid (D) at its Fe (II) binding site (Figure 4.19b). These results suggest that JMJD8 is distinct from typical JmjC domain-containing histone demethylases.

(a)



(b)

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SP|Q9NWT6|HIF1AN_HUMAN   GMEGNVTPAHYDEQ-QNFFAQIKGYKRCILFPPDQFECLYPYPVH------HPC-D---- 96
SP|Q8N371|JMJD5_HUMAN    GPQGTISPLHQDPQ-QNFLVQVMGRKYIRLYSPQESGALYPHDTH------LLH------ 88
SP|A2RUC4|TYW5_HUMAN     SSPGLQLWTHYDVM-DNLLIQVTGKKRVVLFSPRDAQYLYLKGT---------------- 92
SP|Q96EW2|HSPBAP1_HUMAN  GSLGAHTPCHLDSYGCNLVFQVQGRKRWHLFPPEDTPFLYPTRIP------YEESS---- 92
SP|P0C870|JMJD7_HUMAN    GEAAAVTSLHKDHY-ENLYCVVSGEKHFLFHPPSDRPFIP-YELYTPATYQLTEEGTFKV 99
SP|Q96S16|JMJD8_HUMAN    AGAGSGVPFHWH--GPGYSEVIYGRKRWFLYPPEKTPEFHPNKTT------LAW------ 85
SP|Q3TA59|JMJD8_MOUSE    AGAGSGVPFHWH--GPGFSEVIYGRKRWFLYPPEKTPEFHPNKTT------LAW------ 85
SP|Q6AY40|JMJD8_RAT      AGAGSGVPFHWH--GPGFSEVIYGRKRWFLYPPEKTPEFHPNKTT------LAW------ 74

SP|Q9NWT6|HIF1AN_HUMAN   ---------RQSQVDFDNPDYERFPNF-QNVVGYETVVGPGDVLYIPMYWWHHIESLLNG 146
SP|Q8N371|JMJD5_HUMAN    ---------NTSQVDVENPDLEKFPKF-AKAPFLSCILSPGEILFIPVKYWHYVRALDLS 138
SP|A2RUC4|TYW5_HUMAN     ---------KSEVLNIDNPDLAKYPLF-SKARRYECSLEAGDVLFIPALWFHNVISEEFG 142
SP|Q96EW2|HSPBAP1_HUMAN  ---------VFSKINVVNPDLKRFPQF-RKAQRHAVTLSPGQVLFVPRHWWHYVESIDPV 142
SP|P0C870|JMJD7_HUMAN    VDEEAMEKVPWIPLDPLAPDLARYPSY-SQAQALRCTVRAGEMLYLPALWFHHVQQSQGC 158
SP|Q96S16|JMJD8_HUMAN    -------------------LRDTYPALPPSARPLECTIRAGEVLYFPDRWWHATLNLDTS 126
SP|Q3TA59|JMJD8_MOUSE    -------------------LLEIYPSLALSARPLECTIQAGEVLYFPDRWWHATLNLDTS 126
SP|Q6AY40|JMJD8_RAT      -------------------LLEIYPSLAPSARPLECTIQAGEALYFPDRWWHATLNLDTS 115
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Figure 4.19: Phylogenetic analysis of JmjC domain-containing proteins.
(a) Thirty-two sequences of JmjC domain-containing protein were retrieved from UniProtKB and a phylogenetic tree was generated using the Maximum Likelihood method. (b) Multiple sequence alignment of JmjC domains of HIF1AN, JMJD5, TYW5, HSPBAP1, JMJD7 and JMJD8 (human, mouse and rat). Fe (II) binding site was highlighted in red.

4.8 Signal peptide of JMJD8 is essential for dimerization or oligomerization.

To examine whether JMJD8 forms oligomer, a co-immunoprecipitation assay was performed. FLAG-HA-tagged and eCFP-tagged JMJD8 were co-expressed in HEK293T cells and was immunoprecipitated JMJD8 with a HA-specific antibody followed by immunoblotting using a JMJD8-specific antibody. FLAG-HA-tagged JMJD8 interacted

with eCFP-tagged wild type and ΔTM-JMJD8, but not with eCFP-tagged Δ45-JMJD8 (Figure 4.20a). To further confirm the dimerization or oligomerization of JMJD8 *in vivo*, a gel-filtration chromatography (Superdex 200) analysis was performed from a cell lysate prepared from HeLa S3 cells stably expressing a JMJD8-FLAG-HA (HeLa-JMJD8-FLAG-HA). Consistently, the majority of JMJD8 eluted from the gel filtration column in fractions presented as monomers and dimers in the cells, which corresponded to a molecular weight of 48 and 76 kDa, respectively (Figure 4.20b). Moreover, a small population of JMJD8 eluted as high molecular weight species between 118-686 kDa (the estimated molecular weight for fraction 9-13). These results suggest that the signal peptide of JMJD8 is required for its dimerization or oligomerization.

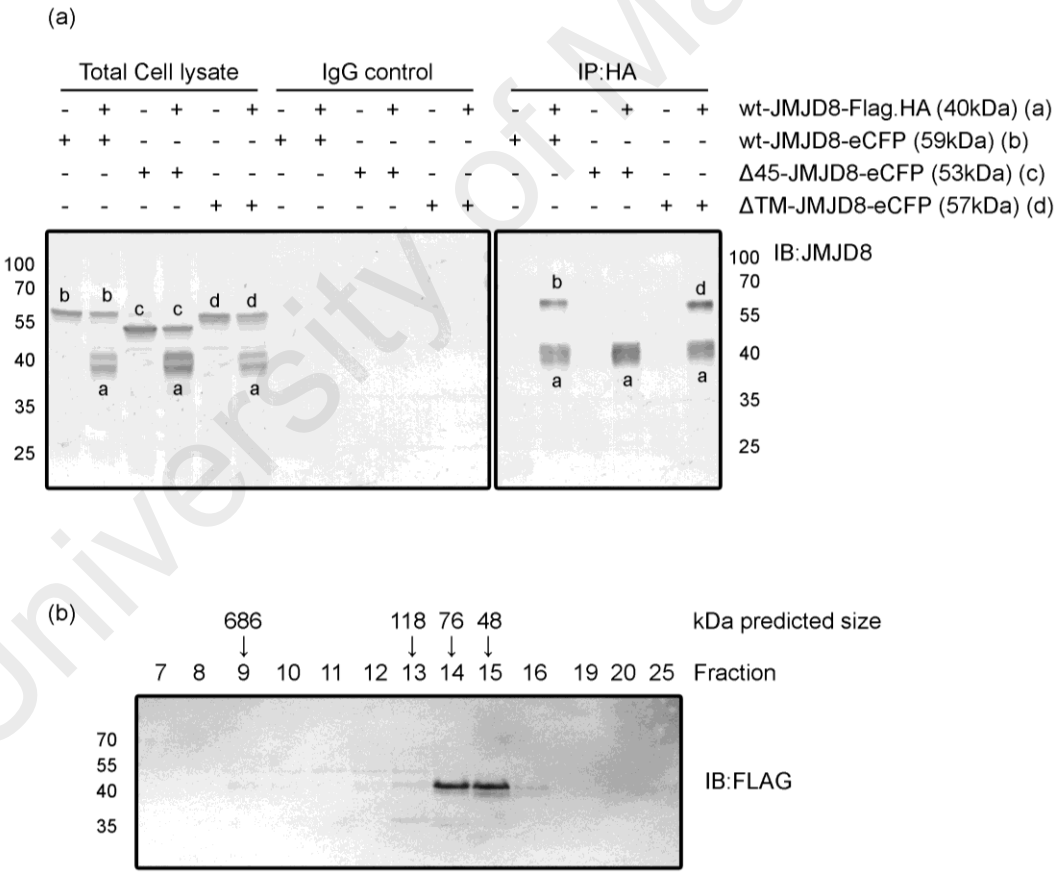

Figure 4.20: JMJD8 forms dimers or oligomers.
(a) HEK293T cells were transfected with plasmids that express the indicated proteins. The interaction between wild-type and mutant JMJD8 was determined with an immunoprecipitation assay using an anti-HA antibody or mouse IgG. Total cell lysates and immunoprecipitated products were immunoblotted with the indicated antibodies (n = 3). (b) Total cell lysate from HeLa S3 cells stably expressing a JMJD8-FLAG-HA (HeLa-JMJD8-FLAG-HA) was subjected to gel filtration (Superdex 200) analysis. The fractions were collected and immunoblotted with anti-FLAG antibody. The "n" represents the number of repeats.

4.9 JMJD8 may be involved in protein complex assembly and protein folding.

In previous observation, JMJD8 functions as a positive regulator of TNF-induced NF-κB pathway was presented. To better understand the biological functions of JMJD8, the interacting partners of JMJD8 were examined. An unbiased mass spectrometry analysis was employed to identify the proteins that bound to JMJD8. According to subcellular fractionation assay showed that endogenous JMJD8 is enriched in the Nw fraction (Figure 4.16). Therefore, HEK293T-JMJD8-FLAG-HA cells were subcellular fractionated to obtain Nw fraction for immunoprecipitation to minimize undesired background protein and to target only ER-localized JMJD8. A list of JMJD8 potential interacting partners was obtained. By applying an established stringent filtering protocol as previously described by Kriegsheim's group (Turriziani et al., 2014), thirty-five statistically significant targets that interact with JMJD8 in the cells were identified (Figure 4.21a and Appendix J). To determine if any biological processes are enriched among the 35 identified targets, all the 35 targets was further examined with PANTHER, a gene ontology analysis software (Mi et al., 2016). Interestingly, the majority of the JMJD8 interaction partners were grouped under metabolic and cellular processes which are in line with the previous study (Boeckel et al., 2016) (Figure 4.21 and Appendix K). According to PANTHER, among the 35 proteins, 19 proteins are involved in metabolic processes (GO: 0008152) that can be further assigned to more detailed processes, including lipid metabolic process (GO: 0006629) (5%), cellular amino acid metabolic process (GO: 0006520) (15%), protein metabolic process (GO: 0019538) (75%) and carbohydrate metabolic process (GO: 0005975) (5%). Fifteen of them are classified into protein metabolic processes such as proteolysis (GO: 0006508) (9.1%), translation (GO: 0006412) (9.1%), protein complex assembly (GO: 0006461) (31.8%), protein folding (GO: 0006457) (45.5%) and cellular protein modification process (GO: 0006464) (4.5%). Majority of the target proteins clustered into protein complex assembly (7 targets) and

protein folding (10 targets) (Figure 4.21b-e and Appendix K). These results demonstrate that JMJD8 may form complexes that regulate protein complex assembly and protein folding.

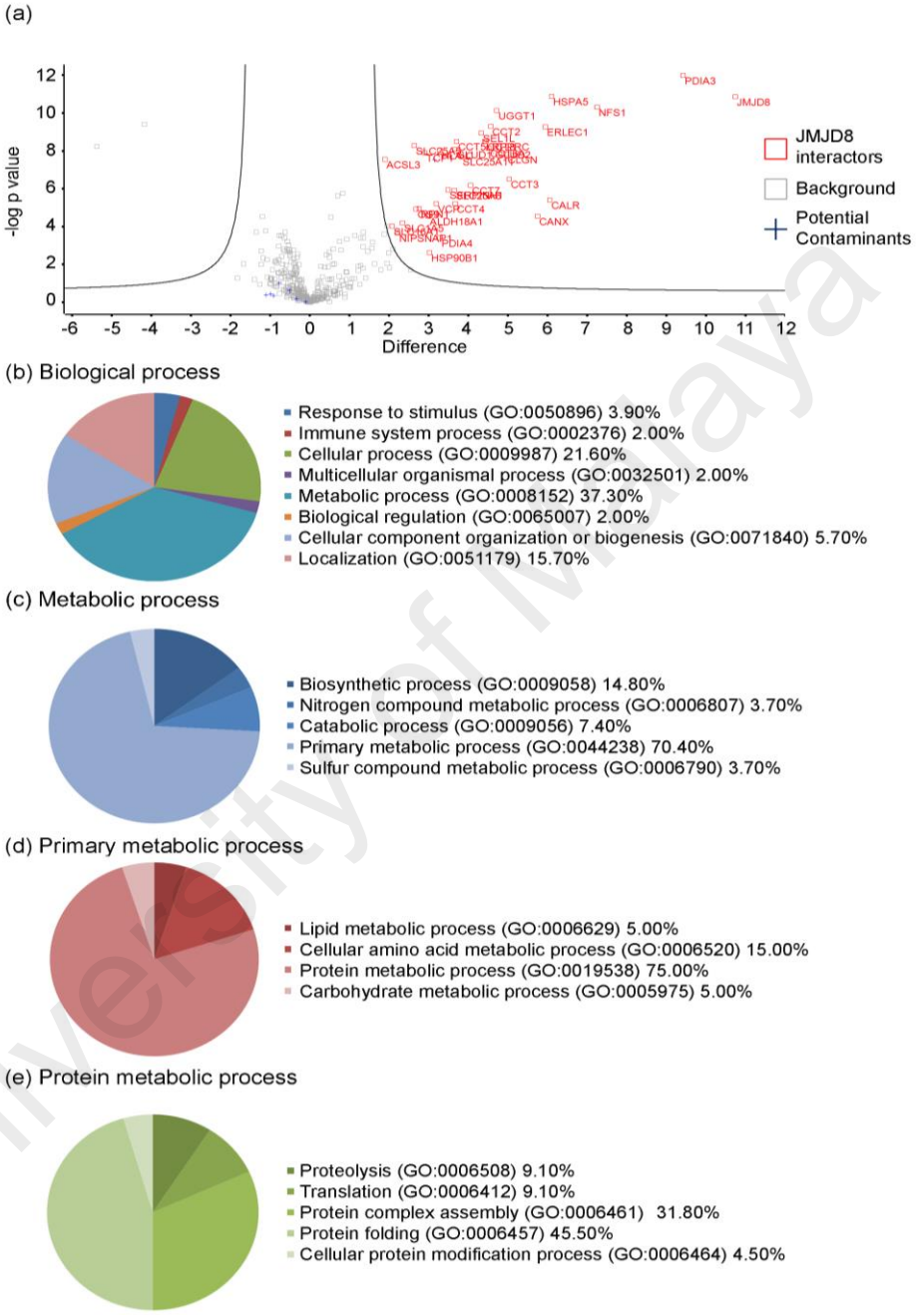

Figure 4.21: Interaction partners of JMJD8.
Nuclear wash (Nw) fractions generated from HEK293T-JMJD8-FLAG-HA cells were subjected to immunoprecipitation using FLAG-agarose beads. The beads were digested and subjected to mass spectrometry analysis. (n=6) (a) Volcano plot represented all identified targets. Statistically significant targets with a FDR<0.01 and a fold change greater than 1.5 folds were shown in red. (b) Potential JMJD8 targets were subjected to gene ontology analysis and the pie chart was generated from the percent of gene hit against a total number of genes in process hits. Distribution of identified proteins was further divided into a subset of biological processes such as (c) metabolic processes, (d) primary metabolic processes and (e) protein metabolic processes. The "n" represents the number of repeats.

CHAPTER 5: DISCUSSION

5.1 JMJD8 is a positive regulator of TNF-induced NF-κB signaling.

To date, this is the first report that demonstrates a functional role of JMJD8 in TNF-induced NF-κB signaling. Knockdown of JMJD8 expression in HEK293T cells results in reduced TNF-induced NF-κB-dependent genes expressions, IκBα degradation and p65 nuclear translocation. The upstream RIP1 ubiquitination, phosphorylation of IKK, IKK kinase activity, and MAP kinase activation are also suppressed with the depletion of JMJD8 expression. Furthermore, TNF-induced apoptosis is enhanced in the knockdown of JMJD8.

IκBα degradation is regulated by the phosphorylation of its two serine residues by IKK kinase (Brown et al., 1995; Chen et al., 1995a; Scherer et al., 1995). Results from current study suggest that less IKK kinases are activated in JMJD8-deficient cells. Furthermore, in parallel with *in vitro* kinase assay, less phosphorylation of IKK was observed in JMJD8-silenced cells (Figure 4.8). Consequently, less phosphorylation of IκBα leads to less IκBα degradation (Figure 4.6). In addition, TNF-induced MAPK pathways are defective in JMJD8-deficient cells (Figure 4.7). Moreover, TNF induced less ubiquitination of RIP1 in the knockdown of JMJD8. Co-immunoprecipitation assay suggests that RIP1 does not interact with JMJD8. Thus, the defect in NF-κB activation in JMJD8 knockdown cells may lie upstream or at the level of RIP1 ubiquitination. Interestingly, depletion of JMJD8 also greatly suppressed Sendai virus-induced *IFNβ* expression which suggests that JMJD8 may be essential for the type I interferon pathway as well. On the contrary, overexpression of JMJD8 however did not lead to enhanced NF-κB activation in 293T-luc cells.

Several studies have previously reported the involvement of different methylation of p65 subunit in NF-κB signaling pathway (Carr et al., 2015; Chang et al., 2011; Ea & Baltimore, 2009; Harris et al., 2014; Levy et al., 2011; Lu et al., 2013; Perkins, 2006;

Yang et al., 2009, 2010). However, reduced IKK kinase activity and p-IKK results (Figure 4.6 and Figure 4.8, respectively) in JMJD8 knockdown cells would argue against the possibility of p65 subunit, which is downstream of IKK, being the regulatory target of JMJD8. On the other hand, TRAF proteins for example TRAF2, 5 and 6 are adaptors for activation of various NF-κB signaling pathways. These proteins have been implicated to act as essential intermediates during TNFR complex formation in TNF-induced signaling as well as during virus infection-dependent NF-κB activation (Gil et al., 2004; Liu et al., 2013). Furthermore, JMJD6 which is structurally highly similar to JMJD8, was reported to demethylate TRAF6 in response to Toll-like receptor ligands (Tikhanovich et al., 2015). Therefore, it is possible that JMJD8 may regulate TRAF proteins similar to JMJD6 in TNF-induced signaling and antiviral response in the cytoplasm. In this study, the results show that JMJD8 depletion promotes TNF-induced apoptosis (Figure 4.12). In general, TNFR1 is able to form two distinct complexes that lead to different cell fates. While TNFR1 complex I is important for pro-survival, TNFR1 complex II is required for pro-apoptotic function (Micheau & Tschopp, 2003). Based on these results, the shift in the activation of TNFR1 complex I to II that eventually leads to enhanced apoptosis in JMJD8-deficient cells may be due to the failure or inability of TNFR1 complex I to form appropriately. This is further supported by the reduction of RIP1 ubiquitination in JMJD8-silenced cells (Figure 4.9) which is consistent with previous studies that RIP1 ubiquitination is essential for TNF-induced NF-κB signaling (Kanayama et al., 2004; Lee et al., 2004; Legler et al., 2003). In contrast, non-ubiquitinated RIP1 would serve as a pro-apoptotic signaling molecule that facilitates the formation of complex II (O'Donnell et al., 2007). As a result, the incomplete activation of the pro-survival pathway may lead to the promotion of pro-apoptotic pathway. Together, these results imply that JMJD8 may be required for TNFR1 complex I formation.

5.2 JMJD8 is a novel luminal endoplasmic reticulum protein with a JmjC domain.

This is also the first report to demonstrate that JMJD8 is a luminal ER resided JmjC domain-only protein (Figure 4.13c and Figure 4.18a). EndoH digestion assay further strengthen the observation that JMJD8 is a luminal ER protein as N-glycosylation happened only in the lumen of ER (Figure 4.18b). The JMJD8 was showed to contain an N-terminal signal peptide that is required for its ER localization (Figure 4.13). Intriguingly, the signal peptide of JMJD8 is still intact in the mature protein based on the observed protein size after endoH treatment that matches the predicted molecular weight of JMJD8-FLAG-HA (39.9 kDa) (Figure 4.18). There are examples of uncleaved signal peptide found on other proteins, such as CD18 and prion protein (Shanthalingam & Srikumaran, 2009; Stewart et al., 2001). The uncleaved signal peptide of the prion protein tethers the protein to ER membrane within the lumen. Thus, it is possible that the uncleaved signal peptide of JMJD8 anchors JMJD8 to ER membrane with the rest of JMJD8 resides in the lumen of ER. Consistently, a recent study by Boeckel et al. (2016) showed that JMJD8 localizes to the extranuclear region instead of the nucleus. Interestingly, ubiquitinated NF-κB signaling components which are required for optimal activation of NF-κB including RIP1, Bcl10 and TRAF2, have been shown to be recruited to ER (Alexia et al., 2013). Therefore, this finding that JMJD8 is a positive regulator of TNF signaling pathway as well as an ER protein further underscores the importance of ER as a signaling platform for NF-κB pathway. Further experiment is required to test whether the ER localization of JMJD8 is essential for its function. Sequence analysis of JMJD8 also predicted that JMJD8 contains a TM domain. However, deletion of TM domain does not affect the ER localization of JMJD8. It is possible that the prediction was inaccurate and JMJD8 is an ER lumen protein instead of ER membrane bound

protein. Alternatively, the ΔTM-JMJD8 may form dimer or oligomer with endogenous JMJD8 and thus retain its ER localization.

5.3 JMJD8 forms monomer and dimer.

The co-immunoprecipitation and gel filtration chromatography studies suggest that JMJD8 forms predominantly monomers and dimers, and a small population of JMJD8 forms oligomers or high molecular weight complexes with other proteins (Figure 4.20). Consistently, JMJD6 has also been shown to form homo-oligomer in cells (Hahn et al., 2010; Tibrewal et al., 2007). Moreover, the ER localization is required for dimerization or oligomerization of JMJD8 as Δ45-JMJD8, which localizes to cytoplasmic and nuclear compartments, fails to form dimers or oligomers.

5.4 JMJD8 interacts with the cellular protein folding and complex assembly machinery.

Through the application of high throughput mass spectrometry analysis of JMJD8 bound proteins, 35 statistically significant targets were identified that are involved in various biological processes (Figure 4.21). In line with the previous study by Boeckel et al. (2016), these results also suggest that JMJD8 interacting proteins are mainly involved in the metabolic process and cellular process (Figure 4.21b). In this regard, JMJD8 was shown to play a role in angiogenesis and angiogenesis involves metabolism (Boeckel et al., 2016; Eichmann & Simons, 2013; Fraisl et al., 2009). Furthermore, the proliferation of cancer cells is closely related to metabolism and knocking down of JMJD8 expression suppresses the proliferation of cancer cells (Schulze & Harris, 2012; Zhu et al., 2016).

Interestingly, ten target proteins (Appendix K) belong to the category of protein folding suggesting that JMJD8 may form complexes with these chaperonins (T-complex protein 1) (Dunn et al., 2001) and calcium-binding proteins (Calnexin, Calmegin and

Calreticulin) (Ellgaard & Helenius, 2003) to modulate protein folding. However, caution should be taken when dealing with overexpression of an ER protein since ER is a protein folding compartment and is sensitive to changes in protein homeostasis (Kaufman, 1999). Ectopic expression of JMJD8 may overload the ER and lead to misfolding of JMJD8. Misfolded JMJD8 may interact with the cellular protein folding machinery, which may give rise to false positive interacting proteins that are irrelevant to the physiological function of JMJD8. Nonetheless, together with the results from Boeckel et al. (2016) which showed that JMJD8 interact with phosphofructokinase 1, JAK1, CANX and PKM2 cytoplasmic proteins, these observations indicate that JMJD8 is an ER protein that may regulate protein outside of nucleus which is different from typical Jumonji domain-containing proteins that target the histone.

Consistent with the previous study from Boeckel and co-authors, HSPA5, calnexin, and SEL1L were detected as JMJD8 interacting partners. However, pyruvate kinase M2 (PKM2) was unable to detect in the mass spectrometry analysis (Boeckel et al., 2016). The difference in the identified targets may be attributed to the differences in the expression systems and cell lysates used in JMJD8 purification. In their study, JMJD8 was transiently expressed in HEK293 cells and total cell lysates were used for immunoprecipitation of JMJD8. On the contrary, this analysis focused on ER-bound JMJD8 because of the biochemical fractionation from HEK293T cell extracts showed that majority of endogenous JMJD8 is localized to ER that co-purified with the nuclear fraction (Figure 4.16). Among the 35 JMJD8 interacting proteins, 15 are ER proteins (lumen or membrane, see Appendix L), which are consistent with these findings that JMJD8 is a luminal ER protein.

5.5 JMJD8 may not be a hydroxylase or demethylase

Phylogenetic analysis suggests that JMJD8 belongs to a cluster containing JMJD7, HSPBAP1, JMJD5, TYW5 and HIF1AN (Figure 4.19), which implies that JMJD8 may function similarly to this group of proteins. However, this group of JmjC domain-containing proteins has very diverse biological functions. For instance, TYM5 is a hydroxylase that is responsible for the biosynthesis of $tRNA^{phe}$ (Noma et al., 2010) while HIF1AN is an asparaginyl hydroxylase that regulates the transcriptional activity of hypoxia-inducible factor (Lando et al., 2002) and modifies ankyrin repeats in IκB proteins (Cockman et al., 2006). On the other hand, JMJD5 is a H3K36me2 histone demethylase that regulates p53 and cell proliferation (Hsia et al., 2010; Huang et al., 2015; Ishimura et al., 2012) whereas the biological function of JMJD7 and HSPBAP1 remains to be identified.

There is some evidence showing that Jumonji domain-containing proteins can function without their demethylase enzymatic activity in the cells. For example, JMJD3 was found to act as an adaptor for PHF20 to recruit Trim26, an E3 ligase for K48-linked polyubiquitination and mediates PHF20 proteasomal degradation (Zhao et al., 2013). Moreover, a typical JmjC domain contains three conserved residues (H, E/D, H) that interact with Fe (II) and α-ketoglutarate (Klose et al., 2006). Binding of these cofactors is important for the hydroxylase or demethylase activities of JmjC domain-containing proteins. In this regard, JMJD8 may not be a hydroxylase or demethylase since the conserved aspartic acid or glutamic acid residue is substituted with a histidine residue in JMJD8, which may affect its binding to the cofactors.

5.6 Limitations and future directions

Despite showing that JMJD8 is a positive regulator of TNF pathway, several questions remain to be answered: 1) the exact mechanism on how JMJD8, which is an ER protein, regulates RIP1 polyubiquitination remains to be elucidated. The fact that RIP1 is not interacting with JMJD8 (Figure 4.10), suggests that JMJD8 may regulate RIP1 ubiquitination indirectly. Previous study by Alexia et al. (2013) shows that ER-anchored protein metadherin (MTDH) is responsible for the accumulation of ubiquitinated NF-κB signaling components on the cytosolic leaflet of ER membrane. However, it is unable to detect the interaction between JMJD8 and MTDH in the mass spectrometry results. In the future, mass spectrometry analysis of TNF-induced HEK293T-JMJD8-FLAG-HA cells Nw fraction should be carried out in order to determine whether JMJD8 interacts with any proteins that regulate RIP1 ubiquitination. On the other hand, all secretory and membrane protein like TNFR1 are co-translationally imported into ER for post-translational modification and proper folding (Araki & Nagata, 2012). Thus, JMJD8, as a luminal ER protein, may somehow involve in regulating these processes for proper TNFR1 post-translational modifications and folding in ER and in turn regulating TNFR1 activity, although the TNFR1 protein level at the cell surface did not change (Figure 4.11). However, further study will be required to verify this speculation. 2) Does JMJD8 act as a transcriptional repressor of the negative regulator of NF-κB, such as CYLD and A20 (Draber et al., 2015; Kovalenko & Chable-bessia, 2003; Wertz et al., 2004, 2015). According to Khoueiry et al. (2017), JMJD8 translocates into the nucleus and acts as a transcriptional repressor when the truncated JMJD8 without its signal peptide (27-271 aa) is overexpressed (Khoueiry et al., 2017). Although the full-length JMJD8 localizes to ER (IFA and subcellular fractionation assay) whereas only Δ45-JMJD8 localizes to cytoplasmic and nuclear compartments (Figure 4.14 and 4.15), this study still cannot rule out that an undetectable amount of full-length JMJD8 might be present in the nucleus,

thus the role of JMJD8 as a transcriptional repressor needs further validation. Immunofluorescence assay to visualize the localization of endogenous JMJD8 protein will be important. Unfortunately, currently commercially available antibodies against JMJD8 are not suitable for immunofluorescence assay. 3) Does JMJD8 possess any demethylase enzymatic activity? Since JMJD8 possesses a C-terminal JmjC domain, it is possible that JMJD8 may act as a demethylase that regulating proteins function via lysine or arginine demethylation. However, there is no direct evidence from this study showing that it is indeed a demethylase. Interestingly, in a preliminary test done to detect the possible metals that would incorporate into the β-barrel like structure of JmjC domain of JMJD8 through microscale thermophoresis technology, nickel (Ni^{2+}) and cobalt (Co^{2+}) were detected to interact with recombinant JMJD8 but not iron (Fe^{2+}). However, this observation will need further verification. It will be a novel finding if JMJD8 acquires Ni^{2+} or Co^{2+} instead of Fe^{2+} as cofactor compared to the typical JmjC domain-containing protein, which further strengthens that JMJD8 may function differently compared to typical JmjC domain-containing protein. 4) Last but not least, it will be useful to generate a knockout system to evaluate and further strengthen the observed phenomenon in this study. Since JMJD8 is not an essential gene for development (Blomen et al., 2015; Wang et al., 2015), thus it will be possible to generate a JMJD8 knockout cell, mouse or zebrafish system to examine the functional roles of JMJD8.

CHAPTER 6: CONCLUSION

In conclusion, JMJD8 acts as a positive regulator of TNF-induced NF-κB signaling that regulates RIP1 polyubiquitination. However, the precise mechanism of action and target of JMJD8 remains unknown. Further studies will be required to pinpoint the exact target of JMJD8 to fully elucidate its role in TNF-induced NF-κB signaling. Besides, these findings reveal that JMJD8 is a JmjC domain-only protein that localized to the lumen of endoplasmic reticulum whereby the signal peptide is important for its ER localization and dimerization or oligomerization. In addition, JMJD8 may form protein complexes that are involved in protein folding. Taken together, these findings indicate that JMJD8 represents the first JmjC domain-containing protein found in the lumen of endoplasmic reticulum that may function in protein complex assembly, protein folding and positive regulation of TNF-induced NF-κB activation.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

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1. **Yeo, K. S.**, Tan, M. C., Wong, W. Y., Loh, S. W., Lam, Y. L., Tan, C. L., Lim, Y.-Y., & Ea, C.-K. (2016). JMJD8 is a positive regulator of TNF-induced NF-κB signaling. *Scientific Reports*, *6*, 34125.

2. **Yeo, K. S.**, Tan, M. C., Lim, Y.-Y., & Ea, C.-K. (2017). JMJD8 is a novel endoplasmic reticulum protein with a JmjC domain. *Scientific Reports*, *7*(1), 15407.

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1. **Yeo, K.S.**, Tan, M.C., Tan, C. L., Wong, W.Y., Loh, S. W., Lim, Y.Y., Ea, C.K. "JMJD8 is a positive regulator of TNFα-induced NF-κB signaling." 20th Biological Sciences Graduate Congress. 9-11th December 2015, Bangkok, Thailand. (International)