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Understanding Ten-Eleven Translocation-2 in Hematological and Nervous Systems

Feng Pan
fpan001@fiu.edu

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

UNDERSTANDING TEN-ELEVEN TRANSLOCATION-2 IN HEMATOLOGICAL
AND NERVOUS SYSTEMS

A dissertation submitted in partial fulfillment of
the requirements for the degree of
DOCTOR OF PHILOSOPHY

in

BIOLOGY

by

Feng Pan

2015

To: Dean Michael R. Heithaus
College of Arts and Sciences

This dissertation, written by Feng Pan, and entitled Understanding Ten-Eleven Translocation-2 in Hematological and Nervous Systems, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

Mingjiang Xu

John Makemson

Dietrich Lorke

Lidia Kos, Co-Major Professor

Ophelia Weeks, Co-Major Professor

Date of Defense: December 3, 2014

The dissertation of Feng Pan is approved.

Dean Michael R. Heithaus
College of Arts and Sciences

Dean Lakshmi N. Reddi
University Graduate School

Florida International University, 2015

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DEDICATION

This dissertation is lovingly dedicated to my parents, for their heartfelt support and encouragement throughout my life.

This dissertation is also dedicated to my beloved brilliant wife, Sophie Che and our little angel Ada Pan.

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First and foremost, I would like to thank my major professor Dr. Ophelia I. Weeks, for helping me receive the Ph.D. and for helping to make my academic dream come true. Dr. Mingjiang Xu for providing me such a wonderful opportunity to work as a research assistant in his lab. I would also like to thank the following people who helped me to develop and execute my projects Drs. Feng-Chun Yang, Peng Jin, Xuekun Li. Next, I would like to thank all my committee members: Drs. Lidia Kos, John Makemson and Dietrich Lorke, for all their professional help and support throughout my Ph.D. years. I appreciate all the past and current lab members of Dr. Weeks' and Dr. Xu's labs. Without your patience, kindness and understanding, I won't have grown and matured to accomplish my aims in a timely manner.

ABSTRACT OF THE DISSERTATION
UNDERSTANDING TEN-ELEVEN TRANSLOCATION-2 IN HEMATOLOGICAL
AND NERVOUS SYSTEMS

by

Feng Pan

Florida International University, 2015

Miami, Florida

Professor Ophelia Weeks, Co-Major Professor

Associate Professor Lidia Kos, Co-Major Professor

I proposed the study of two distinct aspects of Ten-Eleven Translocation 2 (TET2) protein for understanding specific functions in different body systems.

In Part I, I characterized the molecular mechanisms of Tet2 in the hematological system. As the second member of Ten-Eleven Translocation protein family, TET2 is frequently mutated in leukemic patients. Previous studies have shown that the TET2 mutations frequently occur in 20% myelodysplastic syndrome/myeloproliferative neoplasm (MDS/MPN), 10% T-cell lymphoma leukemia and 2% B-cell lymphoma leukemia. Genetic mouse models also display distinct phenotypes of various types of hematological malignancies. I performed 5-hydroxymethylcytosine (5hmC) chromatin immunoprecipitation sequencing (ChIP-Seq) and RNA sequencing (RNA-Seq) of hematopoietic stem/progenitor cells to determine whether the deletion of Tet2 can affect the abundance of 5hmC at myeloid, T-cell and B-cell specific gene transcription start sites, which ultimately result in various hematological malignancies. Subsequent Exome

sequencing (Exome-Seq) showed that disease-specific genes are mutated in different types of tumors, which suggests that TET2 may protect the genome from being mutated. The direct interaction between TET2 and Mutator S Homolog 6 (MSH6) protein suggests TET2 is involved in DNA mismatch repair. Finally, *in vivo* mismatch repair studies show that the loss of Tet2 causes a mutator phenotype. Taken together, my data indicate that TET2 binds to MSH6 to protect genome integrity.

In Part II, I intended to better understand the role of Tet2 in the nervous system. 5-hydroxymethylcytosine regulates epigenetic modification during neurodevelopment and aging. Thus, Tet2 may play a critical role in regulating adult neurogenesis. To examine the physiological significance of Tet2 in the nervous system, I first showed that the deletion of Tet2 reduces the 5hmC levels in neural stem cells. Mice lacking Tet2 show abnormal hippocampal neurogenesis along with 5hmC alternations at different gene promoters and corresponding gene expression downregulation. Through the luciferase reporter assay, two neural factors Neurogenic differentiation 1 (NeuroD1) and Glial fibrillary acidic protein (Gfap) were down-regulated in Tet2 knockout cells. My results suggest that Tet2 regulates neural stem/progenitor cell proliferation and differentiation in adult brain.

TABLE OF CONTENTS

CHAPTER	PAGE
INTRODUCTION	1
1.1 DNA METHYLATION AND DEMETHYLATION	2
1.2 5-HYDROXYMETHYLCYTOSINE AND TET FAMILY PROTEINS.....	7
1.3 TET2 GENE IN HEMATOPOIESIS AND HEMATOLOGICAL DISORDERS	12
1.4 TETs AND 5-HYDROXYMETHYLCYTOSINE IN NEUROGENESIS AND NEURODEVELOPMENT.....	17
 TET2 PROTECTS GENOMIC STABILITY THROUGH INTERACTING WITH MSH6.....	 37
2.1 ABSTRACT	38
2.2 INTRODUCTION	39
2.3 MATERIALS AND METHODS	42
2.3.1 Analyses of mice	42
2.3.2 Tumor transfer assay	43
2.3.3 Generation of FLAG-V5 Tag knock-in ES cells	43
2.3.4 Purification of TET2-associated proteins	45
2.3.5 Constructs	45
2.3.6 RNA interference.....	46
2.3.7 Real-time PCR.....	47
2.3.8 Western blot and co-immunoprecipitation assays.....	48
2.3.9 Exome sequencing and mutational confirmation	49
2.3.10 DNA Mismatch repair assays.....	50
2.3.11 Microsatellite instability and HPRT mutability analyses.....	50
2.3.12 Confocal Microscopy and immunofluorescence analysis	51
2.3.13 Bisulfite-Seq and TAB-Seq.....	52
2.3.14 Statistical analysis.....	53
2.4 RESULTS AND DISCUSSION	53
2.4.1 Tet2 ^{-/-} mice develop lymphoid malignancies.	53
2.4.2 Acquisition of mutations in Tet2 ^{-/-} tumors and increased mutational frequency in loci marked by Tet2-dependent 5hmC	56
2.4.3 TET2 interacts with MSH6.....	60
2.4.4 TET2 loss causes a mutator phenotype in cells.....	61
2.5 CONCLUSIONS.....	64
 TET2 REGULATES ADULT BRAIN NEUROGENESIS.....	 98
3.1 ABSTRACT	99
3.2 INTRODUCTION	100
3.3 MATERIALS AND METHODS	103
3.3.1 Generation of Tet2 ^{-/-} mice and LacZ staining.....	103
3.3.2 Adult NPCs isolation and culture	104
3.3.3 Proliferation and differentiation analyses of cultured adult NPCs.....	104

3.3.4 Real-time PCR.....	105
3.3.5 Confocal Microscopy and immunofluorescence analysis	105
3.3.6 Dot blot.....	105
3.3.7 DNA plasmids and luciferase assay	106
3.3.8 Statistical analysis.....	106
3.4 RESULTS AND DISCUSSION	107
3.4.1 TET2 is highly expressed in the central nervous system.....	107
3.4.2 Hydroxylation of 5-methylcytosine affects stem cell proliferation and differentiation in dentate gyrus of the hippocampus	108
3.4.3 TET2 regulates the expression of NeuroD and GFAP transcriptionally	109
3.5 CONCLUSIONS.....	110
CONCLUSIONS AND FUTURE DIRECTIONS.....	121
REFERENCES	126
VITA.....	144

LIST OF FIGURES

FIGURE	PAGE
Figure 1 DNA methylation and demethylation pathways.....	22
Figure 2 The dynamic methylome landscapes of different genomic elements.....	23
Figure 3 TETs are the homolog of JBP.....	24
Figure 4 Quantification of x in Purkinje cells and granule cells.....	25
Figure 5 The protein structures of TET family.....	26
Figure 6 Assays for mapping 5mC and 5hmC at single-base resolution.....	27
Figure 7 TET2 mutations detected in individuals with different tumors.....	28
Figure 8 Histogram of TET2 mutations.....	29
Figure 9 Circos plots demonstrating 12 coexisting genes in a cohort of patients with myeloid malignancies.	31
Figure 10 Tet2 ^{-/-} mice develop lethal myeloid malignancies.....	33
Figure 11 TET2 background mutations cause various hematological malignancies.....	34
Figure 12 Adult neurogenesis.....	35
Figure 13 Transcriptional epigenetic regulators in adult neurogenesis.....	36
Figure 14 Tet2 ^{-/-} mice develop lethal lymphoid malignancies involving both T- and B-cell lineages.....	68
Figure 15 Tet2 ^{-/-} mice with T-cell malignancy.....	69
Figure 16 Tet2 ^{-/-} mice with B-cell malignancy.....	71
Figure 17 Lymphoid malignancies in Tet2 ^{-/-} mice are transplantable to secondary recipients.....	73
Figure 18 Array-based CGH ideograms of Tet2 ^{-/-} tumors.....	74
Figure 19 Acquisition of mutations in Tet2 ^{-/-} tumors.....	75

Figure 20 Notch1 mutations were identified from Tet2 ^{-/-} mice with T-cell malignancy..	76
Figure 21 5hmC distribution and enriched regions with genomic features.	77
Figure 22 Changes of 5hmC among the genes with different expression levels.	78
Figure 23 Increased mutational frequency in loci marked by Tet2-dependent 5hmC.....	79
Figure 24 Validated mutational signatures found in Tet2 ^{-/-} tumors.....	80
Figure 25 Dynamic changes of 5mC and 5hmC surrounding the mutation sites.	81
Figure 26 Generation of Flag-V5 tag knock-in ES cells.....	82
Figure 27 Flag-TET2 is expressed as a 240kD protein and localized in the nucleus.	84
Figure 28 TET2 associates with MSH6.	85
Figure 29 MSH6 co-immunoprecipitates with TET2 N-terminal.....	86
Figure 30 Immunostaining identified TET2 and MSH6 co-localization.	87
Figure 31 TET2 and MSH6 co-localization at different cell cycle.....	88
Figure 32 Tet2 is not physically involved in MMR activity in vitro.	89
Figure 33 Knockdown of TET2 in HeLa cells using human TET2-specific shRNA.....	90
Figure 34 Increased mutational frequency in TET2 knockdown HeLa cells.	91
Figure 35 Increased mutational frequency in shTet2-NIH3T3 cells.	92
Figure 36 MSH6 foci formation and localization in control- and shTET2- HeLa cells ...	93
Figure 37 Analysis of PCR product patterns of microsatellite markers in bone marrow cells from WT and Tet2 ^{-/-} mice.....	94
Figure 38 Analysis of PCR product patterns of two microsatellite markers in control- and shTET2-HeLa cells.	95
Figure 39 Mutation numbers in MDS patients with or without TET2 mutations.....	96
Figure 40 Proposed model for loss of Tet2 induced hematological malignancies.	97
Figure 41 Immunostaining of 5hmC in two brain regions in mice.....	112

Figure 42 Tet1, Tet2 and Tet3 expression levels in P7 and 6-week old cerebellum.	113
Figure 43 Tet2 is expressed in the nervous system.....	114
Figure 44 Detection of Tet2 in WT and Tet2 ^{-/-} hippocampi.	115
Figure 45 Quantification of 5hmC in P7 and adult cerebellum.	116
Figure 46 Cultured adult neural progenitor cells expressed representative cell markers	117
Figure 47 Quantification of 5hmC in proliferating and differentiating cells from WT and Tet2 ^{-/-} adult NPCs.....	118
Figure 48 Loss of Tet2 leads to increased adult NPCs proliferation and decreased adult NPCs differentiation.	119
Figure 49 The NeuroD1 and Gfap promoter regions are altered by Tet2.....	120

LIST OF ABBREVIATIONS AND ACRONYMS

2-OG	2-oxoglutarate
5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
6-TG	6-thioguanine
aCGH	Array comparative genomic hybridization
AID	Activation-induced deaminase
AITL	Angioimmunoblastic T-cell lymphoma
AML	Acute myeloid leukemia
	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
APOBEC	
ASXL1	Additional sex combs like transcriptional regulator 1
BDNF	Brain-derived neurotrophic factor
BER	Base excision repair
BM	Bone marrow
CFU	Colony-forming unit
CGI	CpG islands
ChIP-Seq	Chromatin immunoprecipitation sequencing
CMML	Chronic myelomonocytic leukemia
CpG	Cytosine linked with a phosphodiester bond to guanine

DG	Dentate gyrus
DLD-1	DL Dexter, Dukes' type C, colorectal adenocarcinoma
DMEM	Dulbecco's Modified Eagle's Medium
DMR	Differentially methylated regions
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DSBH	Double stranded beta helix
EBF1	Early B-cell factor 1
ESC	Embryonic stem cell
EZH2	Enhancer Of Zeste Homolog 2
FBS	Fetal bovine serum
FGF	Fibroblast growth factors
FLT3	Fms-like tyrosine kinase 3
GCL	Granule cell layer
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
H&E	Hematoxylin and eosin
HEK293T	Human Embryonic Kidney 293 cells with Tantigen of SV40
HNPCC	Hereditary nonpolyposis colorectal cancer
HPC	Hematopoietic progenitor cell
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HSC	Hematopoietic stem cell

IDAX	Inhibition Of The Dvl And Axin Complex
JBP	J-binding-protein
KD	Knock-down
KI	Knock-in
LC-MS	Liquid chromatography–mass spectrometry
LK	Lineage negative, c-kit positive
LSK	Lineage negative, Sca1 positive, c-kit positive
MBD2	Methyl-CpG binding domain protein 2
MDS	Myelodysplastic syndromes
MMR	Mismatch repair
MPN	Myeloproliferative neoplasm
MS	Mass spectrometry
MSH2	MutS homolog 2
MSH6	MutS homolog 6
MSI	Microsatellite instability
NeuroD1	Neurogenic differentiation 1
NIH3T3	NIH Swiss mouse embryo; adherent fibroblastoid cells
NOTCH1	Neurogenic locus notch homolog protein 1
NPC	Neural progenitor cell
NSC	Neural stem cell
OGT	O-linked N-acetylglucosamine transferase
PB	Peripheral blood

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PGC	Primordial germ cells
PRC2	Polycomb Repressive Complex 2
qPCR	Quantitative PCR
RGs	Radial glial cells
RHOA	Ras homolog gene family, member A
RMS-OB	Rostral migratory stream, olfactory bulb
RNA	Ribonucleic acid
SGZ	Subgranular zone
shRNA	Short hairpin RNA
SNVs	Single nucleotide variants
SRSF2	Serine/arginine-rich splicing factor 2
SS-PBS	Serum, Triton PBS
SVZ	Subventricular zone
T-ALL	T-cell acute lymphoblastic leukemia
TAB-Seq	Tet-assisted bisulfite sequencing
TCGA	The Cancer Genome Atlas
TDG	Thymine-DNA glycosylase
TET	Ten eleven translocation
TSS	Transcription start site

TujI	Neuron-specific class III beta-tubulin
UTR	Untranslated region
VZ	Ventricular zone
WT	Wild type
β GT	β -glucosyltransferase

CHAPTER
INTRODUCTION

1.1 DNA METHYLATION AND DEMETHYLATION

Epigenetics, proposed by Waddington in 1942, is broadly defined and attributed to heritable cellular changes without altering the deoxyribonucleic acid (DNA) sequence^{1,2}. Molecular mechanisms of epigenetic regulation include: DNA methylation, histone modifications and noncoding regulatory ribonucleic acids (RNAs). DNA methylation on cytosine-phosphate-guanine (CpG) islands at promoter regions is considered as one stable epigenetic mark³ and such DNA alternation is sufficient to maintain genome imprinting and X-chromosome inactivation⁴. Deoxyribonucleic acid methylation also occurs in other genomic elements such as gene body, enhancer and untranslated regions (UTR). It may contribute to transcription elongation or gene activation/inactivation⁵. Histone modifications widely exist posttranslationally, and include lysine and arginine methylation, lysine acetylation, ubiquitination and sumoylation, as well as serine and threonine phosphorylation⁶. These modifications regulate gene expression and are responsible for the presence of enhancers or repressors^{6,7}. In mammalian cells, microRNAs regulate gene expression by posttranslational repression of target sequences. Since the expression of microRNAs is also affected by their promoter status, the transcriptional regulation by microRNAs is relatively mild⁸. In recent years, studies on hematological malignancies have revealed a subset of epigenetic regulators as cancer-relevant genes. The corresponding epigenetic alterations lead to changes in specific gene transcriptions, which finally result in abnormal hematopoietic stem cells (HSC) and/or hematopoietic progenitor cells (HPC) self-renewal, proliferation and differentiation.

Methylation of DNA normally indicates modification of cytosine. In mammals, the DNA methyltransferase (DNMT) family comprises five members: DNMT1, DNMT2,

DNMT3A, DNMT3B and DNMT3L. The enzyme DNA methyltransferase 1 (DNMT1) maintains cytosine hemimethylated during DNA replication⁹ (Figure 1). DNA methyltransferase 2 (DNMT2), also termed TRDMT1, is the smallest DNMT whose function is still poorly understood, but the specific protein structure of DNMT2 suggests the possibility of involvement in DNA recombination and DNA damage repair¹⁰. Deoxyribonucleic acid methyltransferase 3 has two family members: DNMT3A and DNMT3B which control de novo cytosine methylation of unmethylated DNA substrates¹¹ (Figure 1). However, their enzymatic activities can be negatively regulated by DNMT3L, which functions mainly during early embryogenesis^{12,13}.

The human genome contains around 28 million CpGs while the mouse genome has approximately 22 million CpGs. These CpGs reside in different genomic elements such as CpG islands (CGIs), gene bodies and enhancers. Only around 20% of CpGs are dynamic, most of the CpGs are relatively stable^{14,15}. Genome-wide methylation studies have revealed detailed architectural profiles for DNA methylation at gene bodies, CGIs, CGI shore, enhancers, canyons, repeats and large differentially methylated regions (DMR)¹⁶ (Figure 2). Cytosine-phosphate-guanine islands are defined as short and CpG-rich regions within the genome, and more than 70% of genes have CGIs¹⁷. When CGIs are unmethylated at the promoter region, that usually signifies transcription initiation in nucleosome-free style, in which the transcriptional levels are regulated by Histone H3 lysine4 tri-methylation and transcription factors association. Whereas, methylation at CGIs may signify long-term gene silencing, as occurs in imprinted genes^{18,19}.

Recently, genome-wide methylome studies have allowed scientists to examine DNA methylation status outside promoter CGIs in both stem and differentiated cells. It has

been reported that tissue- or cancer-specific differentially methylated regions within CGI shores are more dynamic than those regions within CGIs¹⁶. Thus, it is plausible to propose that the methylation status at CGI shores may be involved in epigenetic reprogramming and cancer²⁰. Actually, many genes in lymphoid- or myeloid-specific lineages show frequent methylation changes in CGI shores during cell differentiation²¹.

DNA methylation also occurs in intergenic regions, but little is known about its physiological function. As a critical genetic element, most enhancers reside in such regions. Studies suggest that cell-specific transcription is closely associated with enhancer methylation levels even if the relevant promoter stays unmethylated. Through binding to chromatin modulating factors, these enhancers are able to interact with distal promoters, which result in specific DNA methylation patterns in different cell types²². Genome-wide methylome studies also identify a bunch of differential methylated regions that are enriched at different cellular stages during hematopoiesis and these regions are demonstrated as transcription factor binding sites. Interestingly, in lymphoid stem/progenitor cells, myeloid transcription factor binding sites are hypomethylated. Whereas during lymphoid differentiation, myeloid-specific factors binding sites are hypermethylated²³.

In addition, large hypomethylated regions have been identified serendipitously, during the process of comparing 5mC abundance in differentiated fibroblasts and human embryonic stem cells²⁴. These large canyon regions usually span at 1Mb, and include certain oncogenes and cancer biomarker genes²⁵. The loss of DNA methylation in these regions leads to repressive histone mark acquisition and gene downregulation²⁶. Moreover, methylation canyons are found in regions with very low levels of methylation

in hematopoietic stem cells²⁷. The loss of DNMT3A causes DNA methylation alterations at the edge of canyons²⁸. The methylation changes in canyons may result in transcription alteration and genomic instability that contribute to the progression of cancer.

In addition to being in promoter regions, CGIs are also found within gene bodies¹⁶. Even though there is a small amount of evidence that gene body methylation correlates with gene transcription *repression*, the opposite, gene transcription *activation* has been proposed²⁹. Indeed, actively transcribed genes have been identified as hypermethylated in the gene bodies¹⁶. Several studies infer that gene body methylation regions are the enhancer sites or regulate tissue-specific gene transcription from promoters. Besides, DNA methylation at gene bodies may also facilitate the recruitment of CpG binding proteins and prevent CCCTC-binding factor mediated RNA polymerase II stalling and spliceosome assembly³⁰⁻³³. Obviously, gene body methylation and promoter methylation play distinct roles in transcription regulation. But, the underlying mechanism of gene body methylation still needs to be elucidated in the future¹⁶.

There are also specific genomic regions called repetitive elements with multiple copies in the genome. Methylation of repetitive elements may repress the expression of transposable elements in order to maintain genome integrity³⁴. Bisulfite sequencing from DNMT3B mutant patients exhibit significant changes of DNA methylation in inactive heterochromatic regions, satellite repeats and transposons. And, aberrant expression levels of key genes may result from hypomethylation of repetitive elements, even though ribosomal RNA repeats are free from global hypomethylation³⁵. As the most abundant genes in the genome, ribosomal DNA stays in tandem repetitive clusters. The hematopoietic progenitor cells from MDS patients harbor increased ribosomal DNA

(rDNA) methylation levels with decreased ribosomal RNA (rRNA) expression compared to healthy controls³⁵. When myeloid cells are treated with 5-Aza-2'-deoxycytidine, significant methylation decrease at rDNA promoter regions is observed along with increased rRNA levels³⁶.

It has been reported that about 80% of CpGs are methylated in mammalian genome, which suggest a relatively stable pattern of DNA methylation in the genome. However, DNA methylation can be erased passively or actively through different demethylation pathways. Passive demethylation initiates through the inactivation of DNMT1/3 (downregulation of gene expression, genetic mutations or inhibitors). The methylation of a newly synthesized DNA strand is blocked during DNA replication. Other studies have demonstrated that DNA methylation patterns can be further modified by active demethylation. Active demethylation is mediated by TET family enzymes, which catalyze the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) and further 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) in this pathway³⁷⁻³⁹ (Figure 1).

On the basis of DNA semiconservative replication mechanism, the relative amount of DNA methylation is half diluted when new DNA strands are synthesized. Thus, the methylation status at specific gene loci will disappear in daughter cells upon cell divisions. The above phenotype is the so-called “passive DNA demethylation”⁴⁰ (Figure 1). However, how “active DNA demethylation” initiates is still less understood. Yet, the identification of 5mC glycosylases in plants has thrown some light on the process, since there is a similar occurrence in mammalian cells during zygote fertilization^{40,41}. In primordial germ cells (PGCs), genome-wide erasure of DNA methylation occurs

naturally⁴². It is believed that active DNA demethylation preferentially happens at the specific loci in differentiated somatic cells⁴³. Studies on identification of DNA demethylases have greatly increased in recent years. Methyl-CpG binding domain protein 2 (MBD2) binds the promoter region enriched in CpG and functions as a mediator of methylation signals to activate gene transcription⁴⁴. Additional studies have shown that mammalian thymine DNA glycosylase (TDG) catalyzes the glycosylation of 5mC, even though no *in vivo* data have verified the exact role of TDG in DNA demethylation⁴⁵. But, several potential mechanisms have also been proposed to better explain DNA demethylation. These include deamination by activation-induced deaminases (AID), and apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like (APOBEC) protein deaminases or DNMTs. Briefly, generated T:G mismatch leads to the initiation of base excision repair (BER) and a series of enzymes facilitate the refill of unmodified mononucleotides⁴⁶. Indeed, several reports provide evidence to support BER mechanisms, such as the requirement of BER-related enzymes and low methylation levels in AID deficient PGCs^{47,48}. Even if AID is completely eliminated, demethylation of PGCs still occurs, suggesting the existence of other possible DNA demethylation mechanisms.

1.2 5-HYDROXYMETHYLCYTOSINE AND TET FAMILY PROTEINS

The first discovery of 5hmC was reported in frog and rodent brain DNA in 1971, but this amazing finding did not draw too much attention⁴⁹. In 2009, two labs shed light on the mystery nucleotide in mammalian DNA (Figure 3&4). Kriaucionis and Heintz (2009) detected an unusual nucleotide when comparing the abundance of 5mC in cerebellar Purkinje neurons and granule cells³⁸. Subsequent experiments determined the nucleotide

as 5hmC, but the abundance of 5hmC is quite low with less than 1% of total nucleotides in cerebellar Purkinje neurons and granule cells³⁸ (Figure 4). In another study, Rao and colleagues (2009) showed that the Ten Eleven Translocation (TET) family proteins catalyze the conversion of 5mC to 5hmC (Figure 5), suggesting a novel epigenetic regulation of 5hmC³⁷. In addition, 5hmC expression level was found to be higher in mouse embryonic stem cells (mESC) than other cell types. Both Tet1 and 5hmC levels are downregulated upon the differentiation of mESC, suggesting a potential role of Tet1 and 5hmC in epigenetic reprogramming³⁷. The recurrence of 5hmC has caught the interest of many towards providing detailed characterization of its biological functions. Ten Eleven Translocation 1 protects the promoter region of Nanog in an unmethylated state, which regulates ESC self-renewal and lineage commitment. Once Tet1 is knocked down, the promoter of Nanog returns to a methylated state and finally contributes to gene silencing. In contrast, the overexpression of Tet1 triggers the demethylation of methylated plasmid DNA⁵⁰.

In various human and mouse cells, 5hmC displays differential profiles, suggesting a critical balance between 5mC and 5hmC, which may subsequently maintain the cell in its normal state^{37,38,51}. Except for serving as an intermediate during active DNA demethylation, 5hmC may have other functions in biological systems. The base-resolution level of 5hmC helps to uncover its biological roles. Several techniques have been developed to map 5hmC architecture in the genome. These techniques include cytosine-5-methylenesulfonate sequencing, oxidative bisulfate sequencing, hydroxymethyl DNA immunoprecipitation sequencing and Tet2-assisted bisulfite sequencing⁵²⁻⁵⁶ (Figure 6). Studies using these techniques have characterized 5hmC

profiles in different cells and tissues. For example, 5hmC accounts for 1% of total 5mC in immune cells, 5-10% of total 5mC in ES cells and 40% of total 5mC in neural cells^{37,38,57}. Whereas, 5fC and 5ca represent only 0.03% and 0.01% of total 5mC in mouse ES cells³⁹. Analysis of genome-wide Tet1-dependent 5hmC distribution, has shown that Tet1 and Tet1-dependent 5hmC are enriched at transcription start sites (TSS) along with repressive histone modifications^{58,59}. However, other labs have obtained opposite results, in which 5hmC and histone modifications are enriched at enhancers, promoters and exons in order to regulate active transcription⁶⁰⁻⁶². These differences are plausible for independent mechanisms, different techniques and the variation of 5hmC dynamics from cell to cell during 5hmC signal capture. In addition, 5hmC abundance changes dynamically during human CD34+ cell and erythroid development such that 5hmC increases at erythroid-specific transcription factor binding sites and decreases at repressive genes. Also, rapid DNA demethylation is observed during erythropoiesis^{63,64}. Therefore, it is reasonable to assert that 5hmC regulates transcription activation or repression during cell differentiation. The later discovery of TET family proteins has helped to better understand the importance of 5hmC and 5hmC dynamics in gene expression and cell-fate decision.

Ten-eleven translocation (TET) family proteins contain two key components: Fe (II) and 2-oxoglutarate (2-OG) to catalyze oxidation of the methyl group of 5mC to hydroxymethyl, formyl and carboxyl groups, which generate 5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxylcytosine step-by-step in an enzymatic activity-dependent manner^{39,65}. Presently, the biological functions of 5hmC, 5fC and 5caC are not well-studied, but most of recent studies have pointed potential roles to DNA demethylation,

which regulates gene expression by a series of enzyme catalysis and DNA repair mechanisms³. All three family members of TET: TET1, TET2 and TET3 are present in different mammalian tissues⁵⁷. They participate in diverse biological processes, i.e., gene transcription, embryonic development and cancer initiation/progression.

The discovery of TET proteins originate from analysis of J-binding-protein 1 (JBP1) and JBP2 in the parasite *Trypanosoma brucei* that causes African sleeping sickness⁶⁶. Such species contain base J, a modified thymine in which β -D-glucosyl-hydroxymethyl group is added up to uracil, which enzymatic process is catalyzed by JBP1 and JBP2 which are capable of oxidizing methylthymine to 5-hydroxymethyluracil, and the intermediate is then further glucosylated by an unknown glucosyltransferase^{67,68} (Figure 3). The J-binding-proteins belong to Fe(II) and 2-oxoglutarate dependent dioxygenases. In *T. brucei*, most of base J resides in silent copies of variant surface glycoproteins that help parasites evade host immune defense⁶⁹. When computational screening of JBP homologues were used to predict nucleic acid modifying dioxygenases, TET family proteins were identified in eukaryotic cells and bacteriophages (Figure 3). Ten-eleven translocations are present in all metazoans but not in *Caenorhabditis elegans* because of a loss of methylation function⁷⁰. On the basis of the chemical similarity between thymine and 5mC and the protein structure of TET, it is possible that TET proteins may oxidize 5mC in mammalian genome.

All three TET proteins contain a Cys-rich domain and a carboxy-terminal catalytic domain that forms a double-stranded β helix (DSBH). Evolutionary studies indicate that the ancestor TET gene triplicated to TET1, TET2 and TET3⁷¹ (Figure 5). Furthermore, TET2 underwent chromosomal inversion, which resulted in the detachment of CXXC

domain, thereby encoding a separate gene called IDAX⁷². Many chromatin-binding proteins contain CXXC domains. The CXXC domain of IDAX preferentially binds to unmethylated CpG in CGIs. Strangely, TET1 CXXC domain does not bind DNA regardless of whether CpG is methylated or not⁷³. On the other hand, TET3 CXXC domain binds to unmethylated cytosine even if the resulting nucleotide is not a guanine (supported by X-ray crystallography)⁷⁴. Ten-eleven translocation 2 no longer has a CXXC domain, but instead IDAX (Figure 5). The loss of IDAX in ES cells prevents TET2 from being degraded in a caspase-dependent destruction mechanism during ES cells differentiation while the depletion of TET2 in human myeloid cell line upregulates TET2 and 5hmC levels. If CXXC domain of IDAX was mutated, IDAX lost the control of TET2 and 5hmC suggesting IDAX recruits TET2 to chromatin and TET2 binds to DNA through a Cys-rich domain⁷² (Table 1.2).

The C-terminal of TET2 catalytic domain is composed of a Cys-rich domain and DSBH domain⁷⁵. The crystallized TET2 catalytic domain made it possible to uncover a detailed inspection of TET2 DSBH bound to methylated DNA. According to the crystal structure of TET2, two Zinc fingers stick Cys-rich and DSBH domains together to form a catalytic domain. The Cys-rich domain stabilizes DNA substrate with proximity to DSBH. 5mC stays in the catalytic cavity in which a methyl group is pointed to Fe(II) for further oxidation⁷⁶. The structural features of TET2 catalytic domain helps to better understand the mechanisms of TET-mediated DNA interaction and oxidation.

1.3 TET2 GENE IN HEMATOPOIESIS AND HEMATOLOGICAL DISORDERS

All the DNMTs contribute to hematopoiesis¹⁶. The deletion of Dnmt1 in hematopoietic stem cells (HSC) and progenitor cells (HPC) leads to defects in hematopoiesis at early stages, characterized by an insufficient myeloid cell population and a decreased myeloid progenitor pool^{77,78}. The loss of Dnmt3a and/or Dnmt3b in mouse HSC shows a deficiency in HSCs self-renewal, with no lineage commitment of HSC shift. However, serial transplantation studies have shown that Dnmt3a is required to maintain HSC differentiation potential^{79,80}. In addition, exome sequencing of patients with acute monocytic leukemia (AML-M5) indicate that DNMT3A mutations occur in 20.5% of cases⁸¹ (Table 1.1). All of these studies demonstrate that DNMT3 are critical for maintaining normal hematopoiesis.

In addition to identified biological roles in epigenetic reprogramming, stem cell self-renewal and embryonic development, several studies have reported the genetic alterations of TET2 in myeloid malignancies, including in myelodysplastic syndrome (MDS), myeloproliferative neoplasms (MPN), acute myeloid leukemia (AML) and chronic myelomonocytic leukemia (CMML), while TET1 and TET3 are rarely mutated in hematological malignancies⁸². A large number of single/double -copy defects, nonsense and frameshift mutations of TET2 throughout the gene are commonly found in clinical patients⁸³ (Figure 7 & 8). The above high genetic alteration frequency of loss-of-function mutations indicates a critical role for TET2 in the cause and/or progression of leukemia. And if it is true, at least biological functions of TET2 could be determined by genetic tools. Previously, Xu and other groups showed that Tet2, a potential tumor suppressor gene, has multiple biological effects on the development of myeloid malignancies⁸⁴⁻⁸⁷.

Although the targeting strategies were different, all of the Tet2^{-/-} mice developed similar phenotypes including an elevated hematopoietic progenitor cell population, and HPC differentiation skewed toward myelomonocytic lineage.

To fully unearth and characterize the nature of Tet2 in myeloid malignancies, four research groups generated Tet2 knockout murine models by different strategies. For example, Li et al (2011) inserted nLacZ/nGFP instead of partial exon 3 of Tet2 gene since exon 3 is the first and largest coding exon in Tet2 and is also highly mutated in myeloid malignancies⁸⁴. Through a specific interbreed crossing, a global knockout mouse model (Tet2^{-/-}) was obtained (Figure 10). On the other hand, Moran-Crusio and colleagues (2011) generated a conditional knockout mouse model in hematopoietic compartment (vav-cre; Tet2^{f/f})⁸⁷. However, the other two groups' disruption approaches were slightly different. Quivoron and colleagues (2011) generated two models, including a gene-trap targeting intron 9 (Tet2^{LacZ/LacZ}) and a conditional knockout targeting exon 11 (Mx1-cre; Tet2^{Lox/Lox})⁸⁶. Both of these two models lost the DSBH domain, which eliminated dioxygenase activity in vivo. In addition, Ko and colleagues (2011) deleted Tet2 exon 8-10 globally to fully remove enzymatic activity⁸⁵. Together, these global or conditional Tet2 knockout murine models were viable and fertile and their progeny was in accordance with Mendelian ratio.

Accordingly, detailed phenotypic analyses were performed on these knockout mice. We have examined hematologic parameters of WT, Tet2^{+/-} and Tet2^{-/-} as early as 2 to 5 months of age⁸⁴. Notably, increased WBC counts and abnormal numbers of monocytes and neutrophils were observed in Tet2^{-/-} peripheral blood. Besides the appearance of significant hepatosplenomegaly in most of the studies, we also found pale footpads in

Tet2^{-/-} that may indicate anemia and this was consistent with the decrease in erythrocytes and platelets counts in Tet2^{-/-}⁸⁴, suggesting a possible occurrence of monocytosis and/or neutrophilia in Tet2^{-/-} mice. In addition to these mentioned findings in Tet2^{-/-} mice, other changes include increased bone marrow (BM) cellularity, splenomegaly and hepatomegaly, as well as increased numbers of colony forming unit granulocyte, monocyte (CFU-GM) in BM and spleen. A resulting diagnosis of these Tet2^{-/-} mice is CMML.

The Tet2^{+/-} and Tet2^{-/-} mice died from 3 to 18 month as a result of spontaneous lethal hematological malignancies. Compared with age-matched WT mice, postmortem examination of Tet2^{-/-} mice show remarkable splenomegaly and hepatomegaly, which are 8-15 times and 2-5 times larger, respectively (Figure 10). Histologic studies reveal massive erythroid and/or myeloid infiltration in spleen and liver. Histopathology and cytopsin studies identify specific cell types as myeloblasts, monocytes/macrophages and neutrophils⁸⁴.

Flow cytometric analyses of BM and spleen cell from Tet2^{-/-} mice is consistent with the results from the histologic analyses. Located in the infiltrated BM and spleen are large numbers of erythroblasts at different stages and various myeloid cell populations. Also, a large peritoneal mass and a few white nodules in the liver are observed, which contains Gr-1⁺CD11b⁺ cells, suggesting the existence of myeloid sarcoma⁸⁴.

To determine whether Tet2 alterations contribute to HSC compartment, previous studies have used a range of cells. These included analyzing BM from 6-7 weeks old Tet2^{-/-} mice, with the emphasis on lineage-negative (Lin-), ScaI-positive (Sca+), c-kit – positive (c-kit+) (LSK) and LK cell populations. But even though results showed

increased numbers of LSK cells, no obvious myeloid malignancies were observed. Single-cell clonogenic assays were also performed to determine the effect of Tet2 alterations on proliferation and differentiation of LSK cells. Here, the results showed that Tet2^{-/-} LSK exhibited higher cloning efficiency than Tet2^{+/-} and WT LSK cells along with CFU-GM in the colonies⁸⁴. Taken together, it is reasonable to believe that the deletion of Tet2 stimulated the differentiation and proliferation of HSC/HPC and facilitated the development of myeloid malignancies.

Genome-wide whole exome sequencing from different groups have revealed that TET2 is one of the most frequently mutated epigenetic regulators in CMML (~50%)⁸⁸⁻⁹⁰, AML (~20%)^{83,91-93} and MDS (~20%)⁹⁴⁻⁹⁶ (Table 1.1). In many patients, deletion of TET2 on one allele at 4q24 region occurs with mutations of TET2 on a second allele⁹⁴ suggesting that haploinsufficiency of TET2 may lead to genome instability through DNA damage/repair pathways. More than 700 TET2 mutations have been identified in about 2000 leukemic patients⁹⁷. These harmful mutations impair the enzymatic activity of TET2, which ultimately result in aberrant DNA methylation profiling and decreased 5hmC levels. The majority of TET2 missense mutations are clustered in two highly conserved regions (AA 1104-1478 and 1845-2002) that are considered as the catalytic domain of TET2 protein⁷⁶. The mutations within these regions may influence protein-DNA association, protein-protein interaction or post-translational modifications. The TET2 mutations have also been found in lymphoma including angioimmunoblastic T-cell lymphomas (~76%)⁹⁸ and peripheral T-cell lymphoma not otherwise specified (~38%) along with Ras homolog gene family, member A (RHOA) mutations^{99,100}.

Analyses of clonal architecture and mutation frequency of MDS and CMML indicate that TET2 mutations occur at an early stage of cell differentiation, which leads to clonal expansion of leukemic stem cells^{101,102}. However, the loss of Tet2 itself in mouse models is not able to initiate hematological disorders. In addition, most of TET2 alone mutations have been observed in older individuals with clonal hematopoiesis but without hematological malignancies¹⁰³. Therefore, it is possible to propose that there should be more gene mutations that serve as “driver mutations” which occur during hematological malignancies initiation and/or progression. Through meta-analysis of mutational sites of hematological malignant patients, TET2 mutations co-exist with other gene mutations such as serine/arginine-rich splicing factor 2 (SRSF2), enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) and additional sex combs like transcriptional regulator 1 (ASXL1) in MDS and CMML^{102,104,105} (Figure 9). The SRSF2 belongs to splicing factors that recognize 3' splice-site and mediate ATP-dependent interactions between U1/U2 snRNPs and pre-mRNA. Like TET2, SRSF2 is frequently mutated in myeloid cancers. EZH2 is an H3K27me3 reader and it is the key component of polycomb repressive complex 2 (PRC2) while ASXL1 differentially recruits methylated H3 to promoters and it also mediates deubiquitination of H2AK119ub1^{104,106,107}. In AITL patients, TET2 mutations accompany a recurrent mutation in RHOA (G17V) which is a small GTPase that regulates cell morphology and migration⁹⁹. The DNMT3A mutations also co-exist with TET2 mutations in T-cell lymphomas⁹⁹. Therefore, TET2 mutations happen in the early stages of hematopoietic stem/progenitor cells, but the mutation alone does not lead to malignant transformations. Additional gene mutations must occur, along

with TET2 mutations for oncogenesis to take place, as in the case of various types of hematological malignancies (Figure 11).

In addition to the hematological system, TET genes mutations also exist in solid tumors at quite low frequencies, according to The Cancer Genome Atlas (TCGA) database. For example, all TET genes mutations are found in colorectal cancer, clear-cell renal cell carcinoma and metastatic castration-resistant prostate cancer¹⁰⁸⁻¹¹⁰. It is still unknown if TET mutations contribute to initiation/progression in these solid cancers. Additional studies will help to clarify the “driver mutations” in TET mutant background which trigger cell proliferation and survival during oncogenesis. Crossing TET knockout mice with current cancer-specific mouse models may help increase our understanding of TET functions.

1.4 TETs AND 5-HYDROXYMETHYLCYTOSINE IN NEUROGENESIS AND NEURODEVELOPMENT

Neurogenesis produces various types of functional neural cells from neural stem cells (NSC). Neurogenesis occurs not only at the embryonic stage but also adult stage throughout the life span of the individual. Such could be considered as an essential neural activity/mechanism for regulating learning and memory¹¹¹.

Embryonic neurogenesis in mice usually starts from embryonic day 8 (E8) to E14¹¹². During E8-E10, neuroepithelial cells undergo transformation in the ventricular zone (VZ) and subventricular zone (SVZ), thereby allowing the cells to acquire astroglial properties¹¹³. These radial glial cells (RGs) have “stem” features, which trigger the generation of nascent neurons or intermediate neuron progenitor cells. When RGs initiate symmetrical mitosis, neurons are generated. However, if RGs go through asymmetrical

mitosis, other “stem” cells are produced¹¹⁴. Newly synthesized neurons and glial cells then migrate to the cortex during embryonic development to thicken the neocortex and build up complicated neural networks¹¹¹. At the end of embryonic development, RGs detach from the ventricular zones, and move to the cortical plate and transform into astrocytes. Some RGs quietly remain in the SVZ and are responsible for adult neurogenesis¹¹³.

Adult neurogenesis occurs in two germinal zones of the brain: the subgranular zone (SGZ) in dentate gyrus and subventricular zone (SVZ) in lateral ventricles (Figure 12). Adult neurogenesis is regulated by many cellular activities including neural stem cell (NSC)/neural progenitor cell (NPC) proliferation and differentiation, fate determination, maturation and localization of newborn neurons^{115,116}. And because neurogenesis occurs largely in these two regions, it is reasonable to postulate that neurogenesis may play a major role in regulating learning and memory. In SGZ, two types of NSC have been identified, type I and type II cells. Type I cells are radial glia-like cells, which maintain self-renewal and may differentiate to type II cells, even astrocytes. As well, the morphology and cellular markers of type I and type II cells are slightly different. Other than the common Nestin marker, type I cells are glial fibrillary acidic protein positive (GFAP⁺) while type II cells are GFAP⁻. Two cell types constitute the NSC/NPC in SVZ: type B cells are self-renewal radial glia-like cells that are able to differentiate to type C cells, and these transient amplifying cells can also transform into neuroblasts at a later development period¹¹⁵. Neurogenesis generates various types of neural cells with the same genome but with distinct properties, suggesting a role for epigenetic regulation in neurogenesis (Figure 13). Significant DNA methylation occurs in neurons and astrocytes.

Consider GFAP which is a cellular marker for astrocytes. The promoter region of GFAP is initially hypermethylated, which blocks the differentiation of astrocytes. However, at specific stages during development, the GFAP promoter becomes hypomethylated and then initiates the differentiation of astrocytes. Two additional critical neural factors, brain-derived neurotrophic factor (BDNF) and fibroblast growth factor (FGF), are regulated by Tet1 protein in terms of the demethylation of BDNF and FGF promoters¹¹⁷.

Studies that have mapped genome-wide 5hmC, have shown that 5hmC distributes temporally and spatially during neurodevelopment^{61,118,119}. For example, studies have shown an age-dependent abundance and distribution of 5hmC in the cerebellum and hippocampus. Interestingly, 5hmC acquisition in these regions is different than what would be expected. There is no strong correlation between 5hmC acquisition and 5mC loss in many important neurodevelopment-related genes, therefore one could argue that 5hmC role in gene regulation may be via a mechanism other than by transient oxidation in the brain¹¹⁸. To better understand the role of 5hmC in the brain, more than 6000 differentially hydroxymethylated regions (DhMRs) at different ages have been identified. These DhMRs were only found in adult brain, from 6-week-old brain, but not in postnatal day 7 (P7) cerebellum, indicating long-term modification of 5hmC in the brain^{118,119}. Although the correlation of 5hmC abundance and gene transcription is still unclear, the enrichment of 5hmC and loss of 5mC at gene bodies coincide with gene transcription activation¹¹⁹. In fact, 5hmC regulation of neurogenesis was reported in 2011. Tet1 overexpression in the dentate gyrus (DG) significantly downregulated the methylation levels of *Bdnf* and *Fgf1b*, highlighting the role of 5hmC in neurogenesis¹¹⁷. Several types of neurodevelopment and neurodegenerative disorders have been found closely

associated with 5hmC, such as Rett syndrome, Autism spectrum disorder and Alzheimer's disease¹²⁰.

Because of the extensive roles of 5hmC and TETs, several studies have explored TET proteins functions in neurogenesis^{117,121}. There is evidence that Tet1^{-/-} mice exhibits robust loss (~45%) of NSCs in the SGZ compared with WT mice and neurospheres from Tet1^{-/-} show growth defects. Microarray studies have shown that many genes (Galanin, Ng2 and Ngn3) that are down-regulated during NSC proliferation¹²¹. In addition, Tet1^{-/-} mice show increased hippocampal long-term repression and memory defects¹²¹. Moreover, in *Xenopus*, Tet3 has been found to regulate early eye development and neurodevelopment transcriptionally. Deletion of Tet3 decreases neuronal genes in the eyes⁷⁴. **The role of Tet2 in neurogenesis is still unknown.**

My dissertation begins with a review of the molecular basis of DNA methylation and demethylation with a particular focus on newly identified 5hmC and TET family proteins, along with their contribution to the pathogenesis of hematological and neurological disorders. In chapter 2, I discuss the proposed work on uncovering the role of Tet2 in hematological malignancies. Section 2.3 introduces the methods and approaches being used in this chapter. I then describe the results and discussions I have achieved to date in Section 2.4. Later, in Section 2.5, I draw conclusions about what I have observed. Given the roles of 5hmC and Tet2 in the nervous system, I discuss specific findings of Tet2 in adult neurogenesis in chapter 3. In Section 3.3, I describe the materials and methods being used in this chapter. Section 3.4 presents the results including Tet2 regulation in NPC proliferation and differentiation. Specific conclusions

are drew in Section 3.5. Finally, chapter 5 summarizes all the observations of the study presented in chapter 2 and 3, as well as discusses directions for future research.

Figure 1 DNA methylation and demethylation pathways.

(A) DNMT1 adds methyl group to hemimethylated DNA. DNMT3a/b add methyl group to non-methylated DNA. (B) Passive demethylation occurs through the absence of DNMTs. TETs mediate active DNA demethylation through hydroxylation of 5mC to 5hmC, 5fC and 5caC, which can be recognized and removed by base excision repair. (C) The molecules involved in DNA methylation and demethylation.

Figure 2 The dynamic methylome landscapes of different genomic elements¹⁶. DNA methylation levels vary from low (blue) to high (red). CGI shores, enhancers and canyon edges indicate the most differentially methylated regions.

Figure 3 TETs are the homolog of JBPs.

(A) The biogenesis of base J. JBP1/2 oxidize thymidine to produce 5hmU, which can be further catalyzed to base J by unknown glucosyltransferases. (B) The multiple alignments of JBP1, JBP2, TET1, TET2 and TET3 are performed using Clustal Omega. These five proteins are highly conserved in their oxygenase regions.

Figure 4 Quantification of x in Purkinje cells and granule cells³⁸.

(A) Nucleoside monophosphates separated from genomic DNA in Purkinje and granule cells. (B) Reference map.

Figure 5 The protein structures of TET family.

TETs have a DNA-binding CXXC domain at N-terminal and Cys-rich domain, DSBH domain located at C-terminal. An ancestral TET gene triplicated into three TET family members. Chromosome inversion occurred and separated TET2 from its CXXC domain, which generated IDAX.

Figure 6 Assays for mapping 5mC and 5hmC at single-base resolution.

Traditional bisulfite sequencing separates 5mC from regular cytosine. TET-assisted bisulfite sequencing separates 5hmC from 5mC.

Figure 7 TET2 mutations detected in individuals with different tumors.

(A) TET2 mutations frequently occur in hematological malignancies and solid tumors. (B) Pie chart of TET2 mutations distribution. Data collected from Catalogue of somatic mutations in cancer database.

Figure 8 Histogram of TET2 mutations.

Data collected from Catalogue of somatic mutations in cancer database.

Gene	Malignancy	Mutation %
<i>DNMT3A</i>	AML	12%-22%
	MDS	8%
	MPN	7%-15%
	CMML	4%
<i>TET2</i>	AML	12%-34%
	MDS	20%-25%
	MPN	4%-14%
	CMML	50%

Table1.1 Mutations of DNMT3A and TET2 in hematological malignancies¹⁶. The incidence of DNA modification related gene DNMT3A and TET2 mutations in hematological malignancies.

Figure 9 Circos plots demonstrating 12 coexisting genes in a cohort of patients with myeloid malignancies¹²².

12 genes have been identified from coexisting genes that show mutual mutations. Ribbons connecting distinct gene mutations within this plot reflect the position and frequency between different gene mutations.

Interactor	Function
OGT	TET2 recruits OGT to chromatin to regulate H3K4me3 and gene transcription
IDAX	Promotes degradation of TET2 through caspase pathway
EBF1	Unknown function
Uhrf2	Promotes 5mC hydroxylation in neuronal cells
Vitamin C	Promotes Fe(II) and 2-oxoglutarate dependent dioxygenases
miR22	Decreases Tet2 pre-mRNA expression level

Table 1.2 Identified TET2 interactor¹²³. Several protein complexes, microRNA and small molecule have been shown to interact with TET2.

Figure 10 *Tet2*^{-/-} mice develop lethal myeloid malignancies⁸⁴.

(A) Targeting strategy of *Tet2* knockout mouse model. (B) Appearance of *Tet2*^{-/-} mice with pale footpads, splenomegaly and hepatomegaly.

Figure 11 TET2 background mutations cause various hematological malignancies. TET2 mutations may occur in hematopoietic stem/progenitor cells with aging suggesting that additional genetic events lead to the initiation/progression of the diseases.

Figure 12 Adult neurogenesis¹¹¹.

In subventricular zone, B-cells (Quiescent RGs) are activated and produce C-cells (transient amplifying cells). C-cells transform to neuroblasts and migrate through rostral migratory stream to olfactory bulb as interneurons. In the dentate gyrus, type I progenitors give rise to type II progenitors, which migrate to granule cell layers and transform to neuroblasts. The neuroblasts then become immature neurons and merge into neural circuit through synaptic integration.

Figure 13 Transcriptional epigenetic regulators in adult neurogenesis.

Green columns highlight the positive regulators and red columns indicate the negative regulators. The left columns are the regulators that impair NSC differentiation and neurogenesis. The right columns are the regulators that promote NSC differentiation and aberrant neurogenesis. This figure was modified from (Yao et al. 2014)¹¹¹.

CHAPTER

TET2 PROTECTS GENOMIC STABILITY THROUGH INTERACTING WITH MSH6

2.1 ABSTRACT

Ten-eleven translocation 2 is a member of dioxygenases that catalyze the multi-step 5mC oxidation. Loss-of-function TET2 mutations are frequently associated with hematological malignancies; however, the underlying mechanism remains unknown. Here, I show that Tet2^{-/-} mice develop spontaneous myeloid, T- and B-cell malignancies. The mice with T-cell lymphoma/leukemia are characterized with enlargement of spleen, liver and thymus. The normal architecture of these organs are disrupted by abnormal lymphoid with cells aberrantly expressing CD4 and CD8. Mice with B-cell lymphoma/leukemia display enlarged lymph nodes and atypical lymphoid infiltrates are observed in the small intestines. Cells with B-cell lineage are altered in different tissues, particularly, B220⁺/low IgM/low CD43/CD19⁺ with high forward scatter. To obtain a more detailed view of the role of Tet2 in hematopoiesis, sublethally irradiated F1 mice were transferred with Tet2^{-/-} T-cell and B-cell tumor cells. Both T-cell and B-cell malignancies were transplantable, suggesting the malignant and neoplastic nature of the infiltrating T- or B- lymphocytes in Tet2^{-/-} mice. To explore genome-wide genetic alterations and multiple lineage involvement in Tet2^{-/-} mice, additional exome sequencing of Tet2^{-/-} tumors were performed, which revealed acquisition of numerous mutations, including genes frequently mutated in human hematological malignancies such as Neurogenic locus notch homolog protein 1 (NOTCH1) and fms-related tyrosine kinase 3 (FLT3). The mutation frequency in Tet2^{-/-} tumors is significantly greater at loci containing Tet2-dependent active demethylation. To investigate the underlying mechanism by which Tet2 loss may result in genomic instability, I found that TET2 associates with MutS homolog 6 (MSH6), a key component of DNA mismatch repair

(MMR) using both Tet2-tag knock-in ES clones and human TET2 overexpressing cell line. The association between TET2 and MSH6 was substantiated by immunostaining. The latter study showed that these two proteins bind closely to each other at S-phase. To examine if TET2 loss impairs DNA mismatch repair, I depleted TET2 in HeLa cells. TET2-deficient cells displayed elevated spontaneous mutational frequencies and increased microsatellite instability, characteristic of MMR-deficient cells. Consistently, myelodysplastic syndrome patients with TET2 mutations exhibit significantly higher mutational events than patients without TET2 mutations. Together, these results suggest that TET2 protects genomic stability through interacting with MSH6, unveiling a novel means by which TET2 loss leads to diverse hematological malignancies.

2.2 INTRODUCTION

Ten-eleven translocation (TET) family of methylcytosine dioxygenases (Tet1/2/3) catalyze the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), which could be further oxidized to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), resulting in active DNA demethylation³⁹. The dynamic methylation/demethylation cycle has been shown to occur at a large number of genomic loci across the genome¹²⁴. Several studies suggest that 5hmC may also have DNA demethylation-independent functions and serve as stable epigenetic marks that recruit specific readers, DNA repair proteins and transcription factors, with limited overlap between these TET proteins^{119,125}. TET2 inactivation through mutations is a common event in myeloid and lymphoid malignancies, and has been detected also in solid tumors¹²³. Several TET2 binding proteins have been identified, including O-linked N-acetylglucosamine transferase (OGT), inhibition of the

Dvl and Axin complex (IDAX) and early B-cell factor 1 (EBF1)^{72,126,127} (Table 1.2). Identification of critical novel partners of TET2 and unveiling functional significance of these interactions may provide valuable insights into the mechanism underlying the critical roles of TET2 in tumorigenesis of many cancers.

To fully unearth and characterize the nature of Tet2 in myeloid malignancies, four research groups generated Tet2 knockout murine models by different strategies. For example, Li et al (2011) inserted nLacZ/nGFP instead of partial exon 3 of Tet2 gene since exon 3 is the first and largest coding exon in Tet2 and is also highly mutated in myeloid malignancies⁸⁴. Through a specific interbreed crossing, a global knockout mouse model (Tet2^{-/-}) was obtained. We, and others examined hematologic parameters of WT, Tet2^{+/-} and Tet2^{-/-} as early as 2 to 5 months of age. Notably, increased WBC counts and abnormal numbers of monocytes and neutrophils were observed in Tet2^{-/-} peripheral blood. Besides the appearance of significant hepatosplenomegaly in most of the studies, we also found pale footpads in Tet2^{-/-} that may indicate anemia and this was consistent with the decrease in erythrocytes and platelets counts in Tet2^{-/-}⁸⁴, suggesting a possible occurrence of monocytosis and/or neutrophilia in Tet2^{-/-} mice. In addition to these mentioned findings in Tet2^{-/-} mice, other changes include increased bone marrow (BM) cellularity, splenomegaly and hepatomegaly, as well as increased numbers of colony forming unit granulocyte, monocyte (CFU-GM) in BM and spleen. A resulting phenotype of these Tet2^{-/-} mice is potentially CMML. Histologic studies reveal massive erythroid and/or myeloid infiltration in spleen and liver. Histopathology and cytospin studies identify specific cell types as myeloblasts, monocytes/macrophages and neutrophils⁸⁴. Flow cytometric analyses of BM and spleen cells from Tet2^{-/-} mice are consistent with

the results from histologic analyses. Moreover, a large peritoneal mass and a few white nodules with Gr-1⁺CD11b⁺ cells were observed in the liver, suggesting the existence of myeloid sarcoma⁸⁴. Taken together, it is reasonable to believe that the deletion of Tet2 stimulated the differentiation and proliferation of HSC/HPC and facilitated the development of myeloid malignancies.

Here, my consideration is to uncover the mechanisms of Tet2 and to discover TET2 interactors to better understand Tet2 loss induced tumorigenesis. Specifically, I proposed to: 1) characterize hematological malignancies in Tet2^{-/-} mice; 2) use whole genome analyzing tools to examine Tet2^{-/-} genomic landscape; 3) seek TET2 interactors that facilitate leukemogenesis; and 4) identify Tet2 regulatory network. Tet2^{-/-} mice develop spontaneous myeloid, T- and B-cell malignancies. Both T-cell and B-cell malignancies were transplantable, suggesting the malignant and neoplastic nature of the infiltrating T- or B- lymphocytes in Tet2^{-/-} mice. To explore genome-wide genetic alterations and multiple lineage involvement in Tet2^{-/-} mice, additional exome sequencing of Tet2^{-/-} tumors were performed, which revealed acquisition of numerous mutations, including genes frequently mutated in human hematological malignancies such as Neurogenic locus notch homolog protein 1 (NOTCH1) and fms-related tyrosine kinase 3 (FLT3). The mutation frequency in Tet2^{-/-} tumors were significantly greater at loci containing Tet2-dependent active demethylation. To investigate the underlying mechanism by which Tet2 loss may result in genomic instability, I found that TET2 associates with MutS homolog 6 (MSH6), a key component of DNA mismatch repair (MMR) using both Tet2-tag knock-in ES clones and human TET2 overexpressing cell line. To examine if TET2 loss impairs DNA mismatch repair, I depleted TET2 in HeLa cells. TET2-deficient cells

displayed elevated spontaneous mutational frequencies and increased microsatellite instability, characteristics of MMR-deficient cells. Consistently, myelodysplastic syndrome patients with TET2 mutations have significantly higher mutational events than patients without TET2 mutations. I therefore proposed that TET2 protects genomic stability through interacting with MSH6, unveiling a novel means by which TET2 loss leads to diverse hematological malignancies.

2.3 MATERIALS AND METHODS

2.3.1 Analyses of mice

Peripheral blood (PB) was collected by retro-orbital bleeding of mice and was smeared for May-Grunwald-Giemsa staining, and/or subjected to an automated blood count (Hemavet System 950FS). Morphological analysis and cell differential of BM and spleen samples were performed via cytopins (5×10^5 cells/sample) followed by May-Grunwald-Giemsa staining. For histopathology analyses, femurs were fixed in formaldehyde, decalcified, and paraffin embedded.

Spleens, livers, lymph nodes, thymus and intestine were treated similarly except for the step of decalcification. Sections (4.5 μ m) were stained with hematoxylin/eosin (H&E). Flow cytometric analysis was performed as previously described⁸⁴. Total PB white blood cells were obtained after lysis of red blood cells. Single-cell suspensions from BM, spleen, liver, lymph node, thymus and PB were stained with panels of fluorochrome-conjugated antibodies. Briefly, cells were divided into 40 μ l aliquots containing 2% FBS in PBS in 96-well plates. One microliter of antibodies was added into each well and incubated for 45min at 4°C. Then, the stained cells were re-suspended in 150 μ l 2% FBS

in PBS and processed for flow cytometric analysis. Dead cells were excluded by DAPI staining. The analyses were performed using a BD FACS CantoII or LSRII flow cytometer. All data were analyzed by FlowJo7.6 software.

2.3.2 Tumor transfer assay

Tumor transfer experiments were performed to evaluate the malignant nature of the abnormally infiltrated T- or B-lymphocytes in the moribund/deceased Tet2^{-/-} mice. Briefly, 1x10⁶ spleen cells from primary Tet2^{-/-} or WT mice were transplanted into sublethally irradiated (600 cGy) F1 recipient mice (CD45.1/CD45.2, n=5) through tail veins. Recipient mice were sacrificed when they became moribund or 6 months after injection. Donor cell chimerism in the PB, spleen and BM were examined at the end of the observation period. The mice were also phenotypically analyzed to determine their hematological phenotype and development of T- or B-cell malignancies. None of the recipients receiving WT spleen cells developed pathology or gross evidence of disease within 6 months of transplantation. By contrast, all of the animals receiving spleen cells from Tet2^{-/-} mice with T- or B-cell leukemia/lymphoma developed diseases with similar characteristics as those observed in their respective primary donor animals including elevated WBC counts, lymphocytosis, splenomegaly, enlarged lymph nodes and death.

2.3.3 Generation of FLAG-V5 Tag knock-in ES cells

The targeting vector contained a neomycin (Neo) cassette flanked by two FRT sites, followed by a 0.5 kb genomic fragment upstream of the Tet2 start codon and an ATG/3xFlag/V5 sequence. A 2.2 kb 5' and a 4.8 kb 3' arm genomic fragments were

subcloned into the vector for gene targeting. All the fragments were amplified using KOD Xtreme™ Hot Start DNA Polymerase and ES cell genomic DNA as template.

KOD Xtreme standard reaction:

2X Xtreme Buffer	25 µl
dNTPs (2mM)	10 µl
PCR grade water	10 µl
Primer mix	3 µl
Template DNA	1 µl
KOD Xtreme Polymerase	1 µl
Total volume	50 µl

The three-step cycling was used to amplify the targets: 94°C for 2min, 98°C for 10s, 60°C for 30s and 68°C for 5min. Steps 2-4 were performed for 35 cycles. The targeting vector was linearized by EcoRI or ScaI and electroporated into 129/sv mouse ES cells and screened by southern blot. I then examined the levels of WT- and Tagged-Tet2 transcripts in the Tet2:Tag ES cells by allele specific qPCR (β-actin as internal calibrator). Using Tet2 primers for both WT-Tet2 and Tagged-Tet2, the expression of WT-Tet2 plus Tagged-Tet2 mRNA in Tet2:Tag ES cells was comparable to that in the WT ES cells. While, using the Tagged-Tet2 specific primers, the Tagged-Tet2 mRNA expression was not detected in control ES cells, suggesting the primers are Tagged-Tet2 mRNA specific. These data suggest that Tagged-Tet2 mRNA expression was comparable to WT-Tet2 mRNA expression in the Tet2:Tag ES cells.

2.3.4 Purification of TET2-associated proteins

To search for binding partners of Tet2, I collected 30 plates (10cm) of Tet2:Tag ES cells. Briefly, cells were washed with PBS and scraped off from the plates. Then, cells were fully re-suspended in Buffer A (10mM HEPES pH7.6, 420nM NaCl, 1.5mM MgCl₂, 0.2nM EDTA and 25% Glycerol) in order to separate cytoplasmic protein. Next, the remaining suspension was incubated with Buffer C (20mM HEPES pH7.6, 420nM NaCl, 1.5mM MgCl₂, 0.2nM EDTA and 25% Glycerol) to obtain nuclear proteins. The nuclear extracts were incubated with 0.2 ml anti-Flag M2 beads at 4°C for at least 4h. The beads were washed and associated proteins were eluted twice with 200ng/μl 3xFlag peptide (Sigma). The eluted proteins were analyzed by 10% SDS-PAGE and gel slices were subjected to trypsin digestion followed by liquid chromatography and tandem mass spectrometry (LC-MS/MS) as described previously¹²⁸. WT ES Cells were used as purification control.

2.3.5 Constructs

WT TET2 was cloned into pOZ-FH-N vector. A mammalian codon optimized human TET2 cDNA was cloned into pcDNA3.1 vector. TET2-N (amino acid 1-942), TET2-M (amino acid 943-1322) and TET2-C (amino acid 1323-2002) were cloned into pcDNA3.1 vector. Briefly, the human TET2 fragments were amplified by PCR and the products were sub-cloned to pCR-Blunt II-TOPO for validating sequences.

Fresh PCR products	3 μ l
Salt solution	1 μ l
Water	1 μ l
pCR-Blunt II-TOPO	1 μ l
Total volume	6 μ l

The reaction was mixed gently and incubated for 5 min at room temperature. Normally, 1 μ l mixture was pipetted directly into one vial of competent cells. The vial was incubated on ice for 30min. Then, the vial was incubated in 42°C for 30s and on ice for 2min. Pre-warmed S.O.C. medium was added to each vial and the vial was incubated at 37°C for 1 hour at 225rpm. About 200 μ l mixture was spread on the LB agar plates with kanamycin for colony selection. The sequence confirmed clones were cloned into pcDNA3.1 vector using NheI and XhoI. Each of these vectors contained Flag-tag and nuclear localization signal at their N-terminal.

2.3.6 RNA interference

shRNA sequences targeting mouse Tet2 (5'-GCTTACAGAATGGAGGGATAA-3' or 5'-CGGGTTCATATTTGAATCCTT-3') were inserted into the pLKO.puro.IRES.mCherry vector. Generation of lentivirus was carried out as previously described. shRNA sequences targeting human TET2 (CCGGCCTTATAGTCAGACCATGAACTCGAGTTTCATGGTCTGACTATAAGG TTTTTTG) and human MSH6 (CCGGGCCAGAAGAATACGAGTTGAACTCGAGTTCAACTCGTATTCTTCTGGC TTTTTTG) were cloned into pLKO.1-puro vector.

The tissue culture 6-well plates were treated with 0.01% poly-L-lysine for 5min at RT. HEK293T cells (1×10^6) were seeded in each well for 24h using Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin. When cells confluence reached 90%, transfection mixture was prepared. 3.75 μ l Lipofectamine® 3000 was diluted by 125 μ l Opti-MEM® Medium. Next, 500ng pLKO construct, 50ng pCMV-VSVG and 450ng psPAX2 were mixed with 125 μ l Opti-MEM® Medium and 5 μ l P3000™ Reagent. Diluted DNA was added to diluted Lipofectamine® 3000 reagent at RT for 5min. The DNA-lipid complex was dropped to cells. The virus containing supernatant was collected after 24 and 48h for instant lentivirus titer test using Lenti-X™ GoStix™. 1×10^6 HeLa or NIH3T3 cells were re-suspended in 500 μ l lentivirus supernatant and transferred to 24-well plates. Polybrene (8 μ g/ml) was added to each well and the plate was centrifuged at 2000rpm for 90min at 37°C. Another 500 μ l complete medium was added to each well for 48h. The viral-infected cells were selected by puromycin (2 μ g/ml) for 72 h and then used for phenotypic and/or functional analysis.

2.3.7 Real-time PCR

Total RNA was isolated and put on ice. The reverse transcription reaction included: 6 μ l 7xgDNA wipeout buffer, 3 μ g RNA template and 33 μ l RNase-free water. The mixture was incubated at 42°C for 2min and placed immediately on ice. Additional 3 μ l quantiscript reverse transcriptase, 12 μ l 5xQuantiscript RT buffer, 3 μ l RT primer mix were added and the tube was incubated at 42°C for 15min and 95°C for 3min. First-strand cDNA was synthesized. Real time PCR was performed using Fast SYBR Green master mix. Briefly, each reaction contained 10 μ l Fast SYBR® Green Master Mix, 2 μ l

primer mix and 2 μ l cDNA template. The plate was applied to Applied Biosystems 7500 fast real-time PCR system following the program: 95°C for 20s, 95°C for 3s and 60°C for 30s, 40 cycles. The PCR amplifications were performed in triplicate for each gene of interest along with parallel measurements of Gapdh or β -Actin cDNA(an internal control). To confirm specific amplification of the desired PCR product, melting curves were analyzed and PCR products were separated on a 3% agarose gel. The data was analyzed by 7500 Fast SDS v1.5.1.

2.3.8 Western blot and co-immunoprecipitation assays

Cells or tissues were disrupted in either whole cell lysis buffer (50mM Tris, pH7.4, 250mM NaCl, 5mM EDTA, 50mM NaF, 1mM Na₃VO₄, 1%NP40 and complete protease inhibitor cocktail) or nuclear extraction buffer. Briefly, cells were washed twice in ice-cold PBS. For each 10-cm dish, 0.5ml ice-cold cell lysis buffer was added to each cell culture dish, and incubated on ice for 30min. The cells were scraped off the dish and transferred to 1.5ml microcentrifuge tubes. The tubes were microcentrifuged for 10min at 4°C, 13,000 x rpm. The supernatant of cell lysate was saved for immunoprecipitation or SDS-PAGE.

Supernatants or nuclear extracts were incubated with 30 μ l protein A/G PLUS-Agarose (Santa Cruz Biotechnology) and incubated with rotation at 4°C for 1 hour. The tube was microcentrifuged for 10min at 4°C to preclear the cell lysate. Primary antibodies FLAG, TET2, MSH6 and so on were added into 400 μ l cell lysate, and incubated with gentle rotation overnight at 4°C. Next day, 30 μ l protein A/G PLUS-Agarose was added to the cell lysate for an additional 3 hours at 4°C. Precipitates were

washed five times with cell lysis buffer, and the pellet was re-suspended with 30 μ l 2XSDS sample buffer. The samples were heated at 95°C for 6min and centrifuged at 13,000 x rpm for 5min. The samples were loaded on SDS-PAGE (4%-15%). The gel was then electro-transferred to PVDF membrane (80V, 120 min). After transfer, the membrane was washed with 25ml TBST for 5min and 25ml blocking buffer (5% non-fat milk in TBST) for 1hour at room temperature. Later, the membrane was incubated in 10ml primary antibody dilution buffer (1:1,000) with gentle rocking overnight at 4°C. Next day, the membrane was washed 3 times for 5 min each with TBST. The membrane was incubated with horseradish peroxidase (HRP)-linked secondary antibody dilution buffer (1:10,000) for 1 hour at room temperature. The membrane was the washed 3 times for 5 min each with TBST, and ECL substrate was added onto the membrane in the dark and exposed to x-ray film. Proper exposure time was performed to detect signals.

2.3.9 Exome sequencing and mutational confirmation

Initial whole-exome sequencing was carried out in order to identify candidate mutations in the exome of genes. The genomic DNA was captured with the NimbleGen mouse exome array according to the manufacturer's protocol and 100bp paired-ended sequencing was performed on the Illumina HiSeq 2000. Raw sequencing reads were mapped to the whole mouse genome (mm10) using the PEmapper (<http://pemapper.sourceforge.net/>) with the default settings, and variant bases were annotated with SeqAnt (<http://seqant.genetics.emory.edu/>).

For the samples subjected to mutational detection, genomic DNA was amplified in selected exons by PCR, and sequenced by means of Sanger sequencing. The location and

types of mutations were then determined by sequencing data.

The exome-Seq was performed and analyzed by Thomas Wingo from Emory University, Georgia.

2.3.10 DNA Mismatch repair assays

In vitro MMR assays were performed essentially as described with some modifications. A circular DNA substrate containing a G-T mismatch and a nick 3' to the mismatch was incubated with 75 µg or 150 µg of whole cell extracts at 37°C for 15 min. DNA samples were digested with Nsil (repair scoring enzyme), followed by polyacrylamide gel electrophoresis and Southern blot analysis to identify repaired and unrepaired molecules.

2.3.11 Microsatellite instability and HPRT mutability analyses

The MSI and HPRT mutability analyses were performed as described previously¹²⁹. For the detection of MSI in mouse bone marrow cells, 5 microsatellite markers (A33, Bat26, Bat66, D6Mit59, and D1Mit62 loci) were used. The D1Mit62 CA repeat loci were used as negative control as these loci are stable. For the human cell lines tested for MSI, independent single cell colonies were isolated in 96-well microtiter plates, and genomic DNA was isolated. Four microsatellite markers (BAT25, BAT26, D2S123, and D5S346) were used for MSI analysis. The HPRT mutation assay was conducted as described previously¹²⁹. Cells (5×10^5) were seeded in triplicate 10 cm Petri dishes for 12 hr and fed with complete medium containing 5 µM freshly prepared 6-thioguanine (6-TG). The plating efficiency was determined by culturing 500 cells similarly in the absence of 6-TG.

After 10 days of culturing, cell colonies were visualized by staining with 0.05% crystal violet. The mutation frequency was determined by dividing the number of 6-TG-resistant colonies by the total number of cells plated after being corrected for the colony-forming ability.

2.3.12 Confocal Microscopy and immunofluorescence analysis

Immunofluorescence analysis was performed as described below. Coverslips were treated by 0.01% polylysine for 5min at room temperature. The remaining polylysine solution was aspirated and the coverslips air-dried for 10min. 10^5 cells were seeded into 6-well plates with coated coverslips overnight. Next day, the medium was aspirated and the monolayer was washed 2X with PBS. Then the cells were fixed in fresh 2.5% PFA at 37°C for 10min. The coverslips were rinsed 2X with PBS and washed 1X with PBS in the dark for 10min. The cells were permeabilized and blocked with SS-PBS buffer (10% normal goat serum and 0.3% Triton™ X-100) at room temperature (RT) for 5min. After SS-PBS buffer was removed, 10µl primary antibody was added directly onto the coverslips for 60min. The well was rinsed 2X with PBS and 1X with SS-PBS in the dark for 10min. 10µl secondary antibody was added onto the coverslips directly after SS-PBS was removed from the well completely. The plate was left at RT in the dark for 30min. The coverslips were rinsed 2X with PBS, 2X with PBS+0.3% Triton™ X-100 and 2X with PBS. After wicking the majority of buffer from the coverslips, the coverslips were mounted using Prolong Gold anti-fade reagent. The coverslips were set overnight in the dark and used for imaging next day. Fluorescence images were obtained using Olympus FV1000 MPE or Leica SP8 MP. The images were analyzed by ImageJ 1.48 software.

2.3.13 Bisulfite-Seq and TAB-Seq

Genomic DNAs (500ng) from WT and Tet2^{-/-} mice LK cells were mixed with 130μl CT conversion reagent. The tubes were placed in a thermal cycler under the following program: 98°C, 10min; 64°C, 150min; 4°C for up to 20 hours. The products were transferred to spin columns with 600μl binding buffer. The columns with collection tubes were centrifuged at 10,000xg for 30s. After washing the column twice with washing buffer, the columns were filled with elution buffer. The eluted DNA was ready for PCR or further analyses.

Genomic DNAs from WT and Tet2^{-/-} mice LK cells were sheared to average 400bp. The β-glucosyltransferase reaction was prepared as follows: 4μg sheared gDNA, 0.4μl 10mM UDP-Glucose, 2μl 10xβGT protection buffer, 0.5μl T4-βGT and Milli-Q water to 20μl. The reaction was performed at 37°C for 1h. The products were then purified using a QIAquick PCR Purification Kit. Next, 500ng gDNA from the last step was incubated with TET1 protein and Tet2 oxidation reagent 1&2. The reaction was carried-out at 37°C for 1h. 1μl Proteinase K was added to the reaction and incubated at 50°C for 1h. The product was purified by Micro-Bio-Spin Columns and then with QIAquick PCR Purification Kit. The target sites were amplified by PfuTurbo Cx DNA polymerase following the manufacture's instructions. Generally, 50μl PCR included 5μl 10xPfuTurboCx reaction buffer, 1μl 10mM dNTP mix, 2μl 5mC control primers, 4μl Bisulfite treated DNA, 1μl PfuTurboCx DNA polymerase and Milli-Q water to 50μl. The verified PCR products were used for either individual clone sequencing or Genome-wide Sequencing analysis.

2.3.14 Statistical analysis

To determine differences between experimental groups, I used the Student's t-test or analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison tests as appropriate. P values < 0.05 were considered significant. For SNV count data, chi-square tests were used as implemented in R (<http://cran.r-project.org/>).

2.4 RESULTS AND DISCUSSION

2.4.1 Tet2^{-/-} mice develop lymphoid malignancies.

The TET2 gene is frequently mutated in human hematopoietic malignancies. Most of these homozygous and heterozygous mutations lead to truncation of TET2 protein and subsequently decreased TET2 enzymatic activity. Several groups generated Tet2 knockout mouse models to determine the role of TET2 in hematopoiesis and hematopoietic diseases⁸⁴⁻⁸⁷. Similar phenotypes were observed from these groups that Tet2^{-/-} mice developed a wide spectrum of myeloid malignancies and the loss of Tet2 was sufficient to promote myeloid transformation. It is still unknown if the deletion of Tet2 is able to cause lymphoid transformation, as previously reported, that TET2 mutations also exist in patients with lymphoid malignancies¹⁰⁰. A long-term investigation was performed to determine if the loss of Tet2 is sufficient to cause lymphoid transformation. I conducted a 2-year study on a cohort of Tet2^{-/-} and 67 wild-type (WT) mice. All the Tet2^{-/-} mice were born with expected Mendelian ratios, were viable, fertile with normal organ development, suggesting Tet1 and Tet3 may have extra roles in embryonic development. No expression level changes in Tet1 and Tet3 were seen in hematopoietic cells after Tet2

deletion, which eliminates the presumption of enzymatic compensation in TET family and endows a unique role for Tet2 in hematopoiesis.

Next, I analyzed details of the incidence, latency, and pathological features of hematological neoplasms that occur in Tet2^{-/-} mice and Tet2^{+/+} littermates. Strikingly, all of the 198 Tet2^{-/-} animals developed spontaneous lethal hematological malignancies with a latency ranging from 3 to 18 months, while no progressive leukocytosis was characterized in the hematopoietic organs of WT animals (Figure 14).

Analyses of these Tet2-deficient mice strengthened the finding that a majority of (>90%) these Tet2^{-/-} mice developed predominantly myeloid malignancies that include CMML-like disease, MDS and MPN with significant hematological phenotypes of leukocytosis (accompanied by neutrophilia and/or monocytosis), hepatosplenomegaly and marked expansion of well-differentiated myeloid cells or erythroid precursors in the bone marrow (BM), spleen, and liver that compromised the normal architecture of these organs. Interestingly, Tet2 loss also affects lymphoid-lineage development. The rest of Tet2^{-/-} mice developed T-cell (7 of 198, 3.5%) or B-cell (9 of 198, 4.5%) lymphoma/leukemia (Figure 14). With age, these mice showed lymphocytosis consisting of atypical lymphocytes, lymphadenopathy, hepatosplenomegaly, and/or enlarged thymus (in most cases of T-cell malignancies).

The mice with T-cell lymphoma/leukemia were characterized with lymphoid infiltration in BM, thymus, spleen and liver (Table 2.1). Histopathological analysis of moribund mice indicated complete effacement of the normal architecture of these organs with lymphoid elements. The morphology of atypical lymphocytes was observed (Figure 14). They were medium sized, immature appearing with irregular nuclei and small to

moderate amount of cytoplasm. The spleen specimen exhibited a diffuse infiltrate within both red and white pulp. The predominant hepatic perivascular and sinusoidal infiltrates were also observed after histological examinations. Flow cytometric analysis confirmed a predominant proportion of T-lymphocytes expressing CD3 and aberrantly co-expressing CD4 and/or CD8 in BM, spleen and thymus (Figure 15).

Atypical lymphoid infiltrate was also identified in the small intestines with lymphoepithelial lesions and lymphoid nodules in Tet2^{-/-} mice diagnosed as B-cell lymphoma/leukemia (Table 2.2). These atypical lymphocytes were medium sized with irregular nuclei, condensed chromatin and small amount of cytoplasm. Nodular lymphoid infiltrate disrupted the normal architecture of the spleen, especially in the white pulp. Requisite perivascular and sinusoidal infiltration occurred in the liver. The B-cell lineage was also altered in different tissues, particularly, B220^{+/low}IgM^{+/low}CD43⁺CD19⁺ with high forward scatter (FSC) (Figure 16).

To obtain a more detailed view of the role of Tet2 in hematopoiesis, I transferred Tet2^{-/-} spleen cells from Tet2^{-/-} mice with T- and B-cell malignancies to sub-lethally irradiated WT mice. Two weeks later, transplanted mice were analyzed. Both T- and B-cell malignancies were transplantable, suggesting the malignant and neoplastic nature of the infiltrating T- or B-lymphocytes in these Tet2^{-/-} mice (Figure 17).

Taken together, these results show that besides myeloid malignancies, Tet2^{-/-} mice also develop lethal lymphoid malignancies involving both T- and B-cell lineages. These findings are consistent with the clinical observations that loss of function mutations of TET2 are frequently associated with T- and B-cell malignancies. The kinetics of disease onset suggests that additional genetic lesion events may occur along with Tet2 ablation to

cause distinct types of hematological malignancies, either in myeloid or lymphoid lineage. It is reasonable to speculate that the deletion of Tet2 may contribute to locus specific hypermethylation. Aberrant methylation at specific myeloid, T-cell and B-cell promoters should correlate with transcription alterations and later disease development.

2.4.2 Acquisition of mutations in Tet2^{-/-} tumors and increased mutational frequency in loci marked by Tet2-dependent 5hmC

To explore if the loss of Tet2 leads to genome-wide genetic alterations, I performed comparative genomic hybridization array (aCGH) and whole-exome sequencing using tumor and/or non-tumor cell genomic DNA from Tet2^{-/-} mice with myeloid-, T- or B-cell malignancies. In the myeloid tumor sample, CGH analysis detected a gain of 16q (Figure 18). Both myeloid- and T-cell malignant genomes showed sub-chromosomal copy number alterations. While no significant alterations were seen from B-cell malignant genome (Figure 18).

The newer and current massive parallel sequencing techniques make it feasible to characterize somatic mutations in cancers. The exome (coding regions) sequencing allows us to identify driver mutations in cancer genome. To gain new insight into Tet2 loss induced leukemogenesis and relevant molecular basis underlying the clinical finding of multiple lineage involvement, I performed whole exome sequencing and subsequent Sanger sequencing to identify somatic mutations in Tet2^{-/-} myeloid-, T- and B-cell tumors. I captured and sequenced the exomes from paired samples of Tet2 loss induced tumors. After normalization (remove SNVs from WT and Tet2^{-/-} non tumor samples), I identified potential somatic mutations including 3,309 single-nucleotide variations (SNVs) and 211

small insertions or deletions (indels). I analyzed the alterations affecting protein-coding sequence, and 1,480 non-synonymous replacement sites and 1,829 silent sites per tumor type were found, which influence the integrity of open reading frame. Of the Tet2^{-/-} tumors from each cell origin, I found somatic mutations in 190 genes with recurrent SNVs that appeared to originate in the different tumors including Notch1, Ebf1, Flt3, Nf1, Rhoa and Ikzf3 (Figure 19). Integration of the above data, together with literature searching for these genes which have been frequently found in human hematological malignancies, led to the validation of exome sequencing results. I amplified the corresponding regions from original tumor samples and applied the PCR products for Sanger sequencing.

Non-synonymous mutations of Notch1 were identified in all 3 cases of Tet2^{-/-} T-cell tumors. As a critical transcription factor regulating innate and adaptive immunity, NOTCH1 can direct hematopoietic stem cell differentiation toward T-cells. In addition, NOTCH1 is frequently altered in hematopoietic and solid tumors. Intriguingly, NOTCH1 genetic mutations may lead to either ligand-independent activation or delayed turnover of NOTCH1 protein, depending on the context and mutation induced conformation changes¹³⁰.

In the hematological system, NOTCH1 mutations have been detected not only in ~55% of human T-cell acute lymphoblastic leukemia (T-ALL) but also in a high fraction of murine precursor-T lymphoma/leukemia in which NOTCH1 is a well-characterized oncogene¹³⁰. Previous studies have shown that the HD (heterodimerization) and PEST (proline-glutamic acid-serine-threonine rich) domains of NOTCH1 are mutation hotspots in T-ALL¹³¹. Indeed, the Notch1 mutations identified by exome sequencing of Tet2^{-/-} T-

cell tumors are all within these hotspots (p.Leu1668Pro, p.Arg1673Pro) and the Notch1 mutations in these hotspots were also found in 3 additional cases of Tet2^{-/-} T-cell tumors (p.Thr2346---, p.Ala1639Thr, p.His1598*) (Figure 20), providing novel insight into the T-cell leukemogenesis caused by the loss of Tet2.

Together, Tet2 loss-induced tumors are characterized by the acquisition of SNVs and small indel, but not large chromosomal deletions/amplifications, suggesting that the loss of Tet2 may create a genomic instability state in cells, where gene mutations accrue much more readily than normal. Indeed, hypermethylation states have been shown to be a potential mutagen in tumor cells.

Given the role of TET proteins in the oxidation process of 5mC, I next examined the effect of the loss of Tet2 on genome-wide 5hmC modification. I mapped the genomic distribution of 5hmC in WT and Tet2^{-/-} Lin⁻c-Kit⁺ (LK) BM cells using selective chemical labeling and affinity enrichment procedures (Figure 21). The deletion of Tet2 leads to a significant decrease of 5hmC at gene bodies, which is consistent with previous observations in mouse ES cells with a reduced expression of Tet2. The effects of Tet2 loss engendered a striking dependence on intermediately-expressed, but not highly-expressed genes in LK cells (Figure 22). The majority of Tet2-dependent 5hmC modifications in LK cells are located within the genes. Genome-wide 5hmC profiling of WT and Tet2^{-/-} LK cells identified the shared loci.

The shared loci represent the ones with 5mC oxidation catalyzed by other TET proteins, such as TET1, but not Tet2 (Tet2-independent sites). Both gain and loss of 5hmC loci in the absence of Tet2 may represent loci with Tet2-dependent 5mC oxidation and might reflect distinct role(s) for Tet2 in the 5mC oxidation process. The loci with the

loss of 5hmC in the absence of Tet2 are the ones that are converted from 5mC to 5hmC by Tet2 (Tet2-dependent 5mC conversion to 5hmC), while the loci with the gain of 5hmC more likely represent the regions with further 5mC oxidation (from 5hmC to 5fC/5caC) mediated by Tet2 (Tet2-dependent active DNA demethylation) (Figure 23). I then explored the overlap between the sites with SNVs/indel in Tet2^{-/-} tumors and the three different genomic loci. Using a chi-square test to examine the correlation between mutation sites and three different genomic loci, I observed a significantly greater frequency of SNVs/indel within the loci that require Tet2 for active demethylation, as compared to that of Tet2-independent sites and the loci that require Tet2 for the conversion of 5mC to 5hmC alone. Similar increases were observed with either overall mutations or C to T (or G to A) mutations (Figure 23 & 24). Thus the genetic loci marked by Tet2-mediated active demethylation are likely accessible by Tet2, which could in turn play a role in the maintenance of genomic stability.

I observed a decrease in 5hmC abundance at mutation sites as these regions close to CpG sites, which further indicates that Tet2 loss induced genetic mutations correlate with Tet2-mediated DNA demethylation. To determine the dynamics of 5hmC alterations at mutation sites, I used single-base resolution Bisulfite-Seq and TAB-Seq to examine the changes of cytosine modification between WT and Tet2^{-/-} young mice LK cells, in which I assumed that these mutation sites stay in origin. From representing gene analysis (Akap12), I found the “C” was hypermethylated in Tet2^{-/-} cells but no expected 5hmC changes were observed (Figure 25), suggesting that the C base confers an essential function to maintain genome integrity because 5mC is considered as a mutagen in the genome and the accumulation of 5mC may alter its active enzymatic deamination.

Although I was not able to detect 5hmC changes, I cannot exclude the dynamics of 5hmC because of 1) 5hmC overlapping region controlled by TETs; 2) 5hmC further oxidized to 5fC or 5caC; 3) Technique limitations.

2.4.3 TET2 interacts with MSH6

To investigate the mechanisms by which Tet2 loss may result in genomic instability, I sought the functional Tet2-binding partners. I generated knock-in ES clones in which tandem (Flag and V5) tags were inserted after the Tet2 start codon within exon 3 through homologous recombination (Figure 26). The knock-in allele allows the expression of both Flag-tagged Tet2 and WT Tet2 at roughly 1:1 ratio in the Tet2:Tag ES cells.

I characterized endogenous TET2-interacting proteins by mass spectrometry, and identified a list of TET2-interacting proteins including the known TET2-interacting protein OGT, and unknown associates MSH6 (Table 2.3). These results are intriguing as MSH6 is specifically involved in the DNA mismatch repair (MMR) of base-base mismatches and small insertion/deletion mispairs generated during DNA replication¹²⁹, consistent with the types of genetic alterations identified in the Tet2^{-/-} tumors. To confirm interaction between TET2 and MSH6, I performed co-immunoprecipitation assays using both Tet2:Tag ES cells and Flag-TET2 overexpressing HEK293 cells (Figure 27 & 28). TET2 was also captured in the MSH6 precipitates from mouse BM cells, confirming the endogenous TET2-MSH6 interaction in primary hematopoietic cells. To map the interacting regions between TET2 and MSH6, I generated a series of constructs including Flag-TET2 fragments and Myc-MSH6 fragments. Human TET2 gene was fragmented to four parts: hTET2-N (AA 1-942), hTET2-M (AA 916-1921) and hTET2-C (AA 1323-

2002, DSBH) (Figure 29). On the basis of the secondary structure of human MSH6, the MSH6 gene was dissected to five fragments that are MSH6-PWWP (AA 1-200), MSH6-MutSNter (AA 183-537), MSH6-MutSConnector (AA 525-735), MSH6-MutSCore (AA 700-1102) and MSH6-MutSCter (AA 1075-1360). TET2-N terminal showed interaction with full-length MSH6. To examine if MSH6 directly interacts with TET2 protein, I also co-expressed GST-MSH6 and TET2-N. These two recombinant proteins were co-purified in vitro, suggesting the direct binding between TET2 and MSH6 in vivo and in vitro. Moreover, the co-localization of the two proteins was examined using HeLa cells and immunofluorescence staining. Consistent with previous reports¹²⁹, MSH6 formed distinct foci in the chromatin of HeLa cells, and the localization of MSH6 and TET2 largely overlapped in these foci (Figure 30). These data collectively demonstrate that TET2 interacts with MSH6 in vivo.

2.4.4 TET2 loss causes a mutator phenotype in cells

Mismatch repair (MMR) of DNA corrects base-base and small insertion/deletion generated during DNA replication. Since MMR reduces the DNA replication errors, defective MMR increases the spontaneous mutation rate, which results in the “mutator phenotype”¹²⁹. In mammalian cells, these mispair errors are recognized by human heterodimer proteins hMutS α and hMutS β . MSH2 and MSH6 are major components of hMutS α and they form a heterodimer to repair the mispairs. Previous studies indicate that the alteration of MSH2 and MSH6 expression leads to susceptibility to certain types of cancer, especially hereditary non-polyposis colorectal cancer (HNPCC)¹³². The MMR defects cause the instability of high frequency of insertion/deletion in microsatellites at

the cellular level. Microsatellite instability (MSI) is a hallmark characteristic in hereditary nonpolyposis colorectal cancer (HNPCC), which is associated with mutations in the MMR pathway genes including MLH1, MSH2 and MSH6¹²⁹. In addition to colorectal cancer, MSI phenomenon is also observed in certain types of leukemia such as secondary AML and MDS¹³³.

The above TET2 and MSH6 interaction data prompted the following question. Does TET2 loss impair MMR and subsequently cause the mutator phenotype? To answer these questions, I first examined the *in vitro* MMR activity and MSH6 expression in the nuclear extract of WT and Tet2^{-/-} total BM cells. A functional *in vitro* MMR assay was performed. Surprisingly, both WT and Tet2^{-/-} BM cell extracts restored MMR efficiently (Figure 32), suggesting TET2 is not a critical factor for MMR *in vitro* and Tet2 loss does not alter the expression or function of MSH6 *in vitro*.

However, if the MSH6-TET2 interaction recruits hMutSa to chromatin, I cannot exclude the possibility that MSH6-TET2 interaction is required for MMR *in vivo*. If this is true, TET2 deficient cells should have impaired MMR and displayed an increased mutation frequency. To determine MSH6 chromatin localization, I depleted TET2 by shRNAs in HeLa cells (Figure 33), which is a MMR-proficient cell line. I used double thymidine block to synchronize control of HeLa (scramble shRNAs) and TET2KD HeLa cells in G1, S and G2/M phase. Then, endogenous MSH6 expression levels and chromatin localization were determined by immunofluorescence (Figure 31). Decreased MSH6 foci were observed in TET2KD HeLa cells compared to control HeLa cells (Figure 36), suggesting that MSH6 chromatin localization is facilitated by TET2. Around 10% of MSH6 foci co-localized with TET2 in WT HeLa cells indicating that not all

TET2 recruit MSH6. Like a previous report¹²⁹, decreased MSH6 foci is also seen in G2/M phase, but not because of altered MSH6 expression.

Control and TET2-depleted HeLa cells were then tested for MSI at four microsatellite loci as described previously¹²⁹. No MSI was detected in control HeLa cells, whereas 9-18% sub-clones (n=11) from TET2-depleted HeLa cells showed new microsatellite species at these loci (Figure 38). As a positive control, MSI was also analyzed in MSH6-deficient DLD-1 cells, which exhibited new repeat species in 27-36% sub-clones. Despite a difference in the percent sub-clones showing new repeat species, these data indicate that like MSH6-deficient DLD-1 cells, TET2-depleted HeLa cells display MSI. I also examined the frequency of MSI in BM DNAs from aged WT or Tet2^{-/-} mice. Tet2^{-/-} BM samples showed 43-86% novel alleles at A33, Bat26, Bat66 and D6Mit59 loci (Figure 37), while no novel alleles were detected at these loci in each of the WT BM samples, further demonstrating Tet2 loss displays MSI.

To further confirm that the deletion of TET2 leads to the mutator phenotype, I measured the spontaneous forward mutation frequency in the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene in TET2KD HeLa cells, control HeLa cells (negative control) and DLD-1 cells (positive control). Human HPRT gene encodes HPRT enzyme whose activity is important for DNA synthesis. 6-thioguanine (6-TG) is a toxic nucleoside analog and it is used for cell selection. Cells without HPRT gene mutations are poisoned by 6-TG, while HPRT gene mutations help cells survive and form colonies. As shown in Figure 34, the mutation frequency in TET2KD HeLa cells had a 24-fold increase compared to control HeLa cells and is ~10-fold lower than that in hMSH6-deficient DLD-1 cells (Figure 34). The presence of HPRT mutations was confirmed in

mutant clones of each cell type and most of the mutations were located at HPRT hot spots. This is likely due to both the efficiency of TET2 knockdown and a likely TET2-independent mechanism for MSH6-mediated MMR. Similar results were obtained using control and Tet2-knock down NIH3T3 cells (Figure 35), confirming that TET2 loss causes a mutator phenotype.

Lastly, I analyzed the number and type of mutations in a cohort of human MDS cases. Interestingly, MDS patients with TET2 mutations harbor a significantly higher number of mutational events compared to patients without TET2 mutations (Figure 39). The association of the presence of TET2 mutations with higher mutational frequencies in MDS patients is consistent with the findings that Tet2 loss constitutes genetic instability. Collectively, these observations are consistent with the hypothesis that TET2 is likely involved in recruitment of MSH6 to the chromatin, and TET2 loss impairs MMR *in vivo* thereby enhancing susceptibility to malignant transformation by a multitude of genetic mutations.

2.5 CONCLUSIONS

Studies using different Tet2 knock-out animal models have revealed that Tet2 loss alters the epigenetic landscape with decreased 5hmC and global hypermethylation of the DNA, increases HSC self-renewal and myeloid progenitor expansion with skewed differentiation toward monocytic lineages, leading to the pathogenesis of myeloid malignancies⁸⁴. Here, I show that a fraction of Tet2^{-/-} mice develop lymphoid malignancies of both T- and B-cell origin, demonstrating that TET2 plays an important role in both myeloid and lymphoid malignancies. Leukemogenesis is thought to be a

multi-step process in which a series of genetic mutations may be acquired in a specific clonal lineage. Genome analysis of Tet2^{-/-} tumors allowed not only a better understanding of Tet2 loss-mediated leukemogenesis, but also the identification of candidate secondary gene mutations that lead to diverse disease phenotypes in Tet2^{-/-} mice.

The exome sequencing identified numerous SNVs/indel in Tet2^{-/-} tumors of each cell origin, indicating that TET2 loss constitutes a state of genetic instability. The increased susceptibility of mutational acquisition is likely further accelerated in Tet2^{-/-} HSC/HPCs since TET2-loss induced intrinsic changes in these cells, with increased self-renewal and proliferation. TET2 loss represents an obvious initiating event in the pathogenesis of hematological malignancies. The occurrence of secondary oncogenic gene mutations, presumably in an early hematopoietic progenitor, is likely capable of modifying the disease phenotype and/or promoting the transformation/progression of a specific hematological malignancy in Tet2^{-/-} mice. This is evident by the acquisition of Notch1, Flt3, Nf1, Ebf1, Rhoa, Ikzf3 and other gene mutations in the Tet2^{-/-} hematological malignancies, consistent with the human hematological malignancy induction and maintenance by gene alterations of these critical genes. Consistently, clinical observations also suggest an initiating role for TET2 mutations in the pathogenesis of hematological malignancies.

Analysis of the clonal architecture in CMML showed that TET2 mutations are often the first detected oncogenic event¹³⁴. As well, the prevalence of TET2 mutations is higher in secondary than in de novo AMLs¹²³. Furthermore, single-cell-derived colony genotyping identified TET2 mutation as one of the earliest events in the accumulation of genetic alterations that lead to the leukemic clone expansion. And, a recent exome

sequencing study on angioimmunoblastic T-cell lymphoma showed that 68% of cases had both TET2 and RHOA mutations, and TET2 mutations predated RHOA mutations in most cases as RHOA mutations were specifically identified in tumor cells, whereas TET2 mutations were found in both tumor cells and non-tumor hematopoietic cells⁹⁹.

The MMR maintains genome stability primarily by correcting base-base and small indel mispairs generated during DNA replication. In mammalian cells, these mispairs are recognized by MSH2-MSH6 and MSH2-MSH3 complexes, both of which are required for MMR^{135,136}. Tet2^{-/-} tumors are associated with the acquisition of SNVs and small indel, and a significantly more SNVs were observed in loci containing Tet2-dependent active DNA demethylation, where Tet2 normally binds. MSH6 is a TET2, but not TET1, binding partner and Tet2 loss causes a mutator phenotype--hallmark of MMR defects, as evidenced by increased MSI and mutational frequencies at the cellular level. The data strongly suggest that TET2 is required for MMR in vivo by regulating MSH6 distribution on the chromatin. Therefore, the loss of TET2 could promote the local mutation rate in these Tet2-dependent active DNA demethylation loci by losing MSH recruitment. However, it is also possible that besides altering gene expression, the active DNA demethylation process itself could protect genomic stability. Indeed, 5mC has been shown to be a mutagen in tumor cells¹³⁷.

Interestingly, Li et al (2013) recently showed that H3K36me3 and SETD2 are required for MMR in vivo, since H3K36me3 recruits hMutS onto chromatin through direct interaction with MSH6. Further studies are warranted to determine if H3K36me3 and/or SETD2 play a role in the TET2-mediated recruitment of MSH6. Of note, Tet2-deficient cells that become MMR defective are not malignant per se, but since they

display a consistent increase in mutational rate, they could accumulate driver mutations in target genes leading to malignancies.

My results unveil a **novel role for TET2 in the maintenance of genome integrity**, and provide **significant insight into the mechanisms by which loss-of-function TET2 mutations causes diverse human hematological malignancies** (Figure 40). These important findings might aid the generation of new therapeutic approaches for these hematological malignancies.

Figure 14 $Tet2^{-/-}$ mice develop lethal lymphoid malignancies involving both T- and B-cell lineages.

(A) Kaplan-Meier survival curve of WT (n = 67) and $Tet2^{-/-}$ (n = 198) mice. (B) Pie charts show the percentage of hematological malignancies of myeloid-, T- or B-cell origin in $Tet2^{-/-}$ mice (n = 198). (C) May-Grunwald Giemsa stained PB smears prepared from a representative WT mouse and $Tet2^{-/-}$ mouse with T- or B-cell malignancy.

Figure 15 *Tet2*^{-/-} mice with T-cell malignancy.

(A) The gross morphologies of lymph nodes, spleen, liver, and thymus from a representative *Tet2*^{-/-} mouse with T-cell malignancy. (B) Flow cytometric analysis of the T-cell lineage (CD4/CD8) in BM, spleen and thymus of a representative *Tet2*^{-/-} mouse with T-cell leukemia/lymphoma and an age matched WT mouse. (C) H&E stained histological sections of femurs, spleen and liver from a representative *Tet2*^{-/-} mouse with T-cell leukemia/lymphoma and an age matched WT mouse.

ID	Age, days	Necropsy findings	Thymus weight (g)	Blood Counts			T-Cell Type	Diagnosis
				WBC, K/ μ L	LY, K/ μ L	RBC, M/ μ L		
G3-6	200	Hepatosplenomegaly, Lymphadenopathy	0.388	165.2	148.7	6.23	CD3 ⁺ CD4 ⁺ CD8 ⁺	T cell lymphoma/leukemia
3G-1	615	Hepatosplenomegaly, Lymphadenopathy	0.201	11.7	10.1	7.48	CD3 ⁺ CD4 ⁺ CD8 ⁻	T cell lymphoma/leukemia
G4-17	105	Hepatosplenomegaly, Lymphadenopathy	0.347		N/D		CD3 ⁺ CD4 ⁺ CD8 ⁺	T cell lymphoma/leukemia
G2-105	440	Hepatosplenomegaly, Lymphadenopathy	0.300	15.5	13.3	7.59	CD3 ⁺ CD4 ⁺ CD8 ⁻	T cell lymphoma/leukemia
G3-49	381	Hepatosplenomegaly	0.056 (Normal)	18.5	11.7	5.65	CD3 ⁺ CD4 ⁺ CD8 ⁻	T cell lymphoma/leukemia
G3-24	305	Hepatosplenomegaly	0.238	9.7	5.5	8.61	CD3 ⁺ CD4 ⁺ CD8 ⁺	T cell lymphoma/leukemia
G3-50	323	Hepatosplenomegaly	0.059 (Normal)	68.2	60.3	4.23	CD3 ⁺ CD4 ⁺ CD8 ⁻	T cell lymphoma/leukemia

Table 2.1 Phenotypic characteristics of T-cell malignancies developed in 7 Tet2^{-/-} mice.

Figure 16 *Tet2*^{-/-} mice with B-cell malignancy.

(A) The gross morphologies of lymph nodes, spleen and liver from a representative *Tet2*^{-/-} mouse with B-cell malignancy. (B) Flow cytometric analysis of the B-cell lineage (B220/IgM) in BM and spleen of representative *Tet2*^{-/-} mice with B-cell leukemia/lymphoma and an age matched WT mouse. The numbers indicate the percent cells in each cell population. (C) H&E stained histological sections of femurs, spleen and liver from a representative *Tet2*^{-/-} mouse with B-cell leukemia/lymphoma and an age matched WT mouse.

ID	Age, mo	Necropsy findings	Histology	B Cell Type	Blood Counts				Diagnosis
					WBC, K/ μ L	LY, K/ μ L	RBC, M/ μ L	PLT, K/ μ L	
G3-35	18	Hepatosplenomegaly, Lymphadenopathy	Invasion lymph nodes, intestine, liver	B220 ^{lo} IgM ⁺ CD19 ⁺ CD43 ⁺	12.3	8.2	8.89	451	B cell lymphoma/leukemia
G3-38	18	Hepatosplenomegaly, Lymphadenopathy	Invasion of lymph nodes, intestine, liver	B220 ⁺ IgM ⁺ CD19 ⁺ CD43 ⁺	11.9	7.1	5.76	249	B cell lymphoma/leukemia
G2-8	13	Hepatosplenomegaly, Lymphadenopathy	Invasion of BM, intestines, liver, kidney, lung	B220 ^{lo} IgM ⁺ CD19 ⁺ CD43 ⁺			N.D		B cell lymphoma/leukemia
G5-87	8	Hepatosplenomegaly, Lymphadenopathy	Invasion of spleen, liver, lymph nodes	B220 ^{lo} IgM ^{lo/c} CD19 ⁺ CD43 ⁺ CD5 ⁺	15.7	10.3	6.16	227	B cell lymphoma/leukemia
G3-322	3	Splenomegaly	Invasion of spleen	B220 ^{lo} IgM ⁺ IgD ^{lo} CD19 ⁺ CD43 ⁺ CD5 ⁺	15.7	12.3	9.99	106	B cell lymphoma/leukemia
G2-25	16	Hepatosplenomegaly, Lymphadenopathy	Invasion of spleen, liver, lymph nodes	B220 ^{lo} gM ⁺ CD19 ⁺ CD43 ⁺	41.9	29.8	6.01	324	B cell lymphoma/leukemia
G3-185	9	Hepatosplenomegaly, Lymphadenopathy	Invasion of spleen, liver, lymph nodes	B220 ⁺ IgM ^{lo/c} CD19 ⁺ CD43 ⁺	Very High	Very High	6.45	527	B cell lymphoma/leukemia
G5-50	11	Hepatosplenomegaly, Lymphadenopathy	Invasion of spleen, liver, lymph nodes	B220 ⁺ IgM ⁺ CD19 ⁺ CD43 ⁺ CD5 ⁺	183.7	172.4	6.17	412	B cell lymphoma/leukemia
G2-7	11	Hepatosplenomegaly, Lymphadenopathy	Invasion of BM, intestine, spleen, liver	B220 ⁺ IgM ⁺ CD19 ⁺ CD43 ⁺	54.1	43.7	9.37	774	B cell lymphoma/leukemia

Table 2.2 Phenotypic characteristics of B-cell malignancies developed in 9 Tet2^{-/-} mice.

Figure 17 Lymphoid malignancies in $Tet2^{-/-}$ mice are transplantable to secondary recipients.

(A) Tumor transfer schema. Spleen cells (1×10^6) from a WT mouse or $Tet2^{-/-}$ mice with T- or B-cell lymphoma/leukemia were each injected into sub-lethally irradiated (600cGy) CD45.1⁺/CD45.2⁺ F1 recipients. (B) Kaplan-Meier survival curve of sub-lethally irradiated recipients (5 animals / donor) transplanted with spleen cells from a WT mouse or $Tet2^{-/-}$ mice with T- or B-cell lymphoma/leukemia. (C) Flow cytometric analyses on the T-cell lineage (CD4/CD8) within the donor (CD45.2⁺) cell populations of peripheral blood cells from a representative recipient receiving spleen cells from a WT mouse or a $Tet2^{-/-}$ mouse with T-cell lymphoma/leukemia. Flow cytometric analyses on the B-cell lineage (B220/IgM) within the donor (CD45.2⁺) cell populations of peripheral blood cells from a representative recipient receiving spleen cells from a WT mouse or a $Tet2^{-/-}$ mouse with B-cell lymphoma/leukemia. Data demonstrate that the recipients receiving spleen cells from $Tet2^{-/-}$ mice with T- or B-cell lymphoma/leukemia developed diseases similar to their respective donor mice.

Figure 18 Array-based CGH ideograms of Tet2^{-/-} tumors.

DNA was extracted from 10 Tet2^{-/-} tumors (4 myeloid, 3 T- and 3 B-cell malignancies) and processed for hybridization to 244K Whole Mouse Genome Chip (G4122A; Agilent Technologies). Arrays were scanned with an Agilent scanner and analyzed with the Agilent Feature Extraction software. After processing on the Genomic Workbench system 5.0, normalized array-CGH values were used to calculate the median ratio at each individual chromosome for each tumor. Graphical representation of the data used the Visualization and Analysis of Array-CGH. Chromosomes are listed in numerical order from left to right. Red squares above zero on the y-axis represent genomic gains relative to the diploid control. The yellow lines indicate the absence of numerical alterations relative to the diploid control. The reference DNA was obtained from the bone marrow of a wild-type C57BL/6 female mouse.

Figure 19 Acquisition of mutations in $Tet2^{-/-}$ tumors.

Selected recurrent gene mutations in $Tet2^{-/-}$ tumors involving myeloid-, B- or T-cell lineages as identified by whole exome sequencing.

Figure 20 *Notch1* mutations were identified from *Tet2*^{-/-} mice with T-cell malignancy. Sanger sequence traces of 4 *Tet2*^{-/-} T-cell tumors with heterozygous mutations in *Notch1* are shown in the middle panel. The mutational locations are shown as red asterisks in the mouse *Notch1* protein schematic representation (upper panel). *Notch1* gene mutations identified by exome-sequencing and/or Sanger sequencing in 6 *Tet2*^{-/-} T-cell tumors are summarized in the lower panel.

Figure 21 5hmC distribution and enriched regions with genomic features.

(A) Distribution of averaged 5hmC enrichment at all genes in WT and Tet2^{-/-} LK cells. (B) Association of 5hmC-enriched regions with genomic features in LK cells. The percentage of 5hmC-enriched regions overlapping a defined genomic feature was compared with the percentage expected to fall in that particular region by chance based on the genomic coverage of that region. Values are expressed as fold change from expected.

Figure 22 Changes of 5hmC among the genes with different expression levels. Gene expression values (RPKM) obtained from RNA-Seq were used to divide all the genes into four groups: Highly expressed (top 25%), Intermediate Expressed (25-50%), Lowly expressed (50-75%) and Non Expressed (below 75%). Distributions of averaged 5hmC enrichment at all genes of each group in WT and Tet2^{-/-} LK cells are shown.

Figure 23 Increased mutational frequency in loci marked by Tet2-dependent 5hmC. Significantly greater frequencies of overall SNVs/indel (A, upper panel) or C to T (or G to A) mutations (A, lower panel) within the loci with Tet2-dependent active DNA demethylation, but not the regions with Tet2-dependent 5mC conversion to 5hmC or Tet2-independent 5hmC modification. Example of a genomic view showing the overlapping feature of Tet2-dependent active DNA demethylation, with *Rccd1* mutations (B).

Figure 24 Validated mutational signatures found in Tet2^{-/-} tumors.

(A) Mutational signatures of Tet2^{-/-} tumors characterized predominantly by C>T. (B) The mutated “C” favors formation of NCG.

Figure 25 Dynamic changes of 5mC and 5hmC surrounding the mutation sites.
 Akap12 mutational signature was characterized as CCG. Pie chart depicts the results of bisulfite-Seq and TAB-Seq.

Figure 26 Generation of Flag-V5 tag knock-in ES cells.

(A) Targeting strategy for Tet2:Tag allele ES cells. The targeting vector contained a Neo cassette flanked by two FRT sites, followed by a 0.5 kb genomic fragment upstream of the Tet2 start codon and an ATG/3xFlag/V5 sequence. A 2.2 kb 5' and a 4.8 kb 3' arm genomic fragments were sub-cloned into the vector for gene targeting. Positive ES cells were identified by Southern blot. Square bars indicate exons. E, EcoRI. S, ScaI. (B) Southern blot of ES cell DNA digested with ScaI and hybridized with a genomic fragment external to the 5' arm displayed a WT band of 6.3 kb and recombinant band of 4.0 kb (left panel), and ES cell DNA digested with EcoRI and hybridized with a probe external to the 3' arm displayed a WT band of 10.0 kb and a recombinant band of 8.9 kb (right panel). (C) Comparable WT Tet2 and Tag-Tet2 transcripts in Tet2:Tag ES cells. Data reported as relative expression units to the WT Tet2 expression in WT ES cells using β -actin as internal calibrator.

Protein	Size	No. of Peptide	No. of Peptide (control)
TET2	220	78	0
OGT	110	2	2
MSH6	163	2	9
TOP2A	174	47	32
SIN3A	15	1	1
MSH2	104	1	1
BRCA1	199	4	0
RAD51	37	2	0

Table 2.3 Table of the most relevant proteins identified by mass spectrometry in the purification of TET2-associated proteins using Tet2:Tag knock-in ES cells. Spectral counts for each interacting protein are shown.

Figure 27 Flag-TET2 is expressed as a 240kD protein and localized in the nucleus. pcDNA3.1-hTET2 (TET2) or empty vector (Mock) was transfected into 293 cells using Lipo3000 transfection reagent. (A) Schematic map of pcDNA3.1-hTET2 construct. (B) Western blot was performed with cytosol and nuclear fractions using anti-Flag antibody. A ~240kD band was detected in the nuclear fraction of TET2-transfected 293 cells, but not Mock cells, which agrees with the calculated molecular mass of Flag-TET2 based on its primary structure. (C) Cells were stained with anti-Flag antibody followed by the orange-fluorescent second antibody and green-fluorescent conjugated anti-F-actin antibody. The cells were then stained with DAPI (blue). Merged image artificially expressed in pink indicating co-localization of Flag-TET2 with DAPI, but not F-actin.

Figure 28 TET2 associates with MSH6.

Reciprocal co-immunoprecipitation and western blots confirmed the interaction of TET2 with MSH6 in HEK293T cells overexpressing Flag-tagged TET2. Non-transfected (ctrl) or Flag-TET2 overexpressing (TET2) HEK293 cell nuclear lysates were immunoprecipitated with anti-Flag (A) or anti-MSH6 (B) antibodies. The presence of Flag-TET2, MSH6, OGT and MSH2 in the precipitates was visualized by western blot using indicated antibodies. Whole cell lysate was used as the input. TET2 shows interaction with OGT in both Tet2:Tag ES cells and Flag-TET2 overexpressing HEK293 cell as previously reported.

Figure 29 MSH6 co-immunoprecipitates with TET2 N-terminal.

MSH6 failed to bind to TET2M and TET2C in HEK293 cells overexpressing each Flag-tagged TET2 fragments. Nuclear extracts were immunoprecipitated with anti-MSH6 antibody and probed with anti-FLAG or anti-MSH6 antibodies.

Figure 30 Immunostaining identified TET2 and MSH6 co-localization.

MSH6 foci formation and MSH6-TET2 co-localization in HeLa cells.

Immunofluorescence was performed to determine nuclear distribution of MSH6 and TET2. Cells were stained with anti-TET2 antibody followed by the red-fluorescent second antibody and anti-MSH6 followed by green-fluorescent second antibody. The cells were then stained with Hoechst33342 (blue). Merged image artificially expressed in yellow indicating co-localization of TET2 with MSH6 in the nuclear.

Figure 31 TET2 and MSH6 co-localization at different cell cycle.

HeLa cells were synchronized at G1, S and G2/M phase using double thymidine block. Immunofluorescence was performed to determine co-localization of MSH6 and TET2. Cells were stained with anti-FLAG antibody followed by the red-fluorescent second antibody and anti-MSH6 flowed by green-fluorescent second antibody. Merged image artificially expressed in yellow indicating co-localization of TET2 with MSH6 in the nucleus.

Figure 32 Tet2 is not physically involved in MMR activity *in vitro*.
In vitro MMR assay using nuclear extracts isolated from WT or Tet2^{-/-} BM cells.

Figure 33 Knockdown of TET2 in HeLa cells using human TET2-specific shRNA. Western blot and qPCR analysis showing the expression of TET2 protein and mRNA in HeLa cells stably transfected with a scrambled shRNA or a TET2-specific shRNAs.

A

Cell line	Mutation Frequency (x10 ⁻⁷)	Fold change of Mut.Freq.	p value
HeLa Scramble shRNA	5.8±1.7	1	0.029
HeLa TET2shRNA	137.3±39.8 (SD)	24	
DLD-1	1370.6±66.4 (SD)	236	

Figure 34 Increased mutational frequency in TET2 knockdown HeLa cells.

(A) HPRT mutability in control- and shTET2-HeLa cells. Data presented as mean ± SD. Fold increase in mutation frequency was calculated using the mutation frequency of control NIH3T3 cells as a reference, and a t test was used to estimate a p value. (B) Low density 6-TG survival assay indicating resistance of TET2 knockdown HeLa cells.

B

Cell line	Mutation Frequency ($\times 10^{-7}$)	Fold change of Mut.Freq.	p value
NIH/3T3 Scramble shRNAs	9.3	1	0.027
NIH/3T3 Tet2 shRNA1	142.9 $\pm$ 22.5 (SD)	15	
DLD-1	1370.6 $\pm$ 66.4 (SD)	148	

Figure 35 Increased mutational frequency in shTet2-NIH3T3 cells.

(A) Western blot and qPCR analysis showing the expression of Tet2 protein and mRNA in NIH3T3 cells stably transfected with a scrambled shRNA or a Tet2-specific shRNAs. (B) HPRT mutability in control- and shTet2-NIH3T3 cells. Data presented as mean $\pm$ SD. Fold increase in mutation frequency was calculated using the mutation frequency of control NIH3T3 cells as a reference, and a t test was used to estimate a p value.

Figure 36 MSH6 foci formation and localization in control- and shTET2- HeLa cells

Figure 37 Analysis of PCR product patterns of microsatellite markers in bone marrow cells from WT and *Tet2*^{-/-} mice.

The appearance of novel length alleles of the A33, D6Mit59 loci is indicated (asterisk) for each sample. The D1Mit62 CA-repeat locus was used as a negative control, as this locus is stable. No alterations were found at this stable locus in both WT and *Tet2*^{-/-} samples.

Figure 38 Analysis of PCR product patterns of two microsatellite markers in control- and shTET2-HeLa cells.

The asterisk shows the clone exhibiting new repeat species.

Figure 39 Mutation numbers in MDS patients with or without TET2 mutations. Exome sequencing data from a cohort of human MDS patients were analyzed for the number and type of mutations and were summarized as mean $\pm$ SD in groups with (n=5) or without (n=5) TET2 mutations.

Figure 40 Proposed model for loss of Tet2 induced hematological malignancies.

In wild type individuals, active DNA demethylation can be initiated by TET2-mediated 1) left panel, TET2 catalyzes the hydroxylation of 5mC to 5hmC, followed by AID/APOBEC mediated conversion from 5hmC to 5hmU, which can be further recognized by TDG and repaired through BER pathway. 2) right panel, 5mC is metabolized through deamination to T to generate T:G mismatch, followed mismatch repair mechanism through TET2 recruited MSH2, MSH6 heterodimer complex, which replace T back to C during DNA replication. In Tet2^{-/-} individuals, the loss oxidative enzymatic activity eliminate Tet2-mediated oxidative pathway; while 5mC can still be converted to T. The newly generated T:G mismatch could not be repaired by MSH2, MSH6 heterodimer because of the loss of Tet2. This may eventually lead to disease-causing mutations.

CHAPTER
TET2 REGULATES ADULT BRAIN NEUROGENESIS

3.1 ABSTRACT

Previous studies that have mapped genome-wide 5hmC, have shown that 5hmC distributes temporally and spatially during neurodevelopment^{61,118,119}. Interestingly, 5hmC acquisition in these regions (5mC specific) is different than what would be expected. There is no obvious correlation between 5hmC gain and 5mC loss in many important neurodevelopment-related genes, therefore one could argue that 5hmC role in gene regulation may be via a mechanism other than by transient oxidation in the brain¹¹⁸. In 2011, a study found Tet1 overexpression in the dentate gyrus (DG) significantly down-regulated the methylation levels of Bdnf and Fgf1b, highlighting the role of 5hmC in neurogenesis¹¹⁷. In addition, several types of neurodevelopment and neurodegenerative disorders have been found closely associated with 5hmC, such as Rett syndrome, Autism spectrum disorder and Alzheimer's disease. Because of the extensive roles of 5hmC and TETs, several studies have explored TET proteins functions in neurogenesis^{117,121}. There is evidence that Tet1^{-/-} mice exhibit robust loss (~45%) of NSCs in the SGZ compared with WT mice, and neurospheres from Tet1^{-/-} show growth defects. Microarray studies have shown that many genes (Galanin, Ng2 and Ng2) that are involved in NSC proliferation are down-regulated¹²¹. In addition, Tet1^{-/-} mice show increased hippocampal long-term repression and memory defects¹²¹.

Because DNA methylation is critical for postnatal neurodevelopment, 5hmC may also be involved in 5mC dynamics during aging. I first assessed the abundance of 5hmC in the subventricular zone of the lateral ventricles and there was a substantial overlap between 5hmC+ and NeuN+ cells in the granule cell layer in the dentate gyrus of the hippocampus, whereas I noted a significant decrease of Tet1 mRNA and increase of Tet2

mRNA in an age-dependent manner. Also, I observed the down-regulation of 5hmC abundance at both postnatal day 7 and adult age compared to WT. Furthermore, to explore the function of Tet2 in adult neurogenesis, BrdU incorporation assay and immunofluorescence staining were performed. Interestingly, Tet2 positively regulates neural stem/progenitor cells proliferation and differentiation. As well, luciferase reporter assay indicated that Tet2 regulates the methylation dynamics of NeuroD1 and Gfap promoter regions. Consistent with these observations, mRNA levels of NeuroD1 and Gfap were decreased in Tet2-deficient cells. These results suggest that Tet2 is positively involved in adult brain neurogenesis through epigenetic regulation.

3.2 INTRODUCTION

Adult neurogenesis occurs in two germinal zones of the brain: the subgranular zone (SGZ) in dentate gyrus of the hippocampus and subventricular zone (SVZ) within the walls of the lateral ventricles. Adult neurogenesis can be regulated by many cellular activities including neural stem cells (NSC)/neural progenitor cells (NPC) proliferation and differentiation, fate determination, maturation and localization of newborn neurons^{115,116}. Since neurogenesis exists in these two regions, it is possible to postulate that neurogenesis may regulate learning and memory. As well, adult neurogenesis can be regulated by signaling pathways and epigenetic regulators^{116,138}.

Ten-eleven translocation (TET) family proteins contain two key components: Fe (II) and 2-oxoglutarate (2-OG) to catalyze oxidation of the methyl group of 5mC to hydroxymethyl, formyl and carboxyl groups, which generate 5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxylcytosine in an enzymatic activity-dependent manner^{39,65}.

Studies that have mapped genome-wide 5hmC, have shown that 5hmC distributes temporally and spatially during neurodevelopment^{61,118,119}. For example, studies have shown an age-dependent abundance and distribution of 5hmC in the cerebellum and hippocampus. Interestingly, 5hmC acquisition in these regions is different than would be expected. There is no strong correlation between 5hmC acquisition and 5mC loss in many important neurodevelopment-related genes, therefore one could argue that 5hmC role in gene regulation may be via a mechanism other than by transient oxidation in the brain¹¹⁸. To better understand the role of 5hmC in the brain, more than 6000 differentially hydroxymethylated regions (DhMRs) at different ages have been identified. These DhMRs were only found in adult brain, from 6-week-old brain, but not in postnatal day 7 (P7) cerebellum, indicating long-term modification of 5hmC in the brain^{118,119}. Although the correlation of 5hmC abundance and gene transcription is still unclear, the enrichment of 5hmC and loss of 5mC at gene bodies coincide with gene transcription activation¹¹⁹. In fact, 5hmC regulation of neurogenesis was reported in 2011. Overexpression of Tet1 in the dentate gyrus (DG) significantly down-regulated the methylation levels of Bdnf and Fgf1b, highlighting the role of 5hmC in neurogenesis¹¹⁷. Several types of neurodevelopment and neurodegenerative disorders have been found closely associated with 5hmC, such as Rett syndrome, Autism spectrum disorder and Alzheimer's disease^{119,120,139,140}.

Because of the extensive roles of 5hmC and TETs, several studies have explored TET proteins functions in neurogenesis^{117,121}. There is evidence that Tet1^{-/-} mice exhibits robust loss (~45%) of NSCs in the SGZ compared with WT mice, and neurospheres from

Tet1^{-/-} show growth defects. Microarray studies have shown that many genes (Galanin, Ng2 and Ng2) that are down-regulated during NSC proliferation¹²¹.

The TET2 gene is well-characterized in the hematological system, however, little is known about its biological role in the nervous system. Considering the high abundance of 5hmC in the mouse adult brain, I tested the assumption of a critical role for Tet2 in the adult brain and for changes in neural processes, by proposing to examine roles and relationships of Tet2 in the nervous system using a Tet2 knockout mouse model.

To this effect, I answer the following three questions regarding to TET2 in nervous system: 1) Does Tet2 loss alter 5hmC level in the brain? 2) Does Tet2 regulate the proliferation and differentiation of NSCs? 3) What are the downstream targets of Tet2 in the brain? First, I used Tet2^{-/-} mouse models to examine the expression and localization of Tet2 at an embryonic stage. Because of the association between 5hmC and Tet2, I also measured 5hmC abundance in different brain tissues in the absence of Tet2. During NSC proliferation, DNA methylation can remain with relatively long-lasting effects after several rounds of cell division¹³⁸. As another form of epigenetic mechanism, DNA methylation indeed plays an essential role in regulating adult neurogenesis. Previous studies on Methyl-CpG-binding domain 1 (MBD1) and methyl CpG binding protein 2 (MeCP2) indicated their binding methylated CpG capacity in the promoters, which subsequently suppressed gene expression. Mice with Mbd1-deficiency showed decreased neurogenesis and impaired cognition through behavior studies. The promoter of fgf2 was hypomethylated, which led to decreased neuronal differentiation¹⁴¹. In another study, when Mecp2 was down-regulated, abnormal maturation and synaptogenesis of granular neurons were present. Also, MeCP2 may influence glia-specific gene expressions like

GFAP¹⁴². More recently, a DNA demethylation study of Tet1 showed that Tet1 is involved in neuronal active DNA demethylation and gene expression in mouse dentate gyrus¹¹⁷. And, although Tet2 has been shown to be involved in hematopoietic malignancies, the expression of Tet2 is relatively high in the nervous system than hematological system¹⁴³.

Therefore, I used Tet2^{-/-} mouse model to investigate whether Tet2 can regulate adult neurogenesis. The loss of Tet2 increased the proliferation of NSC and decreased the differentiation of NSC. Since DNA methylation is critical in postnatal neurodevelopment and seems to undergo age-dependent changes in the brain, I collected and examined the hippocampus and cerebellum of mice at postnatal day 7 (P7), 6 weeks and 1 year of age. Proliferation and differentiation of NSC/NPC and the underlying molecular mechanisms were examined to uncover the role of Tet2 in adult neurogenesis.

3.3 MATERIALS AND METHODS

3.3.1 Generation of Tet2^{-/-} mice and LacZ staining

Tet2: LacZ KI mice were generated as previously described⁸⁴. Briefly, the start codon ATG was replaced by LacZ: GFP: Neo cassette. After crossing with Flipase and/Cre mice, Tet2: LacZ and Tet2: GFP mouse models were obtained. The female mice were euthanized according to *The Guide* and as required by IACUC protocol. Embryos were collected from the uterus and extra tissues were removed in PBS. Embryos were fixed in whole mount fixative for 40min on ice and rinsed 3 times (3X) in PBS for 15min each. The fixed embryos were then stained by X-gal staining solution at 37°C O/N in the dark, then rinsed in PBS for imaging.

3.3.2 Adult NPCs isolation and culture

Neural progenitor cells were isolated from 6-week-old male WT and Tet2^{-/-} mice. Briefly, the dentate gyrus (DG) was dissected-out from coronal sections. Enzymatic digestion was used to dissociate the cells. Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 2mM L-Glutamine and 1% P/S was added to each sample. The cells were filtered through 70-µm cell strainer and washed with DMEM. The cells were then loaded onto 50% Percoll for ultracentrifugation at 127k RPM for 30 min at 20°C. NPCs fraction was cultured in DMEM supplemented with 20ng/ml FGF2, 20mg/ml EGF in a 5% CO₂ cell culture incubator at 37°C.

3.3.3 Proliferation and differentiation analyses of cultured adult NPCs

Neural progenitor cells (between passage 4 to 10) were trypsinized and placed on poly-L-laminin coated chamber slides. Each well contained about 50,000 cells and complete DMEM medium supplemented with growth factors (FGF2 and EGF). 20 hours after plating, the culture medium was supplemented with 5µM BrdU for an additional 8h. NPCs were fixed with 4% paraformaldehyde solution (PFA) for 30min at room temperature followed by 1M hydrochloric acid (HCl) for 30min at 37°C and then washed by borate buffer for 30min, for immunohistochemistry. For NPCs differentiation, NPCs were cultured with differentiation medium, DMEM with 5µM forskolin, 1µM retinoic acid and 0.5% FBS. The NPCs were cultured by differentiation medium for 4 days in a 5% CO₂ cell incubator at 37°C for immunostaining later.

3.3.4 Real-time PCR

Total RNA was purified from WT and Tet2 KO brain tissue using RNeasy kit (Qiagen). The RNA was reverse transcribed to cDNA. RT-qPCR was performed in 20 μ l aliquots with 10 μ l Fast SYBR Green Master Mix, 200nM forward and reverse primers mix and 20ng cDNA. The triplicate reactions were applied to ABI 7500 Fast Real-Time PCR System and quantified using SDS 1.4 software. The relative quantification of Tet2 mRNA was determined by threshold cycle value and normalized to the corresponding value of mouse actin.

3.3.5 Confocal Microscopy and immunofluorescence analysis

The protocol has been described in the previous chapter. Briefly, cells were grown in 6-well plates on coverslips. Growth medium was aspirated and cells were fixed with 4% formaldehyde in PBS for 15 min at room temperature. Then, the fixative was discarded and the cells were rinsed with PBS 3 times. The cells were incubated by blocking buffer for 60min and then incubated with primary antibody overnight at 4°C. The primary antibody was removed and fluorochrome-conjugated secondary antibody was added to the specimen for 1h at room temperature in the dark. Coverslips were mounted by DPAI associated mount reagent and observed by fluorescence microscopy.

3.3.6 Dot blot

Genomic DNA was isolated from NSC harvested from WT and Tet2^{-/-} hippocampus and denatured by 0.2N NaOH. Samples were dotted on nitrocellulose membrane. After cross-linking by ultraviolet, the membrane was blocked in 10% nonfat milk and 1%

bovine serum albumin (BSA). The primary antibody anti-5hmC and secondary antibody HRP-conjugated goat anti-mouse were incubated with membrane, respectively. Signals were detected by ECL detection system as described in the previous chapter.

3.3.7 DNA plasmids and luciferase assay

NeuroD1 and Gfap promoter regions were amplified by PCR from mouse brain cDNA. The PCR products were cloned into pGL3 and confirmed by Sanger sequencing. Human elongation factor 1a promoter region served as an internal control. 2×10^6 NPCs were trypsinized and mixed with plasmids, and then applied to electroporation. The cells were placed into proliferation medium and differentiation medium for 24h and 48h, respectively. The cells were then lysated and collected for luciferase assay. About 20 μ l cell lysate was mixed with 100 μ l Luciferase assay buffer II. F-luc activity was measured by SpectraMax M2E plate reader and the mixture was stopped using Glo Buffer with substrate. After normalization with R-luc, the transfection efficiency was calculated based on a commercial manual (Promega).

3.3.8 Statistical analysis

To determine differences between experimental groups, I used the Student's *t*-test or analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison tests as appropriate. P values < 0.05 were considered significant.

3.4 RESULTS AND DISCUSSION

3.4.1 TET2 is highly expressed in the central nervous system

Since DNA methylation is critical for postnatal neurodevelopment in an age-dependent manner, 5hmC may also be involved in 5mC dynamics during aging. To address these issue, I assessed the abundance of 5hmC in anterior regions of the subventricular zone (SVZa), a region of high neurogenesis, and rostral migratory stream in olfactory bulb (RMS-OB) using 5hmC specific antibody. This procedure allowed me to discover the most active neurogenesis regions with 5hmC positive cells, which also helped to define the regions to be studied in the future. Immunohistochemistry of 5hmC in these regions showed no significant co-localization between 5hmC positive cells and NeuN positive cells in SVZa (Figure 41). However, in the granule cell layer (GCL), there was a substantial overlap between 5hmC⁺ and NeuN⁺ cells, suggesting 5hmC is required for neurodevelopment. Because TET family proteins catalyze the oxidation of 5mC to 5hmC³⁷, I speculated that Tets may be required for 5hmC dynamics during neurodevelopment. A significant decrease of Tet1 mRNA levels was seen during aging from P7 to 6 weeks old, in the cerebellum, whereas an increase in Tet2 mRNA levels occurred during aging (Figure 42). Together, the data suggest that both 5hmC and Tet2 are required for age-dependent neurodevelopment.

To determine genome-wide Tet2 function and 5hmC distribution during neurodevelopment and aging, I used a Tet2 knockout mouse model that had been previously generated in Dr. Mingjiang Xu's lab. To examine the localization of Tet2 in vivo, LacZ-GFP-Neo lines was mated with Flipase mice to acquire LacZ-GFP line. The embryos of Tet2: LacZ were collected at embryonic day 13.5 (E13.5) and stained with

regular β -gal (Figure 43). Despite some staining variability between embryos, the LacZ expression was observed in both the cerebellum and spinal cord suggesting a potential role for Tet2 in neural cells.

To further confirm the elimination of Tet2 in Tet2 knockout mouse models, I collected the brains of Tet2: GFP. The brain tissue was disrupted and total RNA was purified from it. After reverse transcription, cDNA of Tet2: GFP was applied to RT-qPCR to examine relative Tet2 expression. Five primers were designed, which aimed at exon3, 4, 6 and 11 (Figure 44). Clearly, Tet2 was completely lost at ATG region exon3 and exon4. However, there was still low abundance of the last several exons that may be due to the different splicing forms of Tet2.

Although I did not see developmental defects in Tet2^{-/-} mice, the role of Tet2 in adult functioning still needs to be elucidated. Given the high abundance of 5hmC in mouse brain, it is reasonable to propose a regulatory function for Tet2-mediated 5mC oxidation in adult neurogenesis. To determine whether Tet2 deficiency affects 5hmC dynamics, I compared the abundance of 5hmC of WT and Tet2^{-/-} mice cerebellum. At P7, 5hmC in Tet2^{-/-} samples decreased to half of the amount of 5hmC in WT samples. In adults, 5hmC in Tet2^{-/-} samples decreased to 3/4 of the amount of 5hmC in WT samples (Figure 45). This suggests that Tet2 is required for 5hmC dynamics in both P7 and adults.

3.4.2 Hydroxylation of 5-methylcytosine affects stem cell proliferation and differentiation in dentate gyrus of the hippocampus

To explore the function of Tet2 in adult neurogenesis, I examined the expression pattern of 5hmC in the dentate gyrus of adult hippocampus. I isolated adult NPC from

dentate gyrus of WT and Tet2^{-/-} mice. As shown in (Figure 46), all cultured adult NPCs showed positive Nestin and Sox, which are the markers for progenitor cells. It is feasible to use these cells for molecular and functional analyses because of their homogeneity. I then examined the proliferation of these NPCs by exposing the cells to BrdU for eight hours. It is obvious that Tet2^{-/-} NPCs exhibited 1.5 fold BrdU incorporation compared to that in WT NPCs.

To determine the effect of Tet2 on NPC differentiation, both WT and Tet2^{-/-} DG NPCs were exposed to differentiation medium for three days. Several independent assays were performed to investigate the phenotypes of WT and Tet2^{-/-} NPCs differentiation (Figure 47). I first stained the differentiated cells with β -III tubulin (Tuj1) and glial fibrillary acidic protein (GFAP) (Figure 48). Tuj1 and GFAP are specific cell markers for neurons and astroglia, respectively. In addition to WT NPCs, Tet2^{-/-} NPCs were also able to differentiate to neurons and astrocytes. Interestingly, Tet2^{-/-} NPCs showed a 70% decrease in neuronal cell differentiation and a 50% decrease in astrocyte differentiation compared with WT NPCs. These data indicate Tet2 negatively regulate NPC proliferation but positively regulate NPC differentiation.

3.4.3 TET2 regulates the expression of NeuroD and GFAP transcriptionally

The downstream targets of Tet2 are still unknown. To validate the immunocytochemical results, I tested the assumption that two neural cell markers may be altered by Tet2 deletion: neurogenic differentiation 1 (NeuroD1) and GFAP. NeuroD1 is a pan-neuronal transcriptional activator, which contributes to transcriptional regulation of neurogenesis. GFAP is an astrocyte specific cell marker during development. The

NeuroD1 and Gfap promoters were cloned upstream of luciferase reporter construct, allowing the detection of transcriptional features of these neural factors. Cells lysates were mixed with luciferase substrate and measured by luminometer. As I expected, the deletion of Tet2 suppressed the Luc activity at 48h post-transfection. Little luciferase expression was shown in Tet2^{-/-} cell lysate. Furthermore, the relative mRNA expression of NeuroD and Gfap in Tet2^{-/-} NSC were much lower than that in WT NSC (Figure 49). Taken together, these data suggest that Tet2 is capable of regulating NeuroD and Gfap expression through promoter suppression.

3.5 CONCLUSIONS

TET family proteins have been well-studied recently, especially their enzymatic function in active DNA demethylation³⁹. However, the physiological function of Tet2 in neural systems remains unknown. Although no significant development defects were observed in Tet2^{-/-} mice from previous reports⁸⁴, my data indicates a transcriptional regulatory role for Tet2 in adult neurogenesis.

To determine if the transcription levels of Tets change during postnatal development and aging, I measured Tet1, Tet2 and Tet3 mRNA levels at two different ages, P7 and 6 weeks of age. I observed a remarkable decrease of Tet1 mRNA and increase of Tet2 mRNA during aging, suggesting potential opposite functions of Tet1 and Tet2 during postnatal development. Indeed, I found that Tet2 was expressed in the brain and spinal cord from Tet2:LacZ mouse models. Immunostaining of 5hmC showed Tet2 regulates 5hmC abundance preferentially in SGZ rather than SVZ. Finally, I found that overall, 5hmC abundance was decreased in both Tet2^{-/-} proliferating cells and Tet2^{-/-}

differentiating cells, suggesting that the loss of Tet2 impairs the oxidation of 5mC and subsequently influences NPC proliferation and differentiation. I also examined proliferation and differentiation of WT and Tet2^{-/-} NPCs *in vitro*. Loss of Tet2 leads to decreased neuronal differentiation and glial differentiation. I speculated these cellular phenotypes might be induced by the alterations of neural-related transcription factors, which results from the hypermethylation in their promoter regions. Notably, I found two genes NeuroD1 and GFAP mRNAs were reduced and their promoter activities were decrease in Tet2^{-/-} cells. Therefore, it is possible that during adult neurogenesis, Tet2 regulates the gene's promoter region and catalyzes the oxidation of 5mC, which allows the transcriptional activation of the genes and balances neuronal and glial cell numbers.

In summary, I characterized Tet2 and 5hmC in mouse cerebellum and hippocampus during postnatal development and aging. Tet2 regulates 5mC oxidation at NeruoD1 and Gfap promoter regions during aging, pointing to a critical role for Tet2 in adult neurogenesis. My study has great potential to improve the understanding of Tet2-mediated DNA demethylation in neurodevelopment and neurodegenerative disorders.

Figure 41 Immunostaining of 5hmC in two brain regions in mice.

(A) 5hmC specific immunostaining in 6-week old anterior regions of subventricular zone.

(B) 5hmC specific immunostaining in 6-week old olfactory bulb.

Figure 42 Tet1, Tet2 and Tet3 expression levels in P7 and 6-week old cerebellum.
Quantification of Tet1, Tet2 and Tet3 mRNA levels in different individuals (A&B) (n = 3, mean $\pm$ SEM.; *P < 0.05, **P < 0.01, ***P < 0.001, ANOVA)

Figure 43 Tet2 is expressed in the nervous system.

(A) Gene targeting strategy. Exons are filled boxes and LacZ-GFP-Neo cassette was introduced into Tet2 start codon region. (B) LacZ staining of Tet2:LacZ embryos showing Tet2 expression in the brain and neural tube.

Figure 44 Detection of Tet2 in WT and Tet2^{-/-} hippocampi.

(A) Schematic of real-time PCR primers used to detect Tet2 mRNA. (B) The sequences of real-time PCR primers. (C) Real-time PCR analysis of Tet2 mRNA levels in the brain. Actin was used for sample normalization.

Figure 45 Quantification of 5hmC in P7 and adult cerebellum.

(A) Measurement of 5hmC in genomic DNA from P7 and adult cerebellum by dot blot using anti-5hmC antibody. (B) 5hmC quantification of (A).

Figure 46 Cultured adult neural progenitor cells expressed representative cell markers. Nestin (cytoplasmic, red) and Sox2 (nuclear, green; DAPI in blue).

Figure 47 Quantification of 5hmC in proliferating and differentiating cells from WT and Tet2^{-/-} adult NPCs.

(A) NPCs were harvested from WT and Tet2^{-/-} mice with splenomegaly. (B) Detection of 5hmC in adult NPCs from WT and Tet2^{-/-} mice. (C) 5hmC dynamic changes in proliferating cells and differentiating cells from WT and Tet2^{-/-} adult NPCs.

Figure 48 Loss of Tet2 leads to increased adult NPCs proliferation and decreased adult NPCs differentiation.

Immunostaining showing BrdU+ (red) cells in the NPCs. Quantitative analyses of proliferating NPCs suggest that Tet2^{-/-} NPCs proliferated more than WT NPCs. Adult NPCs immunostained using cell lineage markers for quantitative cell fate determination. Both WT and Tet2^{-/-} were able to differentiate into Tuj1+ (red) neurons and GFAP+ (green) astrocytes. Quantitative analyses of differentiating NPCs suggest that Tet2^{-/-} NPCs differentiated fewer neurons and astrocytes than WT NPCs. Data presented as mean ± SEM; Student's t-test.

Figure 49 The *NeuroD1* and *Gfap* promoter regions are altered by *Tet2*.

(A) The *NeuroD1* and *Gfap* promoter regions were cloned into luciferase reporter constructs. (B) Luciferase reporter assay showed decreased *NeuroD1* and *Gfap* promoter activities in *Tet2*^{-/-} differentiating NPCs. Renilla luciferase (R-Luc) plasmid served as a transfection control. Real-time PCR showed that *Tet2*^{-/-} NPCs had decreased *NeuroD1* mRNA levels (n = 3, p<0.05), and decreased *Gfap* mRNA levels (n = 3, p<0.05) upon differentiation. The relative mRNA levels were in comparison with *Gapdh* mRNA.

CHAPTER
CONCLUSIONS AND FUTURE DIRECTIONS

Studies using different Tet2 knock-out animal models revealed that Tet2 loss alters the epigenetic landscape with decreased 5hmC and global hypermethylation of the DNA, increases HSC self-renewal and myeloid progenitor expansion with skewed differentiation toward monocytic lineages, leading to the pathogenesis of myeloid malignancies⁸⁴. Identification of critical novel partners of TET2, and unveiling the functional significance of their interactions may provide valuable insights into the mechanisms that underlie critical roles of TET2 in the tumorigenesis of many cancers.

In this dissertation I investigated whether Tet2 loss leads to various types of hematological malignancies other than myeloid malignancies, and what cellular phenotypes Tet2^{-/-} mice may have. Because clinical studies have reported the haploinsufficient role of TET2 in leukemic patients, it was important to understand if Tet2 loss in mice causes leukemic disease phenotypes as in humans. Using a two-year follow-up investigation, I found that Tet2^{-/-} mice also develop lymphoid malignancies, consistent with TET2 mutant patients with lymphoid disorders. Hence, additional genetic lesions is at least in part required for Tet2 loss induced hematological malignancies.

Depending on the methods in genome analysis, diverse Tet2^{-/-} genome landscapes can be depicted via next-generation exome sequencing and aCGH. These techniques make it possible to sequence all the protein-coding genes and detect chromosome abnormalities. Therefore, it was feasible to propose understanding if Tet2 is required for maintaining genome integrity. By using exome sequencing, several “driver mutations” were identified in Tet2 loss background. Because of the biological instability of 5mC, it is plausible to assume that these mutation sites are close to Tet2-dependent 5hmC sites. Indeed, significant correlations between mutation sites and Tet2-dependent 5hmC sites

were found. Next, if Tet2 regulates genome integrity, relying on its enzymatic activity, mutation sites would be expected to be located only at cytosine. Then, it would be important to understand what are the TET2 protein interactors that participate in Tet2-mediated DNA repair mechanism. The present finding that there is a TET2 and MSH6 interaction, strengthen the concept of mutator phenotypes in Tet2^{-/-} mice and TET2 knockdown cell lines. With these observations, a model was proposed that explains how Tet2 can regulate genome integrity through interacting with MSH6. Chapter II determined that:

- ~8% of Tet2^{-/-} mice develops lymphoid malignancies of both T- and B-cell origin, although myeloid malignancies dominates.
- Whole-exome sequencing identified recurrent mutations from Tet2^{-/-} tumors of each cell origin, such as Notch1 mutations in the T-cell leukemia/lymphoma.
- Using protein purification and mass spectrometry, I found that TET2 associates with MSH6, a key protein involved in DNA-mismatch repair (MMR).
- Tet2-depleted HeLa, and NIH3T3 cells display elevated spontaneous mutation frequency, characteristic of MMR-deficient cells.

Also, I characterized Tet2 and 5hmC in mouse cerebellum and hippocampus during postnatal development and aging. Tet2 regulates 5mC oxidation at *NeruoD1* and *Gfap* promoter regions during aging, pointing to a critical role for Tet2 in adult neurogenesis. My study has great potential for improving the understanding of Tet2-mediated DNA demethylation in neurodevelopment and neurodegenerative disorders. Chapter III determined that:

- Tet2 is highly expressed in adult brain compared to postnatal P7 brain.
- Loss of Tet2 leads to increased adult NPCs proliferation and decreased adult NPCs differentiation.
- Tet2 regulates adult brain neurogenesis through 5mC oxidation of NeuroD1 and Gfap promoter regions.

Together, the observations presented here reveal that Tet2 is involved in different regulatory mechanisms in different systems. In the hematological system, Tet2 serves as a tumor suppressor gene to protect the genome from being mutated and is also dependent on the interaction with MSH6 during DNA damage repair. In the nervous system, it is also clear that Tet2 regulates 5hmC dynamics at its specific downstream loci, and subsequently has an effect on adult neurogenesis. A better understanding of Tet2 regulatory network including TET2-binding protein complex and Tet2-binding DNA sequences may prove to be essential for pharmaceutical research, with the goal, to address Tet2-related diseases.

Future research on Tet2 in the hematological system will be focused on several important aims: 1) providing a detailed characterization of hematological malignancies of Tet2^{-/-} mice; 2) determining when mutations occur during cell differentiation; 3) mapping the minimum binding regions between TET2 and MSH6; 4) crossing Tet2^{-/-} and Msh6^{-/-} mice to elucidate the relationship between TET2 and MSH6. Additional questions to be addressed include whether the association between TET2 and MSH6 is disrupted in Tet2-deficient mice; and if genomic instability is a “driver” or a “consequence” of malignant transformation. The latter could be clarified by exome sequencing of premalignant WT, Tet2^{+/-} and Tet2^{-/-} hematopoietic stem/progenitor cells.

For understanding the role of Tet2 in neurogenesis, it makes sense to evaluate Tet2 in the context of spatial learning and memory based on the present findings of impaired neurogenesis in Tet2 mutants, which may greatly influence learning and memory. I can also investigate the epigenetic changes (5mC and 5hmC) of Tet2 downstream target genes and examine their transcriptional regulation and involvement in neurogenesis. The definitive role of Tet2 loss-induced adult-neurogenesis-defects should be investigated using Tet2 conditional knockout mice in which tissue/cell-specific elimination would help to better understand the biological functions of Tet2 in the nervous system.

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VITA

FENG PAN

Born, Haerbin, China

2000-2004

B.S., Biological Sciences
Jilin University
Changchun, China

2004-2007

M.S., Biochemistry and Molecular biology
Jilin University
Changchun, China

PUBLICATIONS AND PRESENTATIONS

Pan F, et al. TET2 protects genome stability through interacting with MSH6. Under revision.

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