Functional Epigenetics Identifies Novel KRAB-ZNF Tumor Suppressors in ESCC, NPC and Multiple Tumors

CHENG, Yingduan

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List of abbreviations

5-Aza-CdR	5-aza-2'-deoxycytidine
5-Aza-CR	5-azacytidine
5'-RACE	5'-Rapid amplification of cDNA ends
7-AAD	7-Amino-Actinomycin
AAP	Abridged Anchor Primer
ALL	Acute lymphoid leukemia
APC	Adenomatous polyposis coli
AUAP	Abridged Universal Amplification Primer
BGS	Bisulfite genomic sequencing
bp	Base pair
BTB	Broad-Complex, Tramtrack and Bric-a-bric
C	carboxyl terminus
C2H2	Cys2His2
CDK	Cylin-dependent kinase
cDNA	Complimentary deoxyribonucleic acid
CGI	CpG island
ChIP	Chromatin immunoprecipitation
CK1α	Casein-kinase 1α
CO2	Carbon dioxide
CpG	Cytosine-phosphate-Guanine
DAPI	4',6-diamidino-2-phenylindole
DKO	Double knockout
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTPs	Deoxynucleoside triphosphates
EDTA	Ethylene-diamine-tetra-acetic acid
ERK	Extracellular signal related kinase
ESCC	Esophageal squamous cell carcinoma

FADD	Fas-associated death domain
FBS	Fetal bovine serum
G	Guanine
GLTSCR2	Glioma tumor-suppressor candidate region gene 2
GSK3ß	Glycogen synthase kinase
HAT	Histone acetyltransferase
HCC	Hepatocellular carcinoma
HDAC	Histone deacetylases
HMT	Histone methyltransferase
IKK	IκBs kinase
JNK	C-Jun N terminal protein kinase
KRAB	Krüppel-Associated-Box
LB	Luria-Bertani
LEF	Lymphoid enhancer factor
LRP	Receptor-related protein
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MDS	Myelodysplastic syndrome
MgCl2	Magnesium Chloride
MiRNA	Micro-RNA
mRNA	Messenger ribonucleic acid
MSP	Methylation specific polymerase chain reaction
N	Amino terminus
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NIK	NF-κB-inducing kinase
NPC	Nasopharyngeal carcinoma

PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCD	Programmed cell death
PCP	Planar-cell polarity
PCR	Polymerase chain reaction
PMSF	Phenylmethanesulfonylfluoride
POZ	Poxvirus and Zinc finger
RACE	Rapid amplification of cDNA ends
RCC	Renal cell carcinoma
RIP	Regulated intramembrane proteolysis
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
SAPK	Stress-activated protein kinase
SCAN	SRE-ZBP, CTfin51, AW-1 and Number18 cDNA
SDS	Sodium dodecyl sulfate
SRE	Serum response element
SRF	Serum response factor
TAE	Tris-acetate-EDTA
TAK/ASK	Transforming growth factor-β-activated kinase/apoptosis signal-regulating kinase
Taq	Thermus Aquaticus
TCF	T-cell factor
TE	Tris-EDTA
TF	Transcription factor
TNFR	TNF receptor
TRADD	TNF receptor-associated death domain
TRAF2	TNF receptor-associated factor 2
TRE	Transforming growth factor-β response element
Tris-HCL	Trishydroxymethylaminomethane-hydrochloride

TSA	Trichostatin A
TSG	Tumor suppressor gene
TSS	Transcription start site
Ub	Ubiquinone
UV	Ultraviolet
ZNF	Zinc finger protein

List of Tables

List of Figures

List of publications

1. **Yingduan Cheng,** Hua Geng, Suk Hang Cheng, Pei Liang, Yan Bai, Jisheng Li, Gopesh Srivastava, Margaret HL Ng, Tatsuo Fukagawa, Xiushan Wu, Anthony TC Chan, Qian Tao, The KRAB Zinc finger protein ZNF382 is a general, pro-apoptotic tumor suppressor repressing multiple oncogenes and frequently silenced in multiple carcinomas, **Cancer Research**, 2010, 70(16):6516-26.

2. Li Fu, Suisui Dong, Yi-Wu Xie, Lai-Shan Tai, Kar Lok Kong, Kuan Man, Dan Xie, Yan Li, **Yingduan Cheng,** Qian Tao, Xin-Yuan Guan. Downregulation of Tyrosine Amino transferase at a Frequently Deleted Region 16q22 Contributes to the Pathogenesis of Hepatocellular Carcinoma. **Hepatology**. 2010 Feb 1; 51(5): 1624 – 1634.

3. Hua Geng, Fan Fong Poon, JinRong Peng, Linda Soo, Wei Wu, **Yingduan Cheng**, Jun Yu, Jianming Ying, Longtao Wu, Hongchuan Jin, Ben CB Ko, Anthony TC Chan, Gopesh Srivastava, Hwee Koon Goh, Joseph JY Sung, Qian Tao, TUSC6 is a novel proapoptotic 1p36 tumor suppressor inhibiting NFkB/AKT signaling and silenced in aerodigestive tumors, 2010, (Submitted).

4. **Yingduan Cheng**, Pei Liang, Hua Geng, Suk Hang Cheng, Ka Man Ng, Margaret H L Ng, Anthony TC Chan, Qian Tao. Two homologous zinc finger proteins are functional tumor suppressors with frequent epigenetic inactivation in multiple carcinomas. 2010 (Manuscript).

List of Conference abstracts

1. **Yingduan Cheng**, Yan Bai, Ada H. Y. Wong, Pei Liang, Jisheng Li, Jianming Ying, Ya Cao, Anthony T.C. Chan, Xiushan Wu, Qian Tao, The KRAB domain-containing Zinc finger protein ZNF382 is a potent tumor suppressor with frequent epigenetic inactivation in nasopharyngeal, esophageal and other carcinomas. 2008, 7th International Symposium on Frontiers in Life Sciences, Changsha, China.

2. **Yingduan Cheng**, Ka Man Ng, Gopesh Srivastava, Anthony TC Chan, Qian Tao, The KRAB domain-containing transcriptional repressor ZFP30 is a potent tumor suppressor with frequent epigenetic inactivation in multiple carcinomas. 2008, Hong Kong Cancer institute/AACR international conference: infection and cancer: Biology, Therapeutics, and Prevention. Hong Kong.

3. **Yingduan Cheng**, Pei Liang, Hua Geng, Suk Hang Cheng, Margaret H L Ng, Anthony TC Chan, Qian Tao, The KRAB-containing zinc finger protein ZNF545 is a functional tumor suppressor exerting proapoptotic and anti-proliferation abilities with frequent epigenetic inactivation in multiple carcinomas. 2010, AACR 101st Annual Meeting, Washington, USA.

4. **Yingduan Cheng**, Pei Liang, Hua Geng, Suk Hang Cheng, Margaret H L Ng, Anthony TC Chan, Qian Tao, Two homologous KRAB-containing zinc finger repressors act as tumor suppressors with frequent epigenetic inactivation in multiple carcinomas, 2010, SKL Retreat, Dongguan, China.

Abstract of Thesis entitled

Functional Epigenetics Identifies Novel KRAB-ZNF Tumor Suppressors in ESCC, NPC and Multiple tumors

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The development of a tumor from a normal cell is a complex and multi-step process. A large number of oncogenes, tumor suppressor genes (TSGs) and signal transduction pathways are involved in this process. Tumor-specific methylation of TSGs in multiple tumors indicated that it could be used as epigenetic biomarker for molecular diagnosis and therapeutics.

The functions of KRAB-containing proteins are critical to cell differentiation, proliferation, apoptosis and neoplastic transformation. A large number of ZNF genes are located in 10 clusters at chromosome 19. Some of the KRAB-ZNF may function as potential TSGs with epigenetic alterations. Thus, I try to identify silenced novel KRAB-ZNF candidate TSGs through screening chromosome 19.

First, expression profiling of ZNFs with CpG islands at 10 clusters of Chr19 was examined in a panel of NPC and ESCC cell lines by semi-quantitative RT-PCR, with

adult normal tissues - larynx and esophagus as controls. Several down-regulated genes were identified, and I further focused on 5 candidates: *ZNF382*, *ZNF545*, *ZFP30*, *ZNFT1* and *ZNFT2*. These genes were frequently downregulated in NPC, ESCC, lung, gastric, colon and breast carcinomas. Their promoters were frequently methylated in multiple downregulated cell lines but less in non-tumor cell lines as revealed by methylation-specific PCR (MSP) and bisulfite genomic sequencing (BGS). Their expression could be restored by pharmacologic or genetic demethylation, suggesting that DNA methylation was directly involved in their silencing. The frequent methylation of these genes indicated they could act as potential biomarkers.

More functional studies were done for *ZNF382* and *ZNF545*, I found that ectopic expression of *ZNF382* and *ZNF545* in tumor cells lacking endogenous expression could inhibit tumor cell clonogenicity, proliferation and induce apoptosis. I found that ZNF382 suppressed tumorigenesis through mediating heterochromatin formation, as ZNF382 was revealed to be co-localized and interacts with heterochromatin protein. For *ZNF545*, I found that it is a transcriptional repressor. I further showed that ZNF545 was located in the nucleus and sequestered in the nucleolus. ZNF545 could inhibit tumorigenesis at least partially through downregulating the transcription of target genes or regulating nucleolus function such as ribosome biogenesis.

In conclusion, several novel candidate TSGs epigenetically silenced in tumor cells were identified in this study. Their downregulation by promoter methylation was tumor-specific, which could be use as epigenetic biomarkers for diagnosis.

论文摘要

從一個正常細胞發展到腫瘤是一個很複雜的過程，很多的原癌基因和腫瘤抑制因子涉及其中。腫瘤抑制因子在腫瘤細胞中特異性的甲基化修飾標志著它們有可能作爲擬遺傳學的標記物用于癌症的分子診斷和靶向治療目標。

含有 KRAB 結構域的鋅指基因在衆多的生命活動中起作用，包括分化，增殖，凋亡和致瘤性轉化。有相當數量的鋅指基因位于人類的 19 號染色體上，並集中于 10 簇中。目前，已經有一些被擬遺傳學調控的 KRAB 鋅指基因被鑒定爲腫瘤抑制因子。所以，本論文的目標就是在 19 號染色體上的 KRAB 鋅指基因中鑒定出新的候選腫瘤抑制因子。

首先，在鼻咽癌和食管癌的細胞中，我檢測了那些在 19 號染色體上具有 CpG 島的鋅指基因的表達情況，並用正常的喉和食管組織作爲對照。通過這種篩選方法，我鑒定了數個候選的腫瘤抑制因子，並挑選了其中的五個基因進行深入的抑癌作用分子機理的研究工作，包括 ZNF382, ZNF545, ZFP30, ZNFT1 和 ZNFT2。這些基因的表達水平在鼻咽癌，食管癌，肺癌，胃癌，結腸癌，乳腺癌和宮頸癌中都有高頻下調。運用甲基化特異性聚合酶鏈式反應和亞硫酸氫鈉測序法，我發現它們的啓動子在表達下調的細胞中被高度甲基化。而這些基因的表達可以通過藥物或者基因組水平去甲基化而得到恢複，這進一步證明 DNA 甲基化調控了基因表達的下調。這種特異性的高甲基化頻率表明他們能夠作爲候選的癌症分子標記物。

對于 ZNF382 和 ZNF545，我還進行了更多的功能分析。我發現在沒有 ZNF382 和 ZNF545 表達的細胞中過表達 ZNF382 和 ZNF545 能夠抑制細胞的克隆形成，抑制增殖同時可以誘導凋亡。我發現 ZNF382 可以和異染色質蛋白共定位並可以相互作用，說明 ZNF382 可以通過調控異染色質的形成來抑制腫瘤發生。對于 ZNF545，我發現它是一個轉錄抑制因子。ZNF545 定位于核並主要集中在核仁中。它可能通過抑制下遊靶基因的表達或者調控核仁的功能比如核糖體發生來抑制腫瘤發生。

在本文中，我鑒定了一些在腫瘤細胞中受到擬遺傳調控而表達下調的候選腫瘤抑制因子。它們在腫瘤中特異甲基化表明其可以作爲在癌症診斷中的擬遺傳學標志物。

Table of contents

Chapter 1

Introduction and Literature Review

1.1. Cancer is a genetic and epigenetic disease

Cancer is caused by the accumulation of alterations of genome, including inactivation of negative regulators of cell proliferation [including tumor suppressor gene (TSG)] and activation of positive regulators (such as oncogenes) (Coleman and Tsongalis, 2006). Traditionally, cancer is thought as the disease of genetic mutations; however it is now widely accepted that epigenetic also plays critical roles in tumorigenesis (Figure 1) (Sawan et al., 2008). It is well accepted that carcinogenesis is driven by the accumulation of genetic and epigenetic changes which result in the uncontrollable balance between cell proliferation and cell death (Hanahan and Weinberg, 2000).

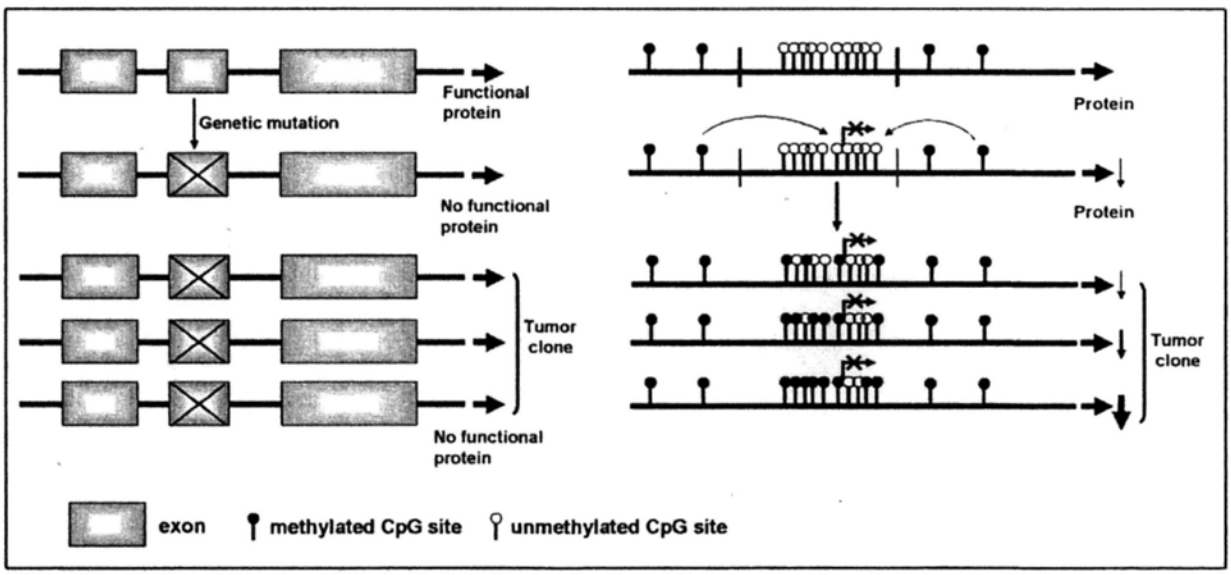


Figure 1. Genetic and epigenetic gene silencing. Left panel: non-functional protein is produced from the mutant allele when a somatic mutation occurs in one round of DNA replication. If a selective advantage is conferred to cells carrying this mutation, the cells with non-functional protein can expand clonally to give rise to a tumor. Right panel: epigenetic mediated gene silencing. This process might start with a bit decrease of mRNA level by epigenetic silencing of a gene which results in the little

reduction of protein production. This downregulation of protein will be fostered by the spreading of DNA methylation through the promoter CpG islands in an emerging clone of tumor cells. But the extent of DNA methylation may variant in individual cells of tumor clone.

1.1.1 Genetic alterations in human cancers

Somatic mutations in cancer cells include changes in chromosome copy number and chromosome alterations such as translocations, deletions, amplifications and minor changes in nucleotide sequences leading to gene mutation. All kinds of alterations frequently co-exist in a single tumor (Sugimura et al., 1992).

With the advance development in sequencing technology and the knowledge of human genome sequence, we could detect the genetic alterations through genome wide screening. A recent study using 11 breast and 11 colorectal cancer specimens suggests that individual tumor may accumulate an average of 90 mutant genes but not all of them contribute to the neoplastic process (Sjoblom et al., 2006). By using stringent criteria to delineate this subset, the author identified 189 genes (average of 11 per tumor) that were mutated at significant frequencies. The mutated genes identified in this study include the well-studied oncogenes and TSGs such as oncogene Ras (gain-of-function) and tumor suppressor TP53 (loss-of-function). In addition, a number of novel genes involved in transcription, cell adhesion and invasion were also identified (Sjoblom et al., 2006).

1.1.2 Epigenetic changes in human cancers

Epigenetics was defined as all heritable changes in chromatin structure and gene

expression without changes in DNA sequence (Bird, 2002). Epigenetic modifications include DNA methylation, histone modifications and RNA-mediated silencing (non-coding RNAs) (Figure 2). All three epigenetic mechanisms allow the stable propagation of gene status from one cell generation to next one. Disruptions of these epigenetic modifications will lead to abnormal gene expression, resulting in tumor formation and other epigenetic related diseases (Herceg and Hainaut, 2007).

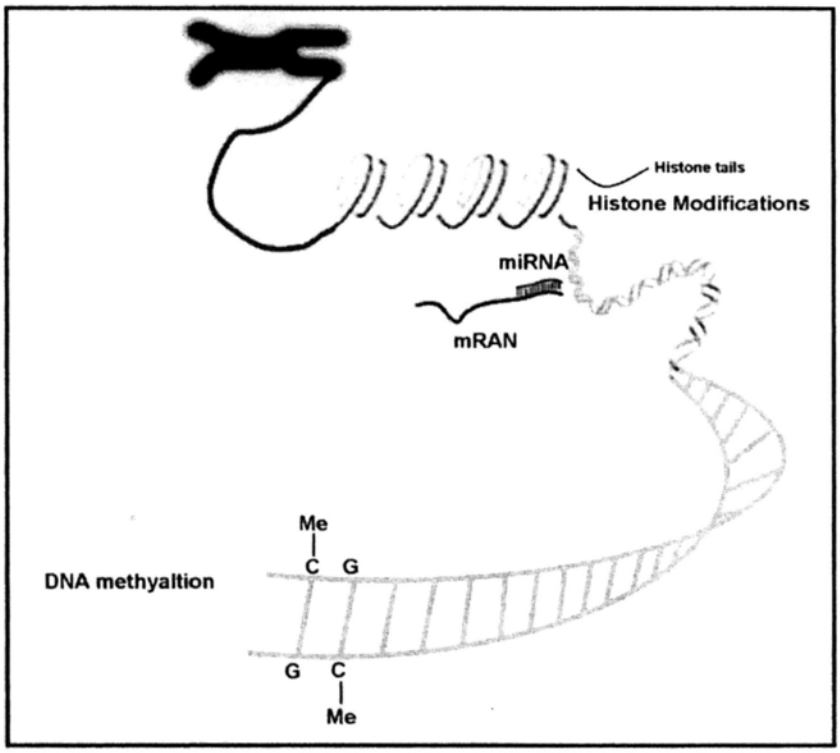


Figure 2. Different types of epigenetic modifications including DNA methylation, histone modification and RNA-mediated gene silencing. DNA methylation happens at the cytosine of a CpG dinucleotide. Histone modification is a covalent modification of four core histones at the N-terminal tails. RNAs in the form of microRNA can also alter the gene expression in a heritable manner.

1.1.2.1 DNA methylation

In mammalian genome, DNA methylation is the best studied epigenetic mechanism. DNA methylation is defined as covalent addition of a methyl group to the 5'-carbon

(C^5) position of cytosine base that is located 5' to a guanosine base in a CpG dinucleotide, which has been progressively depleted from the genome during evolution (Figure 2) (Baylin and Herman, 2000). The CG dinucleotides are less present in genome but rich in CpG islands (short DNA regions, 0.5-4 kb in length) which are frequently located in the proximal promoter regions of almost half of the genes in mammalian genome (Jones and Baylin, 2002). Generally, the CpG islands within TSG promoters are unmethylated in normal cells but hypermethylated in cancer cells which result in abnormal transcriptional silencing of TSGs. The hypermethylation of TSGs in cancer is even more frequent than genetic mutation (Jones and Baylin, 2002).

DNA methylation at 5-carbon (C^5) position of cytosine bases was catalyzed by DNA methyltransferases (DNMTs) by using S-adenosyl-methionine as a methyl group donor (Bird, 2002). Up to now, three families of DNA methyltranferases (DNMTs) have been identified [DNMT1, DNMT2 and DNMT3 (DNMT3a, DNMT3b, DNMT3L)] (Bestor, 2000). DNMT1 is responsible for maintaining DNA methylation. DNMT3a and DNMT3b are responsible for *de novo* methylation (Jones and Baylin, 2002; Herceg and Hainaut, 2007). DNMT2 does not exhibit comparable DNA methyltransferase activity mainly because it lacks the large N-terminal regulatory domain which present in other methyltransferases and DNMT3L has no canonical DNA cytosine-methyltransferase motifs (Yoder and Bestor, 1998; Bestor, 2000).

There are two types of aberrant DNA methylation in human cancers: genome-wide hypomethylation and CpG island hypermethylation (Jones and Baylin, 2002). These two types of methylation are found in all kinds of cancers. Hypomethylation may lead to activation of oncogenes and chromosome instability while hypermethylation of gene promoter results in transcriptional silencing of TSGs. It seems that DNA methylation functions as a double-edged sword. It could promote tumorigenesis through hypomethylation of proto-oncogenes and hypermethylation of TSGs (Jones and Baylin, 2002; Feinberg and Tycko, 2004).

1.1.2.2 Histone modifications

In addition to DNA methylation, histone modifications are defined as covalent post-translational modifications of histone proteins that are critical to chromatin remodeling and gene activation (Jenuwein and Allis, 2001; Peterson and Cote, 2004). The fundamental unit of chromatin is nucleosome. The nucleosome core, which is wrapped around by 146 bases pair genomic DNA, consists of two histone 2A and histone 2B dimers (H2A-H2B). The N-terminal tails of histones are targets of different modifications including methylation, acetylation, phosphorylation and ubiquitination. Combination of all histone modifications defines the concept of "histone code" which could modulate the genetic code (Jenuwein and Allis, 2001). It is now clear that the histone modifications are involved in several cellular processes including gene transcription, DNA repair, recombination and replication (Herceg and Hainaut, 2007).

Acetylation of key histone amino acid residues is maintained by a balance between the activities of histone acetyltransferases (HAT) and histone deacetylases (HDAC). In general, acetylation of histones is associated with nucleosome remodeling which results in transcriptional activation, while deacetylation is associated with chromatin condensation which causes transcriptional repression. Histone methylation of histone lysine and arginine residues is catalyzed by a family of enzyme called histone methyltransferases (HMTs) (Shi and Whetstine, 2007). Lysine (K) of histone can be mono-, di-, and tri-methylated, while arginine of histone can both be monomethylated and symmetrically or asymmetrically dimethylated (Bannister and Kouzarides, 2004; Bedford and Richard, 2005). It is interesting that histone methylation could result in transcriptional activation or repression which is depend on the lysine/arginine methylation and other histone modifications in the surrounding residues or different histone tails (Margueron et al., 2005; Lachner and Jenuwein, 2002). For example, tri-methylation of histone H3 lysine-4 creates a binding site for the chromodomain-containing proteins which recruit HATs, resulting in transcriptional activation (Pray-Grant et al., 2005). In contrast, tri-methylation of histone H3 lysine-9 contributes to the formation of heterochromatin and induces transcriptional silencing (Jenuwein and Allis, 2001).

1.1.2.3 RNA-mediated silencing

RNA-mediated silencing is the most recent class of epigenetic mechanism which

includes RNA interference or non-coding RNA (Figure 2). It plays an important role in gene transcription through cell divisions and functions in a heritable manner (Egger et al., 2004). Micro-RNAs (miRNAs) play roles in several biology processes such as proliferation, development, differentiation and apoptosis (Bartel, 2004). Interestingly, miRNA could act as oncogenes or TSGs through affecting different targets involved in critical biological processes such as differentiation and proliferation. Recent studies suggest that expression of miRNA is also different between normal and cancer tissues and also between different tumors, suggesting that miRNA could be an useful tool in cancer diagnostics and prognosis (Herceg and Hainaut, 2007).

1.1.3 The relationship between genetic and epigenetic during tumorigenesis

With the increasing reports about epigenetics, it is thought that cancer is as much a disease of abnormal epigenetics as a disease of genetic mutations (Jones and Baylin, 2002). Epigenetic modifications often co-exist with genetic changes during the development of cancer. Moreover, the epigenetic changes exist in early tumor and can lead to several genetic alterations (Feinberg and Tycko, 2004). According to the studies on DNA methylation and histone medication, both of them could make genetic changes either separately or in combination (Sawan et al., 2008). The methylation at CpG dinucleotides could induce genetic alterations and result in tumorigenesis. It enhances the binding of carcinogens, which in turn increases the mutability of methylated cytosines and silences the TSGs and DNA repair genes. It is

also known that down-regulated histone modifications could stimulate genetic changes through disrupting normal functions of cellular processes such as gene transcription, DNA repair, cell cycle checkpoints and DNA replication (Sawan et al., 2008).

1.1.4 Clinical implications of epigenetics in cancer

1.1.4.1 Identification of novel TSGs with epigenetic modifications

An important observation of TSG silenced by genetic or epigenetic regulation is that they often locate in frequently deleted chromosomal regions. Epigenetic modification of TSG is even more frequent than genetic mutation. The character for TSG with tumor specific methylation could be used as an epigenetic marker to identify novel TSGs (Jones and Baylin, 2002). There are a growing number of techniques that have been developed for novel TSG identification and a certain number of novel TSGs have been identified with epigenetic silencing in tumors such as *PCDH10*, *OPCML* and *RASAL* (Ying et al., 2006b; Cui et al., 2008; Jin et al., 2007).

1.1.4.2 Tumor specific epigenetic alterations could be used as diagnosis biomarkers

Epigenetic alterations are considered to be early event in tumorigenesis which could be used in cancer risk assessment. With the development of technology for detecting DNA methylation such as methylation-specific PCR (MSP), we could detect the hypermethylated alleles with a high degree of sensitivity. The hypermethylated

alleles could be detected in the DNA derived from biopsy and even the body fluids like sputum, serum, urine and stool in cancer patients (Belinsky, 2004; Dulaimi et al., 2004). For example, the cancer specific methylation can be detected in sputum for lung cancer diagnosis; such methylation can also be detected in urine for bladder cancer, in pancreatic juice for pancreatic cancer and in free DNA from plasma for various cancers. Importantly, the tumor specific methylation of specific genes can also be linked with prognosis and survival. For instance, promoter hypermethylation of *PCDH10* is an early event in cervical cancer and is also associated with shortened survival of patients with gastric cancers (Narayan et al., 2009; Yu et al., 2009). Promoter methylation of $p16^{INK4A}$ and $p14^{ARF}$ is reported to be an early event in tumorigenesis of various cancers (Nuovo et al., 1999; Shen et al., 2003).

The efficacy of a biomarker is related to its sensitivity and specificity. In clinical study, biomarker sensitivity refers to the positive result of biomarker assay in individuals with confirmed disease, whereas the biomarker specificity refers to the negative results of biomarker assay in individuals without the disease (Pepe et al., 2001). The methylation frequency of epigenetic biomarker is different at different tumor stage. The scientists are interested in studying the difference between the different stages of disease progression, such as metaplasia, dysplasia, carcinoma or metastatic tissues (Laird, 2003). The investigation of such difference will lead to identification of useful biomarker associated with disease progression. If the epigenetic abnormalities arise early in normal tissues, the difference between normal

tissues from cancer patients and healthy controls could result in the identification biomarkers for early disease detection (Laird, 2003). The diagnostic biomarker will be more valuable if it can predict disease onset earlier and more accurate.

1.1.4.3 Epigenetic alterations as therapeutic targets

In some sense, epigenetic aberrations are different from genetic alterations because epigenetic aberrations are reversible which revert the gene background of malignant cells to normal state. The utilization of epigenetic drugs in chemotherapy and chemoprevention of cancer is an effective approach. Now there are two main types of epigenetic drugs designed to inhibit either DNA methylation or histone deacetylation (Pray, 2008).

DNA methyltransferase inhibitors: DNMTs can transfer methyl groups (-CH3) to cytosines in CG dinucleotid during cell division. Superabundant DNMT activity will cause plethoric methylation including many TSGs. DNMT inhibitors could function as substitutes for cytidine residues during cell division, which results in blocking and depleting the active DNMTs (Pray, 2008). There are two FDA-approved demethylating agents: 5-azacytidine (5-Aza-CR) and 5-aza-2'-deoxycytidine (5-Aza-CdR). They are powerful inhibitors of DNA methylation and could reactivate the expression of epigenetically silenced genes (Egger et al., 2004). Now, they have been FDA approved for use (Plimack et al., 2007). Patients with myelodysplastic syndrome (MDS) treated with either decitabine or azacytidine might get about an

extra year of life and the AML patients have the similar outcome though such finding is not as well established (Pray, 2008). But these two drugs (5-Aza-CR and 5-aza-CdR) are toxic and instable under physiological conditions; otherwise, the removal of the drug treatment will cause the remethylation and resilencing of demethlylated genes. All these restrain their use in clinical setting (Yoo et al., 2004; Bender et al., 1999). Also several other DNMT inhibitor compounds are in development such as zubularin (derivative of 5-aza-cytidine) (Egger et al., 2004). Zebularine has advantages over other therapeutic drugs including stability, low toxicity and high selectivity for tumor cells, making zebularine an excellent candidate for cancer treatment (Marquez et al., 2005; Holleran et al., 2005). Zebularine can be given continuously at lower dose to maintain demethylation for a prolonged period due to the low toxicity. A combination treatment of 5-Aza-CdR and zebularine (cancer cells were transiently treated with 5-Aza-CdR and then by continuous treatment with zebularine) gave a better DNA demethylation response that remethylation was hindered and gene expression was maintained (Cheng et al., 2004).

HDAC inhibitors: HDACs are enzymes regulating gene expression by removing the acetyl groups (CH3CO-) from histone proteins and inhibiting gene expression. Same as DNMTs, superabundant HDAC activity will cause downregulation of TSGs. Zolinza is the only FDA-approved HDAC inhibitor. It is hypothesized that the hyperacetylation of histones can activate TSGs and repress oncogenes. Inhibitors of

HDAC inhibit HDAC enzymes and also affect other several signaling pathways including NF-κB, C-JNK and BCL2 (Shetty et al., 2005; Dai et al., 2005; Duan et al., 2005). Some cyclin-dependent kinase (CDK) inhibitors such as p21, which is responsible for cell cycle arrest in G1 and G2 phases, are upregulated in tumor cells when treated with HDAC inhibitors (Rocchi et al., 2005).

The DNMT inhibitors and HDAC inhibitors have been used in clinical trails for epigenetic therapy but are far from perfect. It seems that both kinds of drugs work well against hematological cancer but have not shown consistent effectiveness on solid tumors (Best and Carey, 2010). Hence, more investigation is needed. The epigenetic drugs might be used singly or in combination with other therapies, such as chemotherapy, immunotherapy or radiotherapy (Yoo and Jones, 2006). Epigenetic drug treatment is a rapidly developing field, and more studies are needed for better understanding of such approaches.

1.2 Tumor suppression by TSGs

As discussed before, TSGs function in cellular proliferation, apoptosis, differentiation, senescence, autophage, migration, metastasis and tumor angiogenesis (Macleod, 2000; Sherr, 2004). Inactivation of TSGs by genetic mutation or epigenetic modification contributes to tumor initiation and progression. In this section, the functions of TSGs in proliferation, apoptosis and senescence are discussed.

Proliferation refers to the increase in the population number of cells due to transit through the cell cycle. During normal division of cells which is governed by several cyclins and cyclin-dependent kinases (CDKs) (Krug et al., 2002), the genome DNA will be replicated and distributed equally to daughter cells. The cell cycle can be separated into four distinct phases: initial growth (G1), DNA replication (S), a gap (G2) and mitosis (M) (Hirama and Koeffler, 1995). The cell cycle events are supervised at the cell cycle checkpoints that occur at the G1/S boundary, in S-phase, and during the G2/M phases (Pucci et al., 2000). TSGs are the negative regulators of cell cycles. IRX1, which is frequently methylated in gastric cancer, could inhibit cell proliferation in SGC-7901 and NCI-N87 cells (Guo et al., 2010). LARG, a TSG frequently deleted in breast and colorectal cancer, reduces tumor cell proliferation and colony formation (Ong et al., 2009). Functional inactivation of cell cycle regulators- p53 and Rb are also frequently involved in multiple cancers (Krug et al., 2002; Pucci et al., 2000; Macleod, 2000).

There are two mechanisms for complex organisms to suppress proliferation: apoptosis and senescence (Campisi, 2001). It is known that in multi-cellular organisms, some cells must die for proper development so as to maintain health and homeostasis. This cellular activity which is vital for the organisms is termed as "programmed cell death" (PCD). The best description of PCD is apoptosis, a Greek term which means "falling off or away". Apoptosis is one of the most important

tumor suppressive mechanisms of TSG. In response to several stimuli such as DNA damages, hypoxia or other stresses, several tumor suppressors including p53, PTEN and PML could induce apoptosis. Upon DNA damage stress, p53 is activated. The activated p53 will induce the transcription of several pro-apoptotic regulators such as Bax and other BCL2 signaling pathway components including NOXA and PUMA to cause apoptosis (Harris and Levine, 2005). PTEN tumor suppressor, a dual-specificity phosphatase, inhibits the AKT/PKB kinase and promotes apoptosis (Stambolic et al., 1998). PML works in a regulatory network that modulates various apoptotic pathways (Bernardi et al., 2008).

Cellular senescence is a major barrier for malignant transformation of cells (Smith and Pereira-Smith, 1996; Campisi, 2000). Compared to apoptosis that kills and eliminates potential cancer cells, senescence irreversibly arrests cell growth. Several stimuli including DNA damage, chromatin remodeling and strong mitogenic signals could induce normal cells to arrest growth with a senescence phenotype (Campisi, 2000). Cellular senescence causes changes in gene expression patterns and key molecular pathways for tumorigenesis (Campisi, 2000; Campisi, 2001). Consistent with its role in tumor suppression, cellular senescence is controlled by several TSGs. p53 controls the expression of genes involved in cell-cycle arrest or apoptosis in response to genomic damage. pRB controls cell-cycle progression and differentiation through transcription regulation. The pathways controlled by p53 and pRB are essential to cells to establish and maintain the senescence growth arrest in response

to diverse stimuli (Campisi, 2001). YPEL3, a p53 regulated gene, induces senescence in ovarian cancer (Kelley et al., 2010). BRD7 is a candidate TSG required for efficient induction of p53-dependent oncogene-induced senescence (Drost et al., 2010).

1.3 Cancer pathways

1.3.1 Brief introduction of cancer pathways

Several signaling pathways are reported to regulate proliferation, differentiation and survival of normal cells and they are also partly inter-linked. These pathways could transmit and integrate the signals with hormones, growth factors, stress stimulates or cell-cell interaction. Deregulation of these pathways, which involves many proto-oncogenes and tumor suppressors, will turn into "cancer pathways" and induce tumorigenesis. The definition of the cancer pathways is a cellular regulatory system whose overactivation or inactivation by a genetic or epigenetic mechanism will result in the development of at least one cancer. In cancer cells, these pathways are overactive or inactivated, which in turn result in uncontrolled proliferation, blocked differentiation, prevented apoptosis and altered metabolism (Schulz, 2006; Suzuki et al., 2008; Altieri et al., 2009). Several pathways play important roles in various malignant tumors while some are specifically involved in certain cancers.

In this chapter, four cancer pathways related to my studies are discussed, including AP-1-MAPK pathway, NF-κB pathway, p53 pathway and beta-catenin pathway.

1.3.2 AP-1 and MAPK pathway

Mitogen-activated protein kinase (MAPK) pathways control fundamental cellular processes such as growth, proliferation, differentiation, migration and apoptosis (Dhillon et al., 2007). MAPK pathways comprised of a three-tier kinase module in which a MAPK is activated through phosphorylation by a mitogen-activated protein kinase kinase (MAPKK), which is also activated when phosphorylated by a MAPKKK. There are three families of MAPK: the stress-activated protein kinase [SAPK/c-Jun N terminal protein kinase (JNK)], the p38 mitogen-activated protein kinase (MAPK), and the extracellular signal related kinase (ERK) (Wu, 2004). MAPK members could be activated by multiple stimuli such as oxidative stress and DNA damage, leading to diverse cellular response (Figure 3). The activated kinase is named as MAP kinase, being responsible for the activation of ERK1/2 (by MKK1/2), p38 (by MKK3/6) and JNK (by MKK4/7) (Wu, 2004; Zhang and Liu, 2002).

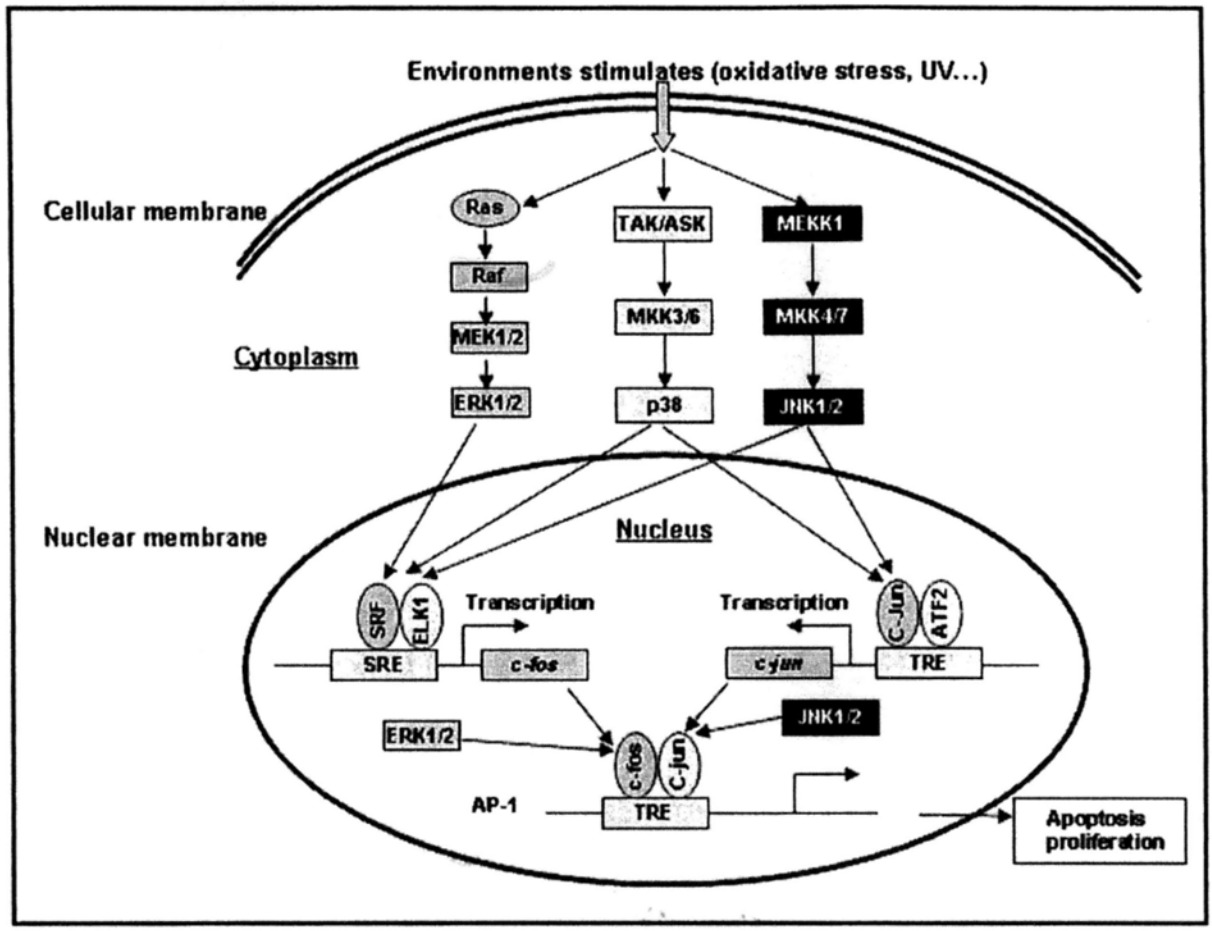


Figure 3. AP-1 and MAPK pathways. Several factors in MAPK pathways such as ERK, p38 and JNK could regulate the transcriptional activities of ELK1, c-Jun and ATF2 which are involved in the transcription of AP-1 gene (including *c-fos* and *c-jun*). The activity of *c-fos* and *c-jun* can also be activated by ERK and JNK pathways, respectively. The MAPK pathway could be activated by pro-inflammatory cytokines, tumor promoters, oxidative stress or UV damage which result in the activation of AP-1 signaling pathway. MKK, MEK kinase; SRE, serum response element; SRF, serum response factor; TAK/ASK, transforming growth factor-β-activated kinase/apoptosis signal-regulating kinase; TRE, transforming growth factor-β response element.

AP-1 is a dimeric transcription factor (TF) and is one of the first identified mammalian TFs. AP-1 composes basic leucine zipper family members, including Jun (c-Jun, JunB, and JunD), Fos (c-Fos, FosB, Fra1, and Fra2), ATF (ATF2, B-ATF, JDP1, and JDP2), and Maf (MafA, MafB, c-Maf, and MafG/F/K) protein families (Eferl and Wagner, 2003). The activity of AP-1 is regulated by dimer composition, post-translational modification of the subunits and the interactions between the

dimers and other proteins (Figure 3) (Shen et al., 2005). Jun and Fos are the major members in this group while Fos cannot homodimerize but can only form heterodimers with Jun proteins to promote the DNA binding activity. Jun and Fos both can efficiently transform cells and the activation of AP-1 is important for tumor promotion (Eferl and Wagner, 2003). It was also found that the expression level of c-fos is correlated with chondrogenic tumor development in mice whereas c-jun plays a critical role in the development of skin and liver cancers (Wang et al., 1991; Domann et al., 1994). However, some proteins of Jun (JunB and JunD) and fos family could suppress the tumor formation (Eferl and Wagner, 2003). AP-1 is a double-edged sword in tumorigenesis and whether AP-1 is oncogenic or anti-oncogenic depends on the antagonistic activity of different Jun proteins, the tumor type and stage, as well as the genetic background of a certain tumor (Eferl and Wagner, 2003; Shen et al., 2005).

1.3.3 NF-κB pathway

NF-κB is also a dimeric TF and is originally recognized in regulating transcription in B cell lymphocytes (Shen et al., 2005). According to the structures and processing, the NF-κB family could be sub-divided into Rel proteins (RelA, RelB, c-Rel) and NF-κB proteins (p50/p105, p52/p100). Only Rel proteins contain C-terminal transactivation domains. Nonetheless, p50 and p52 could bind to specific DNA sequences and recruit other NF-κB subunits leading to gene expression (Dhillon et al., 2007; Shen et al., 2005; Basseres and Baldwin, 2006). The transcription regulatory

effect by p50/p50 or p52/p52 homodimers is mainly dependent on the recruitment of co-activators or co-repressors (Kim et al., 2005).

In unstimulated or resting cells, the NF-κB heterodimer, which is mainly composed by p50 and RelA, is located in cytoplasm through binding to the inhibitor IκBs that masks the nuclear localization sequence of NF-κB. Signals for activating NF-κB signaling pathways will lead to IκBs phosphorylation by IκBs kinase (IKK) complex, which results in subsequent ubiquitination and proteolytic degradation of IκBs (Huang et al., 2000; Johnson et al., 1999). The exposure of the nuclear localization sequence of NF-κB complex triggers the nuclear translocation of NF-κB, leading to transcription activation of NF-κB target genes (Figure 4) (Dhillon et al., 2007).

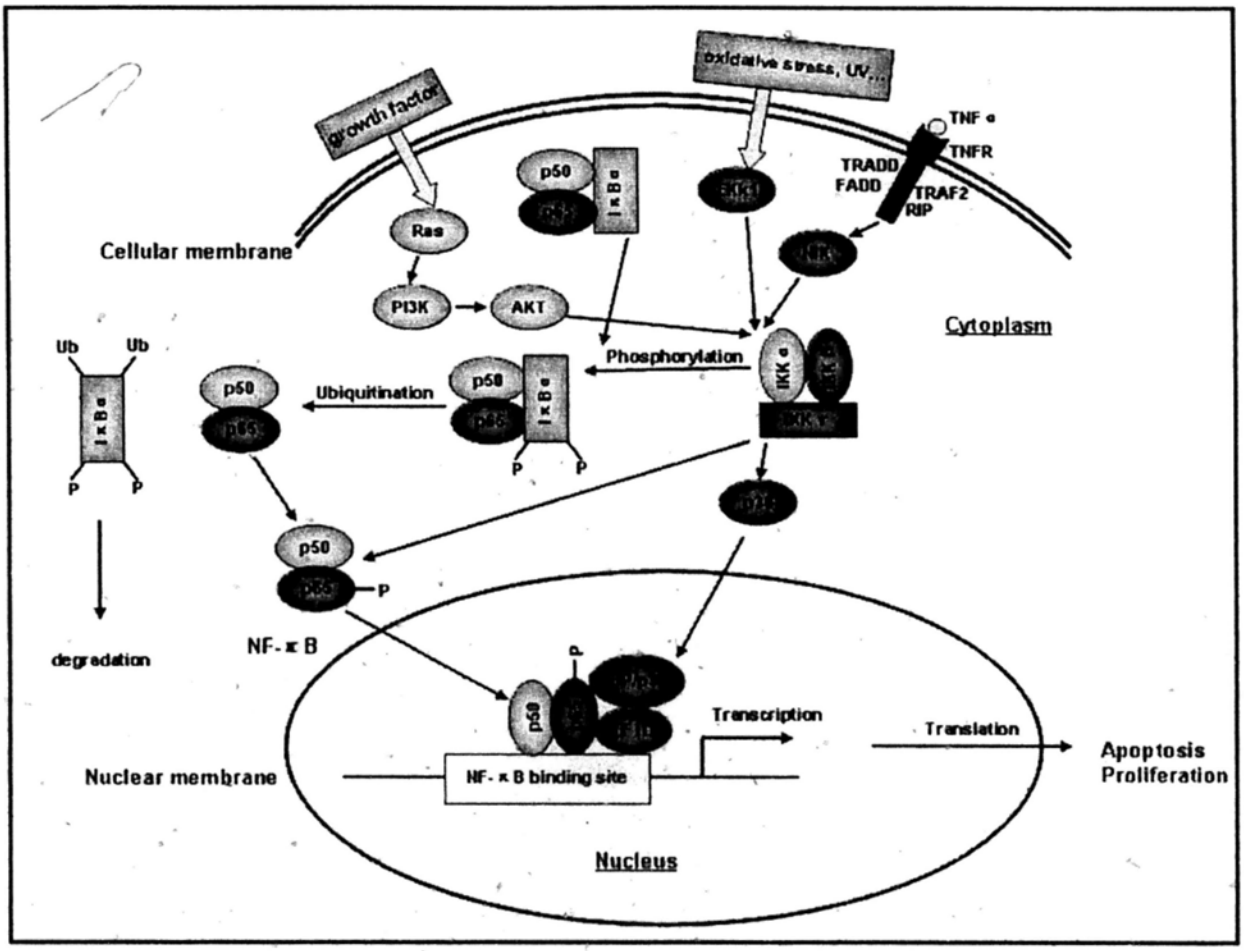


Figure 4. Regulation of NF-κB signaling pathway. Several factors stimulate the activity of NF-κB, including the inflammatory cytokine- and oxidative stress-triggered MAPK cascade, the binding of TNF-α to its receptor, and the growth factor activated PI3K cascade. NF-κB-inducing kinase (NIK) and MEK kinases (MEKKs) will induce the activation of the IκB kinase (IKK), resulting in the phosphorylation and degradation of IκBα and the release of the NF-κB dimer. Then, NF-κB translocate into the nucleus and promote the transcription of target genes. The activity of NF-κB can also be regulated by IKKs through the direct phosphorylation of Rel/p65 subunit. FADD, Fas-associated death domain; RIP, regulated intramembrane proteolysis; TNFR, TNF receptor; TRADD, TNF receptor-associated death domain; TRAF2, TNF receptor-associated factor 2; Ub, ubiquinone.

NF-κB promotes cellular proliferation, angiogenesis, metastasis, and prevent apoptosis (Basseres and Baldwin, 2006). This pathway was regulated by several upstream events. It could be activated by oncogenic H-Ras or oncogenic pathways such as PI3K/Akt dependent signaling (Pianetti et al., 2001; Gustin et al., 2004). In estrogen receptor negative breast cancer cells, NF-κB can be activated by EGF or

some members of EGFR family (Biswas et al., 2000; Gustin et al., 2004). Some TSGs exert their tumor suppressor functions through inhibiting the activity of NF-κB pathways, such as CYLD and CHFR (Hellerbrand et al., 2007; Kashima et al., 2009). The regulation of NF-κB needs more investigations for the better understanding of its oncogenic role in tumorigenesis.

1.3.4 p53 pathway

The p53 pathway is composed of hundreds of genes which could respond to a wide variety of stress signals including DNA damage, hypoxia and aberrant proliferative signals, such as oncogene activation. The response of p53 to stress will lead to apoptosis, prevention of angiogenesis, cellular senescence or cell cycle arrest (Levine et al., 2006; Vazquez et al., 2008; Brown et al., 2009). According to the review of AJ Levine (Levine et al., 2006), p53 pathway was conventionally divided up into five parts. (1) Input signals: input signals can stimulate p53 pathway and trigger or induce it into an activate state. (2) Upstream mediators: the upstream mediators could detect input signals and transmit to p53 protein leading to regulation of p53 activity and protein level. (3) Core set of proteins: the core set proteins include p53, MDM2, p14/p19ARF, E2F1 and so on. This core protein set could directly regulate the activity and function of p53. (4) Downstream events: a lot of genes could be regulated by p53 protein through transcriptional regulation or protein-protein interaction. (5) Output downstream events: all the signals that stimulate the p53 pathways will result in cell cycle arrest, apoptosis or cellular senescence.

1.3.5 Beta-catenin pathway

In the past decade, a lot of studies suggested that beta-catenin pathway was involved in many biological processes including tumorigenesis, cellular development and differentiation, as well as the regulation of embryonic and adult stem cells (Gavert and Ben-Ze'ev, 2007). At present, beta-catenin is not only considered as a cytoplasmic plaque protein involved in linking cadherin family receptors to the actin cytoskeleton but also a co-transcriptional factor. Together with lymphoid enhancer factor (LEF)/T-cell factor (TCF), they function as a core part of Wnt signaling pathway in nucleus (Gavert and Ben-Ze'ev, 2007).

There are two well-defined WNT/beta-catenin signaling pathways: canonical and non-canonical WNT/beta-catenin signaling pathway. Canonical WNT/beta-catenin pathway regulates cell proliferation, behavior and survival through regulating the transcription stability of the co-activator beta-catenin, which in turn activates the expression of target genes (Ying and Tao, 2009). In the absence of canonical WNT signals, Axin and adenomatous polyposis coli (APC) proteins will facilitate casein-kinase 1α (CK1α) and glycogen synthase kinase (GSK3ß), leading to efficient beta-catenin phosphorylation, and ubiquitinylation and proteasomal degradation of beta-catenin. In the presence of canonical WNT signals, WNT binds to Fzd receptors. Then the Dishevelled (Dvl)-depenent phosphorylation of LRP is promoted by low-density lipoprotein receptor-related protein (LRP) co-receptors. Phosphorylated

LRP recruits Axin from the destruction complex to the plasma membrane, which allows beta-catenin to escape from phosphorylation and degradation. Beta-catenin can accumulate in the cytoplasm and translocate to the nucleus where it regulates multiple targets together with members of TCF/LEF family (Ying and Tao, 2009; Gavert and Ben-Ze'ev, 2007). In contrast with the canonical WNT/beta-catenin signaling pathway, non-canonical WNT signaling pathway was exemplified by the Ca^{2+}-dependent pathway, planar cell polarity (PCP) pathway, JNK pathway and both small and heterotrimeric G protein pathways (Veeman et al., 2003; Klaus and Birchmeier, 2008). WNT/beta-catenin is frequently regulated by genetic or epigenetic modifications, and such modification provides useful biomarkers for cancer detection and prognosis prediction about this pathway (Kikuchi and Yamamoto, 2008; Ying and Tao, 2009).

1.4 Zinc finger proteins

In the autumn of 1982, the studies of TFIIIA by Jonathan miller lead to the discovery of a remarkable repeating motif within many proteins, which is called zinc finger motif now (Miller et al., 1985). The sequence of zinc fingers was further confirmed and aligned as a motif of 30 amino acids by computer analysis (Miller et al., 1985). This motif is a self-contained domain stabilized by a zinc ion which was ligated to a pair of cysteines and a pair of histidines in addition to an inner structural hydrophobic core. The consensus sequence Φ-X-Cys-X(2-4)-Cys-X3-Φ-X5-ΦX2-His-X(3,4)-His from which X represents any

amino acid and Φ represents a hydrophobic residue. This classical Cys_2His_2 (C_2H_2) domain can be linearly linked in tandem to bind with nucleic acid sequences of different lengths. This linearly linking pattern increases the binding possibilities for the specific binding on DNA/RNA. Therefore, there is no surprising that C_2H_2 motif is found in 2% of human genes (Tupler et al., 2001). Now, more and more zinc finger types are being elucidated, such as CCHC (C_2HC), Ring, LIM, C_2C_2, CCCH (C_2CH) and TAZ. In this thesis, we focus on C_2H_2 zinc fingers.

In general, the cellular differentiation is regulated by TFs. There are about 30000 human genes in our genome of which about 2000 are TFs. C_2H_2 is the most common DNA binding domain among eukaryotic TFs (Huntley et al., 2006). Recent studies suggested that about 70% C_2H_2-ZNF family are organized in clusters in human genome. Noteworthy, chromosome 19 is particularly enriched with these genes that are present in clusters (Eichler et al., 1998). A study on the evolution of C_2H_2-ZNF between chimpanzee, mouse, rat, dog and human revealed that the evolution of such family is independent between two primates and two rodents (Tadepally et al., 2008). According to the binding specificities and protein function of C_2H_2-ZNF, this evolution could be important to determine the species-specific functions.

1.4.1 Function of C_2H_2 zinc fingers

Many C_2H_2 ZNFs can selectively bind to specific DNA and control gene transcription (Bouhouche et al., 2000). It is also known that this domain is not only

used for protein-DNA interaction but also is used for protein-RNA and protein-protein interaction (Yang et al., 1999; Tian et al., 2009). This motif could bind to the promoter with ββα structure formed by tetrahedral binding of Zn^{2+} ion to the canonical cysteine and histidine residues (Wolfe et al., 2000). Based on the number and the pattern of the zinc finger repeats, C_2H_2 family could be divided into four classes: (A) single C_2H_2, (B) triple C_2H_2, (C) multiple-adjacent C_2H_2, and (D) separated-paired C_2H_2 ZNFs (Figure 5) (Iuchi, 2001). Only the single zinc finger requires an additional non-zinc finger domain to bind to the target DNA (Omichinski et al., 1997).

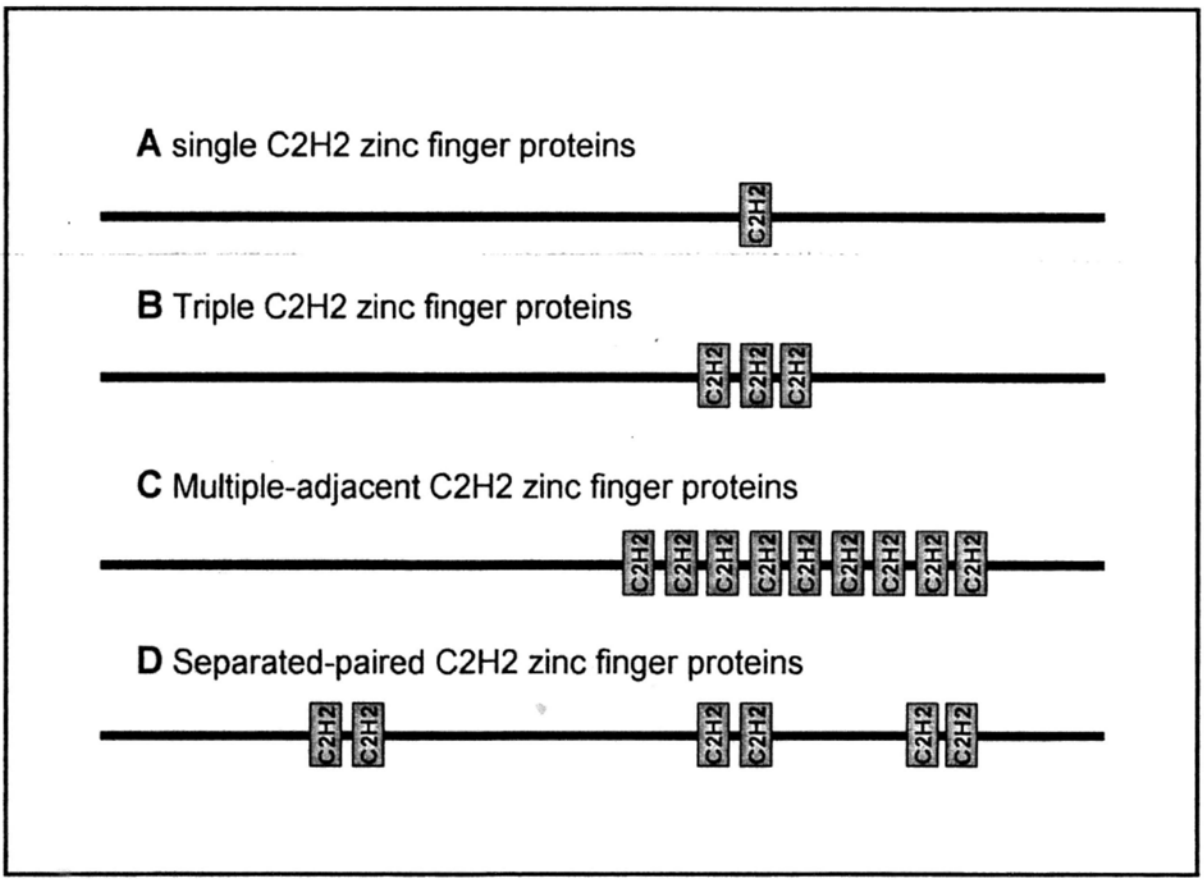


Figure 5. Schematic representation of four classes of C2H2 ZNFs. The pattern and the number of C2H2 zinc fingers indicate how the ZNFs function in DNA binding and transcriptional regulation.

1.4.2 Zinc finger-containing protein

An effector domain is frequently located at the N-terminal of the C2H2 zinc finger. These domains including KRAB (Krüppel-Associated-Box), SCAN (SRE-ZBP, CTfin51, AW-1 and Number18 cDNA) and BTB (Broad-Complex, Tramtrack and Bric-a-bric) domain. The first two domains are vertebrate-specific, while BTB is also present in insects (Zollman et al., 1994).

1.4.2.1 KRAB-containing ZNFs

This type of proteins is characterized by presence of 4-30 zinc finger motifs and a KRAB domain. The KRAB domain consists of either one or both of the KRAB A and B box (or a divergent B box- b box). The KRAB A box and B box are highly conserved in humans (URRUTIA, 2003). The two boxes of the KRAB domain are always encoded by individual exon which is always separated by intron. This composition is very useful for the diversity of proteins through alternative splicing (Figure 6). The zinc finger domain of KRAB- ZNFs is often encoded by a single exon. Also, some ZNFs containing fewer zinc fingers might have more than one exon to encode DNA binding domain such as Sp1-like proteins with 3 zinc fingers (URRUTIA, 2003).

KRAB-ZNFs make up approximately one third of ZNFs in human genome (290/799). Many KRAB-ZNFs are arranged in clusters. Notably, there are about 148 KRAB-ZNFs within a minimal region close to 19q13 but other clusters reside in

telomeric and centromeric regions of other chromosomes. Other KRAB-ZNFs exist individually throughout the genome (Rousseau-Merck et al., 2002; URRUTIA, 2003).

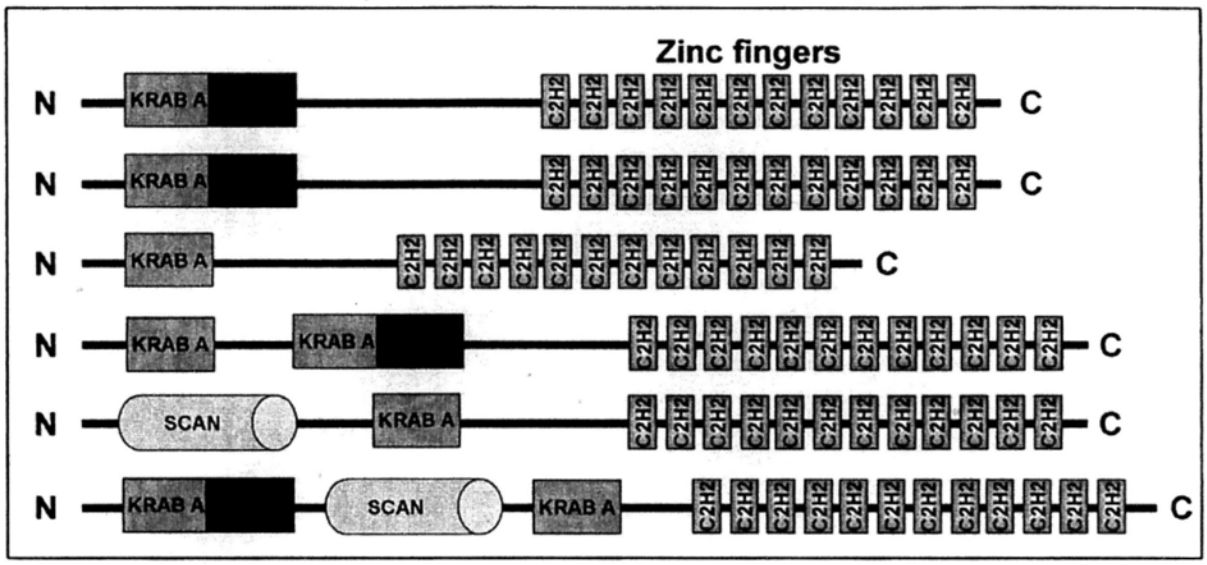


Figure 6. Primary structures of typical KRAB-containing ZNFs. Eight zinc fingers were shown here to imply for the variable number of zinc fingers ranging from 4 to over 34. The KRAB domain consists of A and B boxes; some proteins contain a variant called the b box. A leucine-rich SCAN domain exiting in some members of the family is responsible for the homo- and hetero-dimerization with other SCAN-containing zinc-finger proteins. N, amino terminus; C, carboxyl terminus.

KRAB- ZNFs are involved in the process of cell differentiation, proliferation, apoptosis and neoplastic transformation (URRUTIA, 2003). For instances, APAK interacts with p53 and suppresses p53-mediated apoptosis (Tian et al., 2009); ZNF23 has growth-inhibitory potential (Huang et al., 2007); ZBRK1, a BRCA1- dependent transcriptional repressor, plays roles in cell growth control and survival (Tan et al., 2004). The KRAB-ZNFs are also involved in heterochromatin formation which in turn leads to epigenetic silencing. KRAB domain containing ZNFs bind to specific DNA sequence and recruit KRAB associated protein 1(KAP1), which forms

heterochromatin with HP1, SETDB1 and HDAC to silence the target gene expression (URRUTIA, 2003). Once the heterochromatin is formed, it persists for over fifty times of cell divisions (Ayyanathan et al., 2003). The active form of ZNFs is a critical requirement to initiate heterochromatin formation, thus irregular KRAB-ZNFs disrupt the formation of heterochromatin, and result in the uncontrollable gene expression leading to tumorigenesis.

1.4.2.2 SCAN domain-containing ZNFs

There are about 59 SCAN domain-containing ZNFs in human. The SCAN domain is a highly conserved motif containing 84 residues and is located near the N-terminal of C2H2 ZNFs (Figure 7). This domain is responsible for protein-protein interaction, mediating self-association and selective association with other SCAN domain proteins (Edelstein and Collins, 2005). SCAN domain family plays an important role in regulating expression of genes which are involved in growth, cell survival, differentiation and lipid metabolism. The SCAN domain-containing zinc fingers which constitute approximately 10% of all ZNFs are also located in clusters on 3p21-22, 6p21-22, 16p13.3 and 17p12-13 (URRUTIA, 2003). Several SCAN domain-containing zinc fingers also contain a KRAB domain (Figure 6).

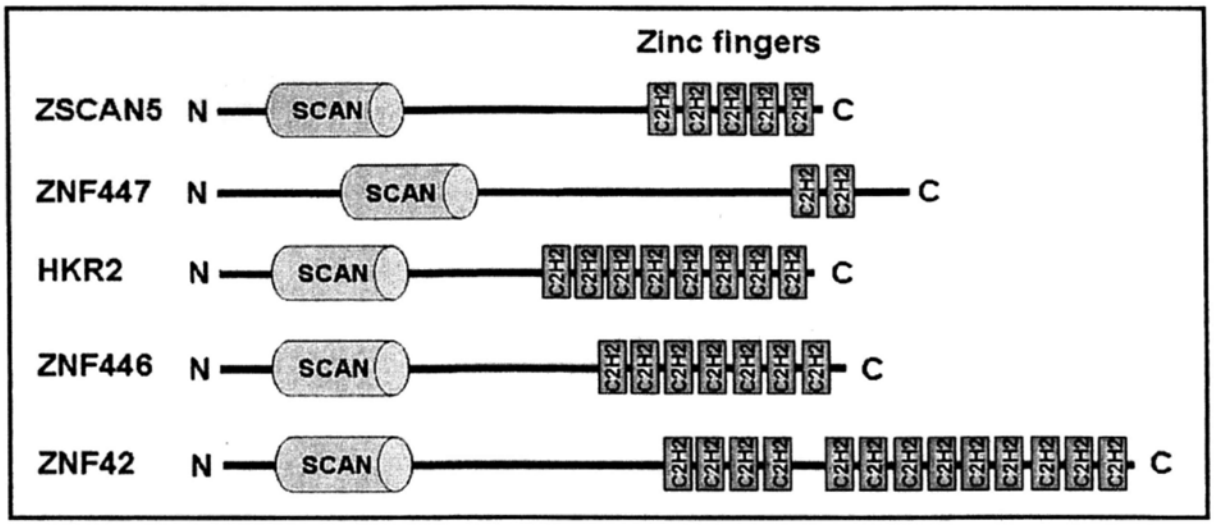


Figure 7. SCAN domain containing ZNFs. Representative show the names and predicted structures of human SCAN domain proteins at chromosome 19.

As mentioned above, SCAN domain is responsible for protein-protein interaction. SCAN family can self-associate or form heterodimers with the SCAN domain but not all the SCAN domains are able to self-associate or homodimerize (Edelstein and Collins, 2005). These interactions are also selective. For example, ZNF174 interacts with several SCAN domains but not with all other SCAN domain-containing proteins. SCAN domain containing-ZNFs were frequently involved in tumor related activities. ZNF307 could suppress p53 and p21 pathway {4376}. ZNF42, a functional tumor suppressor, forms heterodimer with PML nuclear bodies (Noll et al., 2008).

1.4.2.3 BTB domain-containing ZNFs

The BTB domain, also known as POZ (Poxvirus and Zinc finger) domain, is a highly conserved and widely distributed motif which consists of 90-120 residues (Numoto et al., 1993; Zollman et al., 1994). The BTB domain is responsible for protein-protein interaction. The BTB-containing protein can form

homo-oligomerization or hetero-oligomerization, or interact with other non-BTB-containing proteins (Ahmad et al., 1998; Daniel and Reynolds, 1999; He et al., 1998).

The human genome encodes for 205 BTB proteins which could be roughly divided into three subclasses: BTB/ZNFs, BTB/kelch proteins and BTB (T1)/ion channels. The BTB/ZNFs are generally thought as TFs. Studies have shown that BTB proteins PLZF and BCL-6 are involved in cancer. PLZF is a functional tumor suppressor which represses cell growth and leads to cell cycle arrest by inhibiting the expression of cell cycle regulators such as cyclin A and c-MYC (Shaknovich et al., 1998). BCL-6 is a transcriptional repressor and plays a vital role in lymphoma tumorigenesis and the development of the immune system. In lymphoma cells, BCL-6 directly represses the expression of cell cycle mediators, cytokines, chemokines and their receptors (Kusam and Dent, 2007; Crotty et al., 2010). In the immune system, Bcl-6 functions as a critical regulator of specific components and the downreglation of Bcl-6 expression will cause cancer (Ye, 2000).

1.4.3 ZNFs and cancer

Being the largest group of TFs, ZNFs play important roles in cell differentiation, proliferation, apoptosis and neoplastic transformation (URRUTIA, 2003). Several of them are reported as oncogenes or TSGs in different tumors. In case of oncogenic ZNFs, ZNF217 is responsible for the proliferation and invasion of ovarian cancer

(Sun et al., 2008). ZNF639 might be involved in the pathogenesis of esophageal squamous cell carcinoma (ESCC) (Imoto et al., 2003). On the other hand, some zinc fingers are found to be tumor suppressors. For example, ZNF23 could inhibit cell cycle progression (Huang et al., 2007); KLF4 can reduce tumorigenecity of colon, breast and gastric cancers (Dang et al., 2003; Akaogi et al., 2009; Wei et al., 2005); ZBRK1 functions as a BRCA1-dependent transcriptional repressor in cell growth control and survival (Tan et al., 2004); ST18 is a tumor suppressor in breast cancer (Jandrig et al., 2004); ZIPK is a functional tumor suppressor inducing apoptosis and is frequently deleted in gastric cancers (Bi et al., 2009). Notably, a number of zinc finger tumor suppressors are silenced by DNA methylation in different types of tumors (Table 1).

Table 1. A brief summary of methylated zinc finger tumor suppressors in multiple tumors.

Gene name	chromosome	Zinc finger type	Methylated tumors	references
CASZ1	1p36.22	C2H2	neuroblastoma tumours	(Caren et al., 2007)
EGR3	8p21-p23	C2H2	T-cell leukemia	(Yasunaga et al., 2004)
HIC1	17p13.3	C2H2	Breast cancer, lung cancer, hematopoietic neoplasms	(Parrella et al., 2005; Fukasawa et al., 2006)
PEG3	19q13.4	C2H2	Ovarian cancer, Glioma	(Feng et al., 2008; Otsuka et

				al., 2009)
PLAGL1	6q24	C2H2	Ovarian and breast cancer.	(Abdollahi et al., 2003)
PRDM1	6q21-q22.1	C2H2	B cell lymphoma	(Pasqualucci et al., 2006)
PRDM2	1p36	C2H2	Lung cancer, thyroid cancer, neuroblastoma	(Yoon et al., 2007; Lal et al., 2006; Hoebeeck et al., 2009)
PRDM5	4q25-q26	C2H2	Cervical cancer, colorectal and gastric cancer.	(Watanabe et al., 2007; Cheng et al., 2010a)
ST18	8q11.23	C2HC	Breast cancer	(Jandrig et al., 2004)
TNFAIP3	6q23	A20-like zinc fingers	lymphoma	(Honma et al., 2009)
ZIC1	3q24	C2H2	Gastric cancer	(Wang et al., 2009a)
ZIK1	19q13.43	C2H2	Colon cancer, ESCC, gastric cancer	(Borinstein et al., 2010; Oka et al., 2009; Mihara et al., 2006)

Up to date, only few ZNFs, such as ZAC and ST18, are reported as tumor suppressors silenced by DNA methylation, indicating that more epigenetically silenced ZNFs are yet to be identified.

Chapter 2

Aims of This Study

TSGs function through the following mechanisms: protecting the genome from mutagenic events, regulating cell cycle, inducing apoptosis in cells that escape normal cell cycle control, and inhibiting cellular migration and metastasis (Hayslip and Montero, 2006). Cancer is caused by aberrant gene regulation. Genetic alteration is a hallmark of human cancers, while aberrant epigenetic alteration of tumor cells is also important in initiating carcinogenesis and even precedes genetic changes during tumorigenesis (Herman, 1999; Jones and Baylin, 2002). Promoter CpG methylation causes the loss of TSG functions frequently occurring during tumor development and progression (Jones and Baylin, 2002). Clinically, TSG methylation could be used as epigenetic biomarkers for tumor diagnosis and prognosis prediction.

ZNF is the largest family of TFs with their zinc fingers binding to promoters to activate or repress gene expression (Cowger et al., 2007). ZNFs also interact with other proteins to function in various signaling pathways and sometimes even bind to dsRNA (Yang et al., 2006). About one third of ZNFs contain a KRAB domain. KRAB-containing ZNFs are involved in cell differentiation, proliferation, apoptosis and neoplastic transformation (URRUTIA, 2003). A large number of ZNFs are located in clusters at the long arm of Chromosome 19. Genetic deletion of 19q is a frequent event in multiple cancers including malignant glioneuronal tumors, cervix tumor, ESCC and NPC- a prevalent cancer in our locality (Vogazianou et al., 2010; Tsuda et al., 2002; Du et al., 1999; Shao et al., 2001). Some of the KRAB-ZNF may function as potential TSGs with epigenetic alterations (Jandrig et al., 2004; Wang et al., 2009a). Thus, I try to identify novel silenced KRAB-ZNF candidate TSGs

through genome-wide screening on chromosome 19.

2.1 Identify novel silenced KRAB-ZNF candidate TSGs

Expression profiling of ZNFs with CpG islands at 10 clusters of chromosome 19 was first performed in a panel of NPC and ESCC cell lines by semi-quantitative RT-PCR, with adult normal tissues-larynx and esophagus as controls. Several downregulated genes were thus identified. For the candidates I am interested in, I further checked their expression profiles in other tumor cell lines including lung, gastric, colon, breast, kidney, liver, cervix and prostate carcinomas. The methylation statuses of their promoters were revealed in multiple downregulated cell lines and non-tumor cell lines by methylation-specific PCR (MSP) and bisulfite genomic sequencing. To determine whether DNA methylation was directly involved in transcriptional silencing, I examined the expression and methylation status of these candidate genes in cell lines with pharmacologic or genetic demethylation. I further investigated the methylation percentage of these candidate genes in primary tumors of NPC and ESCC, as well as in normal epithelial tissues and paired normal tissues. The frequent methylation of these genes indicated they could act as candidate biomarkers.

2.2 Functional study of candidate KRAB-ZNF TSGs

More functional studies will be done for several candidates with colony formation assay, cell proliferation and apoptosis assay. I will further test the possible signaling pathways and potential targets by luciferase assay or real time PCR. Then I analyze the subcellular localization and proposed a possible model for each ZNFs.

Chapter 3

Materials and Methods

3.1 Cell lines and tumor samples

A series of tumor cell lines were used, including 6 nasopharyngeal (C666-1, CNE1, CNE2, HK1, HNE1, HONE1), 18 esophageal (EC1, EC18, EC109, HKESC1, HKESC2, HKESC3, KYSE30, KYSE70, KYSE140, KYSE150, KYSE180, KYSE220, KYSE270, KYSE410, KYSE450, KYSE510, KYSE520, SLMT1), 13 liver (Hep3B, HepG2, huH1, huH4, huH6, huH7, Mahlavu, PLC/PRF/5, SNU387, SNU398, SNU423, SNU449 and SNU475), 4 colon (HCT116, HT29, LoVo and SW480), 5 lung (A427, A549, H292, H358, H1299), 11 breast (MCF7, T47D, ZR-75-1, MB231, MB468, YCC-B1, YCC-B2, YCC-B3, SK-BR-3, MB435, BT549), 10 gastric (Kato III, YCC-1, YCC-2, YCC-3, YCC-6, YCC-7, YCC-9, YCC-10, YCC-11, YCC-16), 4 cervix (HeLa, CaSki, C33A, SiHa) and 3 prostate (Du145, LNCap and PC3). Nine immortalized normal epithelial cell lines (NP69, NE1, NE3, NE083, HET-1A, HEK293, RHEK1, HMEC and HMEpC) with many features of normal epithelial cells were also included. HCT116 cell lines with genetic knockout of DNA methyltransferases (DNMTs): HCT116 DNMT1$^{-/-}$ (1KO), HCT116 DNMT3B$^{-/-}$ (3BKO) and HCT116 DNMT1$^{-/-}$ DNMT3B$^{-/-}$ (DKO) cells (gifts of Bert Vogelstein, Johns Hopkins) were grown in medium containing either 0.4 mg/ml genecitin or 0.05 mg/ml hygromycin (Rhee et al., 2002). Normal adult and fetal human tissue RNA samples were purchased commercially (Stratagene, La Jolla, CA, USA or Millipore Chemicon, Billerica, MA, USA) (Ying et al., 2006b). DNA Samples of normal nasopharyngeal and esophageal tissues, primary Asian NPC, nude mice-passaged NPC tumors originated from North Africans, and paired esophageal carcinomas (T) and their corresponding surgical marginal normal tissues (N) were

used for methylation study (Ying et al., 2006b; Lee et al., 2008; Cui et al., 2008).

3.2 Drug and stress treatments

Tumor cells (1×10^5 cells/ml) were allowed to grow overnight. Then the culture medium was replaced with fresh medium containing Aza at a final concentration of 10 μM (Sigma-Aldrich Corporation, St Louis, MO, USA) (Qiu et al., 2004). Cells were allowed to grow for 72 h, with changing of 10 μM Aza-containing medium for every 24 h and then treated with histone deacetylase inhibitor Trichostatin A (TSA) for additional 24 hours. Cells were then harvested for DNA and RNA extraction.

For heat-shock treatment of cell lines, cells were first seeded at 3 $x10^5$ cells/ml and allowed to grow overnight. T25 flasks containing cells were incubated in a 42°C water bath for 1 hour followed by recovery in 5% CO_2 incubator at 37°C for 2 hours. The cells were then harvested for RNA extraction.

3.3 DNA and RNA extraction

DNA and RNA were extracted from cell lines or tissues using TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH) according to manufacturer's protocol. Briefly, 5-$10x10^6$ cells were lysed in 1 ml TRI REAGENT by repetitive pipetting until the cell suspension became homogeneous. Then 0.2 ml chloroform was added and the mixture was vortexed for 15 seconds. This mixture was further incubated at room temperature for 3 minutes and centrifuged at 12,000 g for 15 minutes at 4°C. After that, the mixture was separated into an aqueous phase, an interphase and an organic phase. The aqueous phase, which contains RNA, was

transferred to a fresh tube for RNA extraction and the interphase and organic phase were saved at 4 °C for subsequent isolation of DNA. RNA from the aqueous phase was precipitated by mixing with 0.5 ml of isopropanol at 12,000 g for 8 minutes at 4 °C. The RNA pellet was washed with 75% ethanol and subject to centrifugation at 7,500g for 5 min at 4 °C. Finally, the RNA pellet was dissolved in RNASecureTM resupension solution after air dry.

For DNA extraction, 0.3 ml of 100% ethanol per 1 ml of TRI REAGENT was added to the interphase and organic phase for the initial homogenization. The mixture was incubated at room temperature for 3 minutes, and subjected to centrifugation at 2,000 g for 5 minutes at 4 °C. DNA sediment was washed twice with solution containing 0.1 M trisodium citrate in 10% ethanol. After that, the DNA pellets were washed with 75% ethanol. The DNA pellets were air-dried at room temperature and dissolved in 8 mM NaOH by slowly passing through a pipette. The pH of DNA sample was adjusted to 8.4 using HEPES according to the manufacture's protocol (Gibco BRL, USA). DNA quantity and quality were checked by measuring A260 absorbance and the A260/A280 respectively (Nanodrop nd-1000, Wilmington, USA). The DNA samples were stored at 4 °C until further analyses.

3.4 Semi-quantitative RT-PCR and Real time PCR

Reverse transcription (RT) of RNA to cDNA was performed by using the GeneAmp RNA PCR kit (Applied Biosystems, Carlsbad, California, USA) according to manufacture's protocol. RT–PCR was performed for 32 cycles with hot-start Go-Taq (Promega Corporation, Madison, WI, USA). The PCR program started with an initial

denaturation at 95°C for 2 min, followed by 23–37 cycles (94°C for 30 s, 55°C - 60°C for 30 s, and 72°C for 30 s) of amplification, with a final extension at 72°C for 3 min (Tao et al., 2002). The PCR products were visualized under ultraviolet light, photographed with the Bio-Rad digital gel documentation system (Bio-Rad, USA) and the supplied densitometry software Quantity One v4.5. *GAPDH* was used as an internal control, and only 23 cycles of amplification were used.

Real-time PCR was done using ABI SYBR Green Power master mix according to the protocol manufactures (HT7900 system, Applied Biosystems, Carlsbad, California, USA). The expression level of TSGs in normal tissues (larynx and esophagus) was set as a baseline for carcinoma cells, respectively.

3.5 Bisulfite treatment

Sodium metabisulfite (Sigma-Aldrich, St Louis, MO, USA) was used for DNA bisulfite modification. Firstly, 1-5 μg genomic DNA was denatured with 0.3 M NaOH for 15 minutes at 37°C. Solutions of 10 mM hydroquinone and 2.4 M sodium metabisulfite (Sigma, St Louis, MO, USA) should be freshly prepared prior to bisulfite modification. Once the sodium metabisulfite was completely dissolved, 210 μl of freshly prepared 10 mM hydroquinone was added into the sodium metabisulfite solution. Subsequently, 333 μl of sodium metabisulfite/hydroquinone solution was added to the denatured DNA. The reaction was overlaid with mineral oil (Sigma, St Louis, MO, USA) and incubated at 55°C in darkness for 4 hours. Then, the reaction mixture was carefully transferred from beneath the oil to a new 1.5 ml eppendorf tube and the free bisulfite was desalted by QIAEX II DNA Clean-Up System according to the manufacturer's protocol (QIAGEN, Germany). Shortly, 1ml QXI

was added to the sample and then mixed with QX II (16 μl for every 2 μg DNA). These mixtures were incubated at room temperature with rotation for 1 hour, and washed with PE twice. Subsequently, the DNA was dissolved with 50 μl TE buffer and denatured by NaOH at a final concentration of 0.3M for 15 minutes at 37°C. Finally, the bisulfited DNA was extracted using QIAEX II according to the manufacturer's protocol and eluted in TE (20 μl /μg DNA) and stored at -30°C for further analysis.

3.6 5'-Rapid amplification of cDNA ends (5'-RACE)

The 5'-RACE version 2.0 kit (Invitrogen, Carlsbad, California, USA) was used to determine the *ZNF545* and *ZFP30* transcriptional start sites. The first-strand cDNA was synthesized from testis RNA (Stratagene, La Jolla, CA, USA) using primer *ZNF545*RACE2 or *ZFP30*RACE2, respectively. Then, homopolymeric tails were added to the 3'ends with terminal deoxynucleotidyl transferase. PCR was done using Abridged Anchor Primer (AAP) and a second gene-specific primer *ZNF545*RACE3 or *ZFP30*RACE3, The RACE product was enriched by re-amplifying with the Abridged Universal Amplification Primer (AUAP) and *ZNF545*RACE4 or *ZFP30*RACE4. The PCR product was TA cloned and sequenced.

3.7 CpG island and Transcription factor binding sites analysis

CpG island located on the region spanning the core promoter, exon 1 and intron 1, is analyzed by the CpG island searcher (http://www.uscnorris.com/cpgislands/cpg.cgi) and NCBI map viewer. Transcription factors' bindings on putative promoters were predicted by MatInspector (http://www.genomatix.de).

3.8 Methylation-specific PCR (MSP)

Several pairs of MSP primers targeting the methylated or unmethylated alleles of the promoter region were designed and tested. Only the optimal pairs with the best amplification efficiency and specificity were used. These primer pairs were also tested for not amplifying any unbisulfited DNA and thus specific to bisulfite-converted DNA. MSP was performed using AmpliTaq-Gold (Applied Biosystems, Carlsbad, California, USA) and the component was listed in Table 2.

Table 2. Components of each reaction mixture for MSP.

Components	**Volume used (μl)**	**Final Concentration**
10x PCR buffer	1.25	1x
dNTPs (2.5 mM each)	1	0.2 mM
$MgCl_2$ (25 mM)	1	2 mM
Forward M or U primer (10 μM)	0.75	0.6 μM
Reverse M or U primer (10 μM)	0.75	0.6 μM
AmpliTaq Gold (5 U/μl)	0.0625	2.5 U/50μl
Bisulfite-treated DNA	0.5	0.1 μg/100μl
Autoclaved distilled water	7.1875	
Total volume	12.5μl	

The PCR conditions were as follows: initial denaturation and hot start for 10 minutes at 95 °C, then with appropriate cycles consisting of 30 seconds at 94 °C, 30 seconds at annealing temperature (optimized for methylated and unmethylated reactions), and 30 seconds at 72 °C, and a final extension of 5 minutes at 72°C.

3.9 Bisulfite genomic sequencing (BGS)

The bisulfite modification of DNA converts the unmethylated cytosine into uracil except the methylated cytosine. The methylation status of each CpG dinucleotide within a region of our interest can be revealed by BGS. The PCR reaction was the same as the MSP reaction, except for the final extension step was changed to one hour to ensure that all PCR products were 3' adenylated. The PCR products were purified with Costar Spin-X centrifuged tube filter (Cole-Parmer, Vernon Hills, IL) and cloned into the PCR4-TOPO vector which has single, overhanging 3' deoxythymidine (T) residues (Invitrogen, Carlsbad, California, USA). Five to six colonies were randomly chosen and sequenced.

3.10 Plasmid extraction

3.10.1 Mini-scale preparation of plasmid DNA

Bacterial colonies picked from agar plates were cultured at 37°C overnight (~12-15 hrs) in an inoculation tube containing 1.5 ml LB medium and appropriate antibiotics with shaking at 250 rpm. About 1.3 ml cultured medium was spun down at 6,000 rpm for 5 minute. The supernatant was removed by aspiration. The bacterial pellet was resuspended in 150 µl Solution I (50 mM glucose, 25 mM Tris-Cl, 10 mM EDTA and 0.5 mg/ml RNase A) and incubated for 10 minutes at room temperature after vortexing. The bacteria were lysed with freshly prepared 300 µl Solution II (200 mM NaOH and 1% SDS). This mixture was inverted gently for 5-6 times and kept on ice for 3-5 minutes. The lysate was neutralized with 255 µl chilled Solution III (60 ml of 5 M glacial acetic acid added with 11.5 ml glacial acetic acid and 28.5 ml H_2O; pH5.2) and kept on ice for more than 5 minutes. The mixture was centrifuged at 4°C for 15 minutes at 14,000 rpm. After centrifugation, the supernatant was transferred into a new tube. About 330 µl isopropanol was added to the supernatant

and mixed well. This mixture was incubated for 15 minutes at room temperature and centrifuged at 14,000 rpm for 10 minutes at room temperature. The supernatant was removed by aspiration, and the plasmid pellet was washed with 800 µl of 70% ethanol with further centrifugation at 10,000 rpm for 5 minutes. Finally, the plasmid pellet was air-dried and dissolved in 40 µl distilled water. The plasmid was stored at 4°C prior to subsequent analysis and 1µl was subjected to gel electrophoresis for DNA quality analysis.

3.10.2 Large-scale endotoxin-free plasmids extraction

Large-scale endotoxic-free plasmids were extracted by using Endofree plasmid kit (QIAGEN, Germany).

3.11 DNA cycle sequencing

BigDye®V3.1 terminator sequencing (Applied Biosystems, Carlsbad, California, USA) was used for DNA sequence analysis. The sequencing reaction mix was assembled as 1 µl DNA template, 1 µl BigDye and 0.5 µl of 1.6 µM sequencing primer in a total reaction volume of 5 µl (Table 3).

Table 3. Components of each reaction mixture for sequencing

Components	**Volume used (µl)**
DNA template	1
BigDye	1
Primer(1.6 µM)	0.5
Total volume	5 µl

The program was set as 96°C, 15s, 52°C, 15s and 60°C, 2min for 25-30 cycles using MJ DNA Engine Dyad® Thermal Cycler (MJ research). The reaction mixtures were

then subjected to purification with 3 M sodium acetate and 100% ethanol. After that, the mixtures were centrifuged at 14,000 rpm for 15 minutes. The pellets were washed with 100 μl 70% ethanol and were subjected to centrifugation again at 14,000rpm for 5 minutes. After that, the pellets were air-dried at room temperature in darkness and dissolved in 12 μl Hi-Di^{TM} Formamide (Applied Biosystems, Carlsbad, California, USA) for at least 5 minutes in darkness. The samples were then loaded to an ABI optical 96-well plate for sequencing. The ABI 3100 DNA sequencer (Applied Biosystems, Carlsbad, California, USA) was used for sequencing and the sequencing results were analyzed by the ABI Sequencing Analysis software.

3.12 Construction of expression plasmids

3.12.1 Gene cloning and plasmids construction of *ZNF382*

The full length open reading frame (ORF) of *ZNF382* was cloned from adult testis cDNA library by nested-PCR. *ZNF382*CF2/*ZNF382*CR2 was used for the first round PCR, and *ZNF382*CF3/*ZNF382*CR3 was subsequently applied for the second round PCR. All PCR reactions were performed with Pfu polymerase (Stratagene, La Jolla, CA, USA). The PCR product was then digested with B*am*HI, and ligated into pcDNA3.1(+) with sequence and orientation confirmed. The same ORF segment was also subcloned into pIRES-ZsGreen1 for flow cytometry analysis.

3.12.2 Gene cloning and plasmids construction of *ZNF545* and *ZFP30*

The ORF of *ZNF545* or *ZFP30* was cloned from adult testis cDNA library by semi-nest PCR (*ZNF545*: ZNF545CF3/CR3 for first round, ZNF545CF1/CR3 for second round; *ZFP30*: ZFP30CF3/CR3 for first round, ZFP30CF1/CR3 for second round). The PCR product was cloned into PCR-TOPO2.1 and orientation confirmed clones were chosen for sequencing (B*am*HI site in vector sequence was used for the next subcloning). The verified ORF was subcloned into pCMV-BD with B*am*HI. We also added a Flag tag at 3' terminal and a Kozak sequence at 5' terminal by PCR cloning primers with ZNF545CF4/CR4 or ZFP30CF4/CR4 and cloned into pcDNA3.1 with B*am*HI. The same insert was also subcloned into pIRES-zsGreen1 for flow cytometry.

3.13 Subcellular localization

$5x10^4$ of cells were seeded on cover slips in a 6-well plate. Cells were transfected with Flag-tagged plasmids using Fugene6 (Roche, Konzern-Hauptsitz, Switzerland). For ZNF382, we choose COS7 and HCT116 cells; for ZNF545 and ZFP30, we choose COS7, HONE1 and KYSE150 cells. In COS7 cells, ZNF382 was also co-transfected with RFP-*HP1γ* (Fukagawa et al., 2004). Twenty four hours after transfection, cells were fixed with pre-cooled methanol for 15 min and washed with PBS three times. The fixed cell was blocked with 5% normal rabbit serum for 1 hour at room temperature. Cells were then incubated with 0.1mg/ml anti-flag monoclonal antibody (Sigma-Aldrich, St Louis, MO, USA) for 1 hour at 37°C. Subsequently,

cells were incubated with FITC-conjugated rabbit anti-mouse IgG antibody (F0313, DAKO, Produktionsvej, Glostrup, Denmark) for 1 hour and the nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI) for 10 min. Subcellular localization was detected using an inverted fluorescence microscope (Olympus, Japan).

3.14 Colony formation assay

Monolayer culture was performed. Cancer cells (1×10^5/well) were seeded in a 12-well plate and transfected with the target plasmids or the control vector (0.8 μg each), using Fugene 6. Forty-eight hours post transfection, cells were collected and plated at appropriate density in a 6-well plate and subjected to G418 (0.4 mg/ml) selection for 10-12 days, with selective medium replaced every 3 days. Surviving colonies (>50 cells/colony) were counted after staining with Gentian Violet (ICM Pharma, Singapore). Total RNA from the transfected cells was extracted, treated with DNase I and analyzed by RT–PCR to confirm the ectopic expression of the targets. All the experiments were performed in triplicate wells for three times.

3.15 Cell proliferation assay

Cell proliferation assay was measured by flow cytometry (BD Biosciences, San Jose, CA) with DDAO-SE (Invitrogen, Carlsbad, California, USA) staining. pIRES-ZsGreen1 was used for the sorting of GFP positive cells. The cells were transiently transfected with the target genes or control vector. Twenty-four hours after transfection, 1×10^5 cells were replated in a 12-well plate. After another 24 hours,

cells were washed with PBS and incubated with 5 μM DDAO-SE (Molecular Probes) in PBS for 15 min at 37°C. The reaction was stopped by replacing the staining solution with fresh, pre-warmed medium and incubated for additional 30 min at 37°C. Cells were then harvested at the starting time point and 48 hours, and the fluorescence intensity was measured by flow cytometry for GFP positive cells. The experiment was repeated for three times independently.

3.16 Apoptosis assay

Apoptosis was assessed using the Annexin V-PE Apoptosis Detection Kit I (BD Biosciences, San Jose, CA) by flow cytometry. Cell apoptosis and viability were measured by Annexin V-PE and 7-Amino-Actinomycin (7-AAD) staining. Cell populations were counted as viable (Annexin V-, 7-AAD-), early apoptotic (Annexin V+, 7-AAD-), necrotic (Annexin V-, 7-AAD+), and late apoptotic cells (AnnexinV+, 7-AAD+). HCT116 cells transfected with pIRES-ZsGreen1 or pIRES-ZsGreen1-*ZNF382/ZNF545* were harvested at 48 h post transfection. GFP positive cells were sorted by flow cytometry and the apoptotic status was analyzed after staining with Annexin V-PE and 7-AAD. Both late and early apoptotic cells were counted together for relative apoptotic changes. All the experiments were performed three times.

Apoptosis was also determined by DAPI staining. Forty-eight hours post transfection of pIRES-ZsGreen1-*ZNF382/ZNF545* or control vector, cells were fixed with

pre-colded methanol for 10 minutes at -20°C. Subsequently, cell nuclei were stained with DAPI (0.5 μg/ml) for 10 minutes at room temperature and examined under a fluorescence microscope (Olympus, Japan). Condensed or fragmented nuclei indicated apoptotic changes.

3.17 Co-immunoprecipitation and Western blot

For co-immunoprecipitation, HEK293 (1×10^6) cells were co-transfected with 2 μg pcDNA3.1(+)-Flag-*ZNF382* and pEGFP-C1-*HP1β* (gift from Alain Verreault, UK) using Fugene 6. Forty eight hours after transfection, cells were lysed in 200 μl of lysis buffer (50 mm NaCl, 20 mm Tris, pH 7.6, 1% Nonidet P-40, 1×protease inhibitor mixture) for 1 h. The lysates were cleared by centrifugation at 10,000×*g* at 4°C for 10 min. Two hundred μg of total protein of each sample was used for a reaction. Firstly, the total protein lysates were precleared by 20 μl protein-G with incubation at 4°C for 30 min. After a brief centrifugation, the supernatant was transferred to a new 1.5 ml tube. The immunoprecipitation reaction was performed with 2 μg of indicated antibody and 50 μl of protein-G slurry for overnight incubation at 4°C. The precipitated materials were separated by SDS PAGE, transferred to PVDF membrane and immunoblotted (Wang et al., 2009b). For other western blot, forty-eight hours after transfection, cells were harvested and lysed in lysis-buffer (10 mM Tris-HCl pH 7.4, 1% SDS, 10% glycerol, 5 mM $MgCl_2$, 1 mM PMSF, 1 mM sodium orthovandate, 5 μg/ml leupeptin and 21 μg/ml aprotinin). Thirty μg of total cell lysate proteins were separated by SDS-PAGE, transferred to

PVDF membrane. The dilution of primary antibodies was used according to the company's recommendation. Proteins were visualized using the ECL detection system.

3.18 Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation assay was performed according to the protocols of manufacturer (Upstate, Lake Pacid, NY). Specific H3K4me3 antibody was used for ChIP assay (Abcam, Cambridge, MA, USA). As control for ChIP assays and for antibody specificity, equal amounts of control IgG and antibodies were used in parallel reactions. The DNA of input and immunoprecipitation reaction was purified with Qiagen DNA mini kit according to the manufacturer's suggested protocol (Qiagen, Germany). All samples were analyzed by quantitative real-time PCR. The fold enrichment of target sequence was determined using the following formula (fold enrichment=$2^{(\Delta CT \text{ of input}-\Delta CT \text{ of IP'ed DNA})}$).

3.19 Luciferase reporter assay

To screen for signaling pathways modulated by ZNF382 and ZNF545, several signaling pathway luciferase reporters were evaluated in *ZNF382/ZNF545*-transfected HCT116 and HEK293 cells, including NF-κB-luc, p53-luc, AP-1-luc, SRE-luc (Stratagene, La Jolla, CA, USA) and TOPFLASH (kindly provided by Prof. Christof Niehrs, German Cancer Research Center DKFZ, Heidelberg, Germany). Dual-luciferase assays were performed according to the

protocol of Promega (Promega Corporation, Madison, WI, USA). The pRL-SV40 Renilla control vector (Promega Corporation, Madison, WI, USA) was used as an internal control to normalize the different transfection efficiencies in all transfection assays. All the experiments were performed in triplicates.

3.20 Protein translation efficiency assay and cell size analysis

HCT116 or HONE1 cells were transfected with pIRES- ZsGreen1 or pIRES-ZsGreen1-ZNF545. Twenty-four hours after transfection, the GFP intensity was counted by flow cytometry after sorting the GFP positive cells, in parallel the fluorescence signal was also imaged by fluorescence microscope. For cell size analysis, twenty-four hours after transfection (pIRES- ZsGreen1 or pIRES-ZsGreen1-ZNF545), HCT116 cells were replated in a six-well plate at appropriate density. Cells were harvested at day 4 and the cell size of transfection positive cells was examined by flow cytometry.

3.21 Statistical analysis

Data were presented as mean±SD. Statistical analysis was carried out with Student's *t* test. $p< 0.05$ was considered as statistically significant difference.

Chapter 4: Results

4.1 Identification of novel KRAB-ZNF tumor suppressors in NPC and ESCC at chromosome 19.

ZNFs play important roles in cell differentiation, proliferation, apoptosis, and neoplastic transformation (URRUTIA, 2003). As discussed before, about 70% C_2H_2-ZNF family genes are organized in clusters in human. Chromosome 19 is particularly enriched in clusters of these genes (Eichler et al., 1998). There are 25 major zinc finger clusters in human genome and 10 of them are located on chromosome 19 (Huntley et al., 2006).

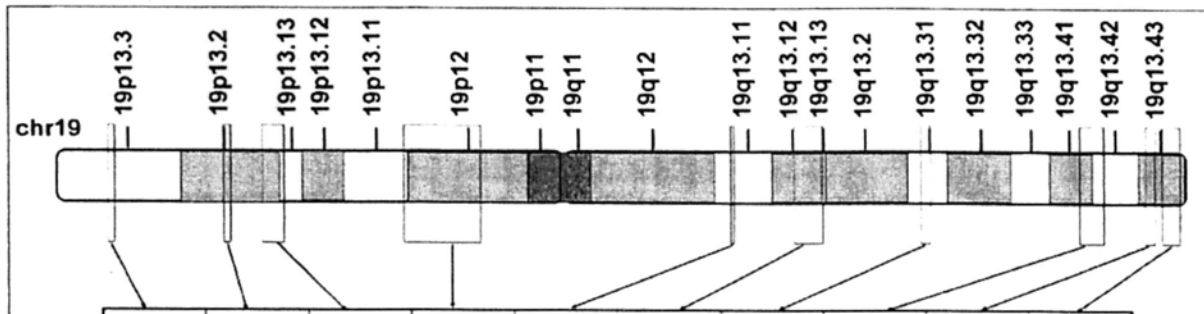
Genetic deletion of 19q is a frequent event in multiple cancers including malignant glioneuronal tumors, cervical tumor, ESCC and NPC - a prevalent cancer in our locality (Ramirez et al., 2010; Tsuda et al., 2002; Du et al., 1999; Shao et al., 2001). Several TSGs are located in chromosome 19. Glioma tumor suppressor candidate region gene 2 (*GLTSCR2/PICT-1*) is localized within chromosome 19q, which is frequently altered in various human tumors, including diffuse gliomas (Kim et al., 2008b). *EMP3* is a candidate TSG on 19q13.3 and frequently methylated in glioma (Kunitz et al., 2007). *ZIK1*, locating at 19q13.43, is a functional TSG in colon, ESCC and gastric cancers (Borinstein et al., 2010; Oka et al., 2009; Mihara et al., 2006), *ZIPK* is another tumor suppressor in 19q with frequent deletion in gastric cancer (Bi et al., 2009). According to the increasing evidence about TSG on chromosome 19, I hypothesize that chromosome19 is a very important TSG loci with several novel zinc

finger TSGs.

Firstly, I checked the CpG islands of all 210 ZNFs promoters located in the 10 clusters of chromosome 19. The genes with strict CpG island (500 bp min length, more than 50% GC content, 0.60 or higher observed CpG / expected CpG ratio) (Takai and Jones, 2002) was chosen for further expression analysis.

4.1.1 RT-PCR screening of candidate zinc finger TSGs in chromosome 19.

There are 10 clusters in chromosome 19 (Figure 8). I firstly checked the CpG of all ZNFs promoters, and then examined the expression profiles of the candidate zinc fingers with strict CpG island by semi-quantitative RT-PCR.



Region D (19p13.3)	Region A (19p13.2)	Region B (19p13.2-p13.13)	Region C (19p13.11-p12)	Region E (19q13.11)	Region O (19q13.12-q13.13)	Region F (19q13.31)	Region G (19q13.41-q13.42)	Region H (19q13.43a)	Region I (19q13.43b)
Z1	Z6	Z14	Z33	Z74	Z78	Z105	Z123	Z156	Z170
Z2	Z7	Z15	Z34	Z75	Z79	Z106	Z124	Z157	Z171
Z3	Z8	Z16	Z35	Z76	Z80	Z107	Z125	Z158	Z172
Z4	Z9	Z17	Z36	Z77	Z81	Z108	Z126	Z160	Z173
Z5	Z10	Z18	Z37		Z82	Z109	Z127	Z161	Z174
	Z11	Z19	Z38		Z83	Z110	Z128	Z162	Z175
	Z12	Z20	Z39		Z84	Z111	Z129	Z163	Z176
	Z13	Z21	Z40		Z85	Z112	Z130	Z164	Z177
		Z22	Z41		Z86	Z113	Z131	Z165	Z178
		Z23	Z42		Z87	Z114	Z132	Z166	Z179
		Z24	Z43		Z88	Z115	Z133	Z167	Z180
		Z25	Z44		Z89	Z116	Z134	Z168	Z181
		Z26	Z45		Z90	Z117	Z135	Z169	Z182
		Z27	Z46		Z91	Z118	Z136		Z183
		Z28	Z47		Z92	Z119	Z137		Z184
		Z29	Z48		Z93	Z120	Z138		Z185
		Z30	Z49		Z94	Z121	Z139		Z186
		Z31	Z50		Z95	Z122	Z140		Z187
		Z32	Z51		Z96		Z141		Z188
			Z52		Z97		Z142		Z189
			Z53		Z98		Z143		Z190
			Z54		Z99		Z144		Z191
			Z55		Z100		Z145		Z192
			Z56		Z101		Z146		Z193
			Z57		Z102		Z147		Z194
			Z58		Z103		Z148		Z195
			Z59		Z104		Z149		Z196
			Z60				Z150		Z197
			Z61				Z151		Z198
			Z62				Z152		Z199
			Z63				Z153		Z200
			Z64				Z154		Z201
			Z65				Z155		Z202
			Z66						Z203
			Z67						Z204
			Z68						Z205
			Z69						Z206
			Z70						Z207
			Z71						Z208
			Z72						Z209
			Z73						Z210

Figure 8. A schematic list of 10 zinc finger clusters in chromosome 19. The regions of ZNFs are marked with rectangle. ZNFs are shown in order of their gene locations.

4.1.2 Expression profiling of ZNFs in NPC and ESCC cell lines.

I examined the expression of the ZNFs in normal tissues (larynx and esophagus are equivalent to the normal tissues of NPC and ESCC). Eighteen cell lines were selected and used for the ZNFs expression screening (NP69, C666-1, CNE1, CNE2, HK1, HONE1, NE1, NE3, EC1, EC18, HKESC1, HKESC2, HKESC3, KYSE140, KYSE180, KYSE220, KYSE410 and KYSE510). For a fast screening, ten carcinoma cell lines were chosen (C666-1, CNE1, HK1, HONE1, EC1, HKESC1, HKESC3, KYSE180, KYSE410 and KYSE510). Several ZNFs are thus identified as downregulated or silenced candidate TSGs in cancer cell lines in contrast to their high expression in normal tissues (Figure 9).

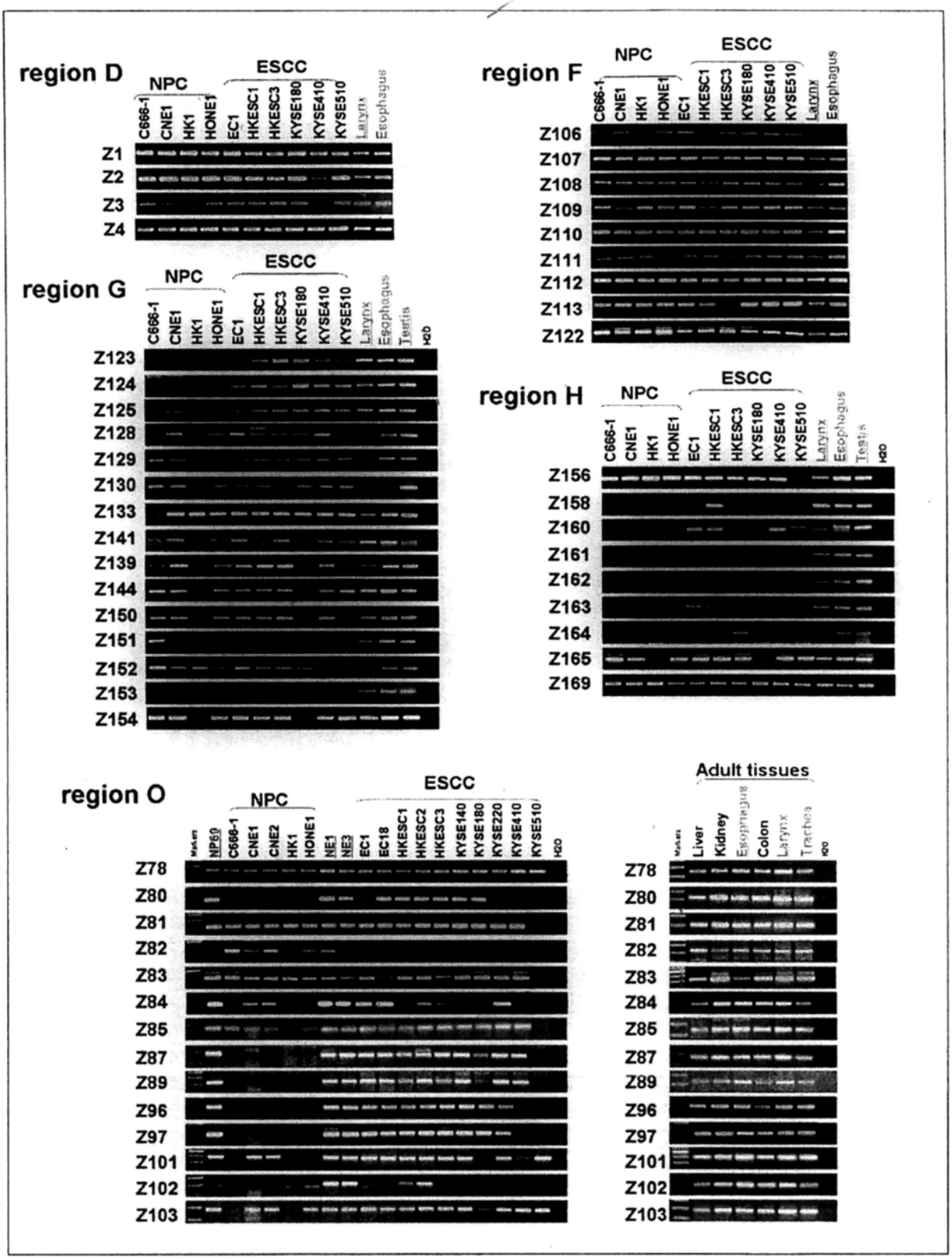


Figure 9. Representative schematic expression pattern of ZNFs in NPC and ESCC cell lines examined by RT-PCR.

In this study, five of candidates were chosen for further studies: *Z84* (*ZNF382*), *Z80* (*ZNF545*), *Z101* (*ZFP30*), *Z96* (*ZNFT1*) and *Z97* (*ZNFT2*). Since *ZNF545* and

ZFP30 share a high similarity at the protein level, while *ZNFT1* and *ZNFT2* are regulated by a bidirectional promoter, these two pairs of ZNFs are combined to study, respectively.

4.2 The KRAB zinc finger protein ZNF382 is a general, pro-apoptotic tumor suppressor repressing multiple oncogenes and frequently silenced in multiple carcinomas

Here, I investigated the potential tumor suppressor function of *ZNF382*, a novel 19q13.12 gene which we identified previously (Luo et al., 2002). I found *ZNF382* was epigenetically inactivated in common carcinomas including NPC and ESCC. I further demonstrated that ectopic expression of *ZNF382* suppressed the clonogenicity, proliferation and induced apoptosis in tumor cells lacking endogenous ZNF382 expression. ZNF382 also repressed NF-κB and AP-1 signaling, and inhibited the expression of multiple oncogenes including NF-κB upstream factors STAT3, STAT5B, ID1 and IKBKE through heterochromatin silencing.

4.2.1 Expression profiling of *ZNF382* in normal tissues and tumor cell lines

We previously identified a novel KRAB-ZNF, *ZNF382*, at 19q13.12 (Luo et al., 2002). By semi-quantitative RT-PCR which is more sensitive than previous Northern blot, I found that *ZNF382* was expressed in all normal adult tissues and fetal tissues examined, as well as normal peripheral blood mononuclear cells (PBMCs), though with varying expression levels (Figure 10B). In contrast, *ZNF382* expression was frequently silenced or reduced in multiple carcinoma cell lines of nasopharyngeal, lung, esophageal, colon, stomach, breast and cervical cancers, but infrequently in liver and prostate cancer cell lines (Figure 11). This expression pattern was further confirmed by real-time PCR in some representative cell lines (Figure 12). *ZNF382*

was also highly expressed in 7 immortalized normal epithelial cell lines examined (NP69, NE1, NE3, NE083, Het-1A, HMEC and HMEpC). These results suggested that *ZNF382* is a broadly expressed gene but frequently disrupted in multiple carcinoma cell lines.

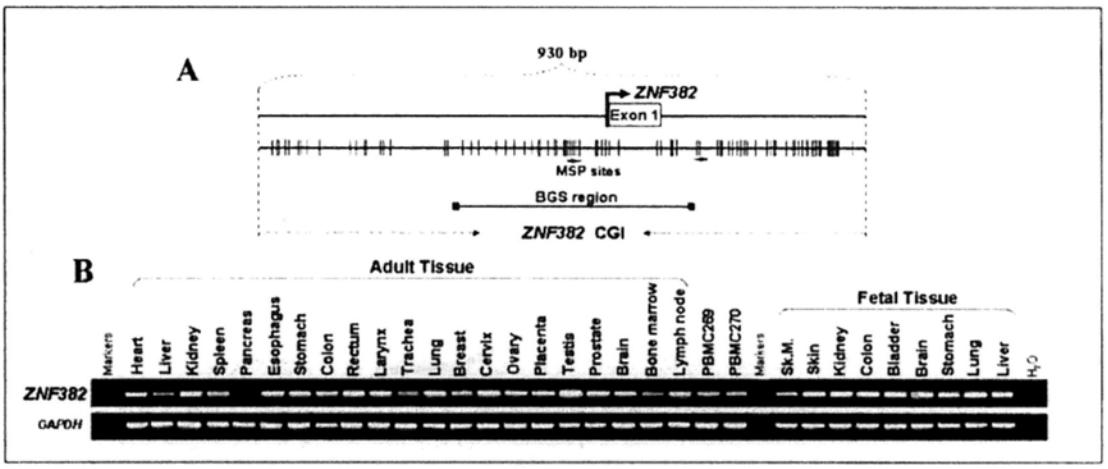


Figure 10. CpG island structures and expression profile in human normal tissues of *ZNF382*. (A) The *ZNF382* CGI. Transcription start site (TSS) is indicated by a curved arrow. The CGI, MSP and BGS regions analyzed are indicated. (B) Expression profile of *ZNF382* in human normal adult and fetal tissues by semi-quantitative RT-PCR with *GAPDH* as a control.

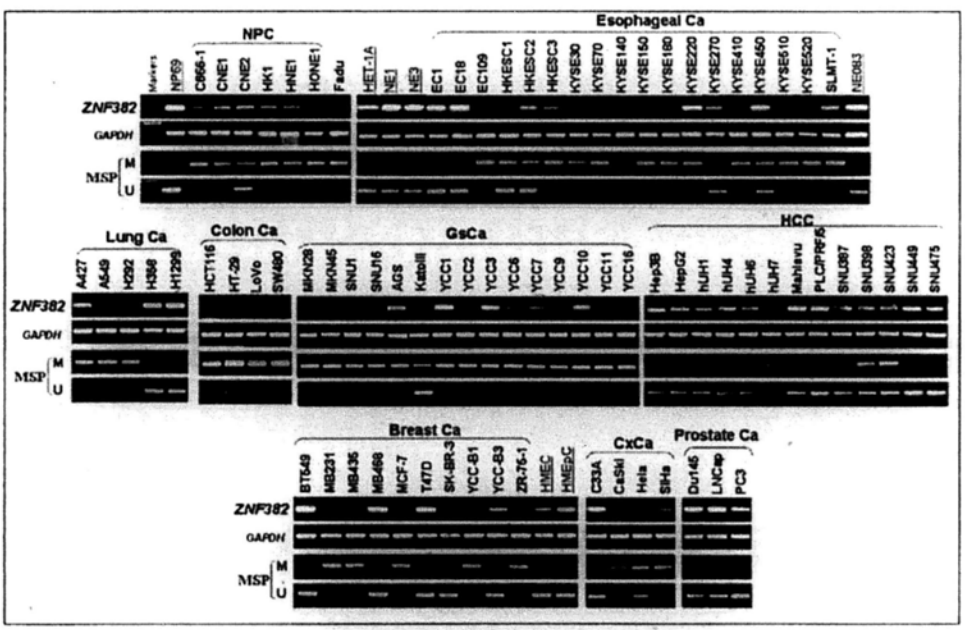


Figure 11. Expression profile and methylation study of *ZNF382*. Representative analyses of *ZNF382* expression and promoter methylation in tumor cell lines and immortalized epithelial cells. M, methylated; U, unmethylated.

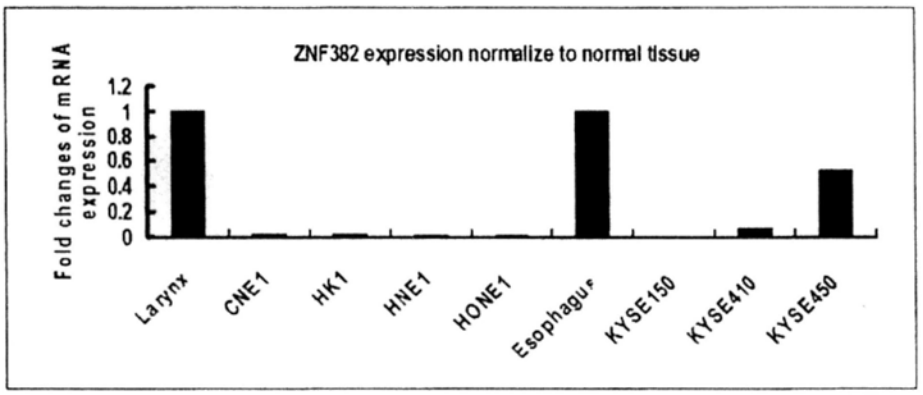


Figure 12. Real-time PCR analysis of *ZNF382* expression in several representative cell lines. The expression level of *ZNF382* in NPC cell lines was normalized to that in normal larynx, while expression in esophageal carcinoma cell lines was compared to that in normal esophagus, respectively.

4.2.2 Frequent inactivation of *ZNF382* by promoter CpG methylation

Sequence analysis revealed that *ZNF382* promoter contains a CGI (Figure 10A), indicating CpG methylation may be a major mechanism silencing its expression in cancer cells (Qiu et al., 2004). MSP analysis showed that the *ZNF382* promoter was methylated in 6/6 NPC, 1/1 hypopharyngeal, 3/5 (60%) of lung, 16/18 (89%) esophageal, 4/4 colon, 15/15 (100%) gastric, 4/13 (30%) hepatocellular, 5/10 (50%) breast, 3/4 cervical, and with no methylation in 3 prostate cancer cell lines (Figure 11). In contrast, obvious *ZNF382* methylation was not detected in 7 immortalized normal epithelial cell lines (Figure 11), suggesting the methylation of *ZNF382* is tumor-specific. A good correlation between the down-regulation and methylation of *ZNF382* was observed in these carcinoma cell lines.. We further examined *ZNF382* methylation in detail by high resolution BGS analysis (Figure 13). The results confirmed the MSP analysis.

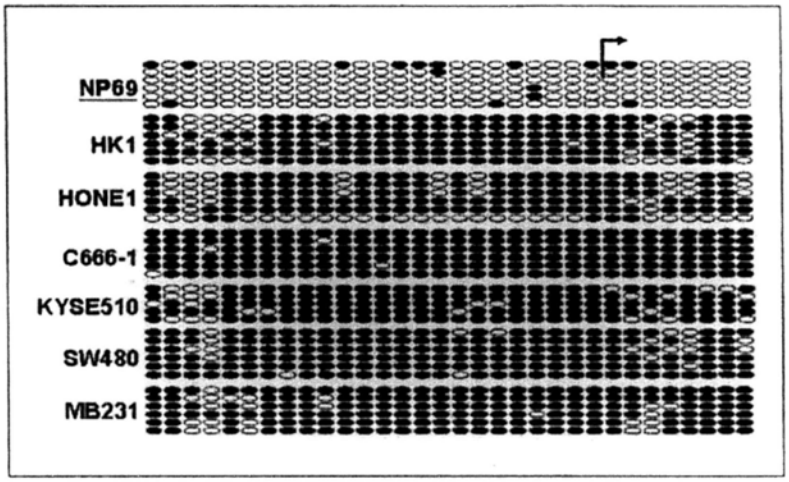


Figure 13. BGS analysis of *ZNF382* in tumor cell lines. High-resolution methylation analysis of the *ZNF382* promoter by BGS in tumor cell lines.

4.2.3 Pharmacologic and genetic demethylation restores *ZNF382* expression

To determine whether CpG methylation directly mediates *ZNF382* downregulation, several carcinoma cell lines, C666-1, HK1, HNE1, HONE1, HCT116, EC109, KYSE510, KYSE520, MB231 and MB435 were treated with the DNA methyltransferase inhibitor Aza alone or combined with histone deacetylase inhibitor TSA. The treatment restored *ZNF382* expression, accompanied with a decrease of methylated promoter alleles and increased unmethylated alleles (Figure 14A). *ZNF382* expression could also be activated by genetic demethylation through double knockout (KO) of both DNMT1 and DNMT3B (DKO cell line), but not single KO of DNMT1 or DNMT3B (1KO or 3BKO cell line) in a colorectal cancer cell line model (Figure 14B), indicating that the maintenance of *ZNF382* methylation was mediated by DNMT1 and DNMT3B together, like other TSGs we have examined (Cui et al., 2008; Ying et al., 2006b). Detailed BGS analysis confirmed the demethylation of *ZNF382* (Figure 14C). These results indicated that promoter methylation directly mediated the transcriptional silencing of *ZNF382*.

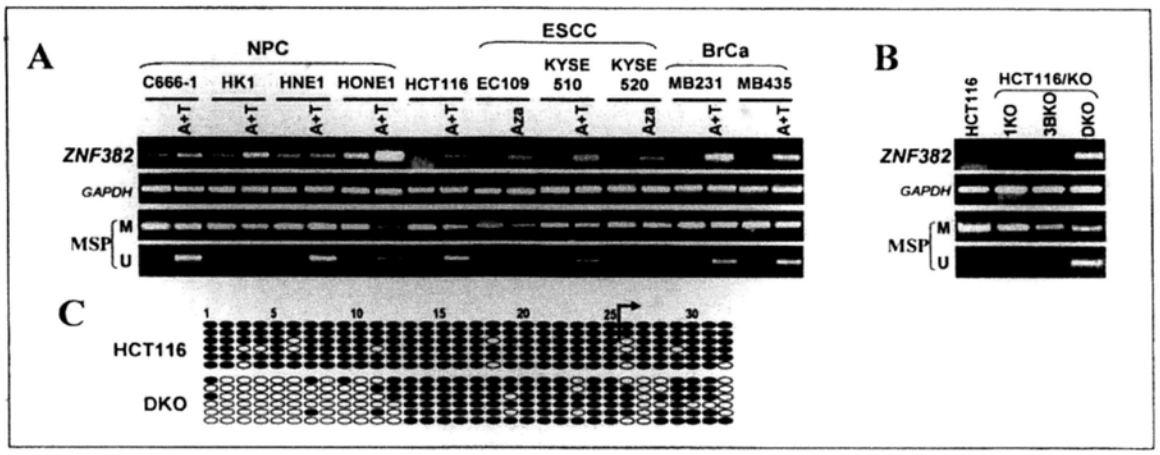


Figure 14. Restoration of *ZNF382* by demethylation. (A) Pharmacological demethylation with Aza or Aza combined with TSA (A+T) activates *ZNF382* expression in tumor cell lines, accompanying with demethylation of the promoter. (B) Genetic demethylation with double knockout of *DNMT1* and *DNMT3B* in HCT116 cells restores *ZNF382* expression, but not in single knockout *DNMT1* (1KO) and *DNMT3B* (3BKO) cells. (C) Detailed BGS analysis confirmed the demethylation of *ZNF382* promoter in HCT116-DKO cell line.

4.2.4 Promoter methylation disrupts the stress response of *ZNF382*

Three heat shock factor binding sites (HSF) were predicted in the *ZNF382* promoter (one localizes within the CpG island while the other two adjacent to the CpG island) (MatInspector: http://www.genomatix.de), indicating that *ZNF382* might be a stress-responsive gene (Figure 15). I thus examined the response of *ZNF382* to heat shock stimulation in several cell lines. *ZNF382* expression was significantly increased upon heat shock treatment in HK1, CNE1 (weakly unmethylation) and CNE2 (hemi-unmethylation) cell lines. This response was, however, abolished in completely methylated HCT116 cells. In contrast, heat shock-activated *ZNF382* expression was rescued in pharmacologically demethylated HCT116 cells (Figure 15). Taken together, these results demonstrate that *ZNF382* is indeed stress-responsive and promoter methylation disrupted its cellular protective response

to environmental stresses.

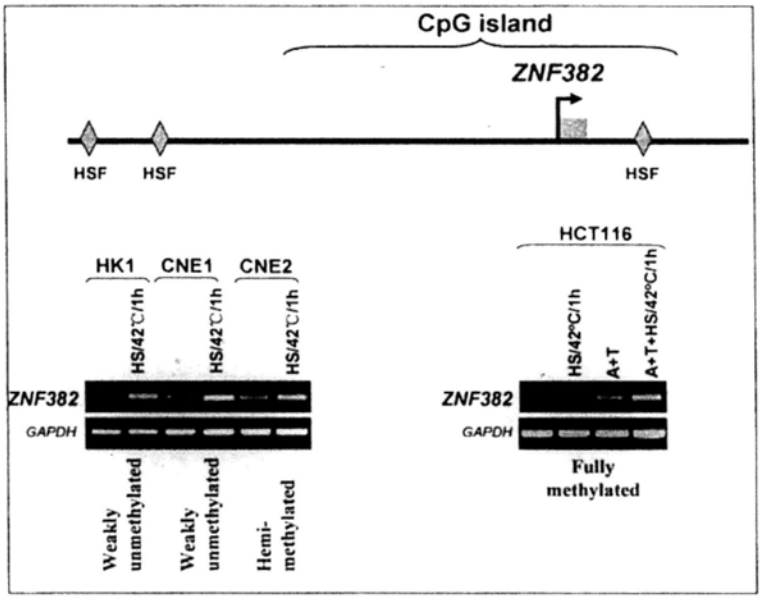


Figure 15. Epigenetic silencing of *ZNF382* results in the disruption of its response to heat shock stress. Upper panel: locations of heat shock factor (HSF) binding sites and the *ZNF382* promoter CpG island are indicated; Lower panels: *ZNF382* expression is upregulated in weakly unmethylated (HK1 and CNE1) or hemi-unmethylated (CNE2) cell lines but not in fully methylated cell line (HCT116) upon heat shock treatment, and pharmacologic demethylation of HCT116 could restore this heat shock response of *ZNF382*.

4.2.5 Frequent *ZNF382* methylation in primary tumors

We further examined *ZNF382* methylation in multiple primary tumors and the corresponding normal tissues. *ZNF382* was not methylated in normal nasopharyngeal (0/10) and esophageal epithelial tissues (0/7), but frequently methylated in 88.9% (48/54) of Asian Chinese primary NPC, 2/3 nude mice-passaged undifferentiated NPC tumors from North African (C15, C17 and C18), 71.4% (20/28) of esophageal, 72.7% (8/11) of colon, 63.6% (7/11) of gastric, and 18.2% (2/11) of breast tumors (Figure 16A &B). Aberrant methylation was also detected in 10.7% (3/28) paired surgical marginal esophageal tissues from esophageal carcinoma patients, which

could be due to the presence of premalignant lesions or infiltrating tumor cells. The transcript level of *ZNF382* was reduced in 19/21 methylated primary tumors but not in unmethylated primary tumors as examined by semi-quantitative RT-PCR (Figure 17), indicating a good correlation between *ZNF382* promoter methylation and its transcriptional silencing in primary tumors. It should be noted that primary tumors, if not microdissected, would also have background expression of *ZNF382* transcripts due to infiltrating normal cells, which could explain the situation of the two not downregulated, methylated cases (#62, #73). Nevertheless, these results demonstrated that *ZNF382* methylation is a common event in multiple tumorigenesis.

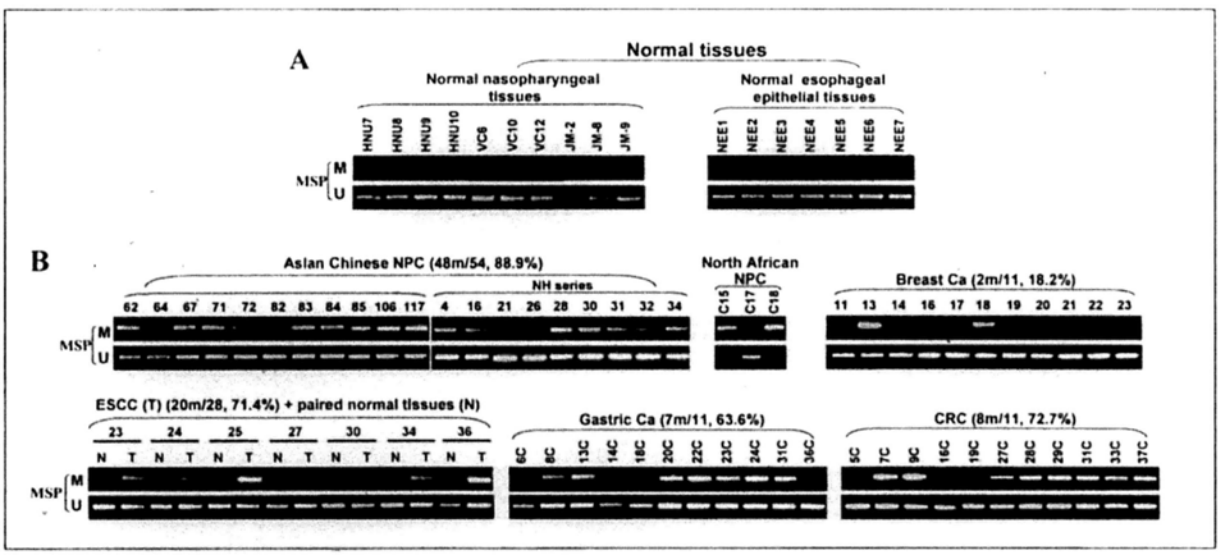


Figure 16. Representative analysis of *ZNF382* methylation in normal tissues and primary tumors by MSP. M, methylated; U, unmethylated. (A) Normal epithelial tissues. (B) Primary NPC, esophageal carcinomas (T) and their surgical marginal normal tissues (N), and other carcinomas.

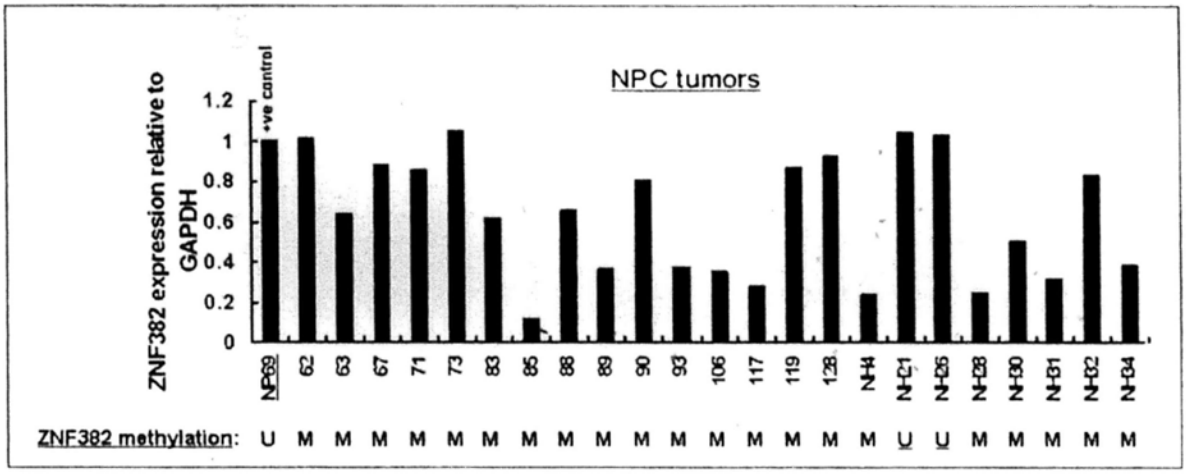


Figure 17. Semi-quantitative RT-PCR analysis of *ZNF382* expression in methylated primary NPC. The expression levels of ZNF382 in NPC tumors were relative to *GAPDH* expression levels and normalized to that in normal control NP69.

4.2.6 ZNF382 is a nuclear protein inhibiting NF-κB and AP-1 signaling pathways

KS1, the rat homolog of human *ZNF382*, is a nuclear protein as a transcription repressor (Gebelein et al., 1998; Gebelein and URRUTIA, 2001). I examined the subcellular localization of ZNF382. Indirect immunofluorescent staining showed that Flag-tagged ZNF382 was also a nuclear protein (Figure 18). Furthermore, through screening several signaling pathways by luciferase reporter assays, I found that ZNF382 significantly suppressed NF-κB and AP-1 reporter activities in HCT116 tumor cells, but in an immortalized normal epithelial cell line HEK293, it only significantly inhibited AP-1 activity (Figure 19A). I further confirmed the inhibitory effect of ZNF382 on NF-κB activity in two other tumor cell lines: KYSE150 and HNE1 (Figure 19B). Both NF-κB and AP-1 signalings are important in cell proliferation, survival, apoptosis and malignant transformation (Basseres and Baldwin, 2006; Eferl and Wagner, 2003). Thus, ZNF382 could induce apoptosis and inhibit cell proliferation through suppressing both NF-κB and AP-1 signaling

pathways.

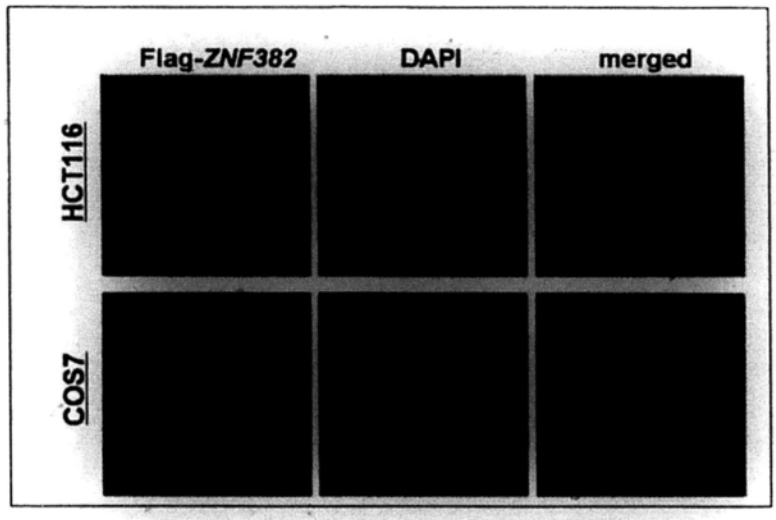


Figure 18. Nuclear localization of ZNF382 by indirect immunofluorescence in HCT116 and COS7 cell lines. Left panel is the FITC green fluorescence to Flag-tagged ZNF382; middle panel is the DAPI-stained cell nuclei; right panel is the merged images.

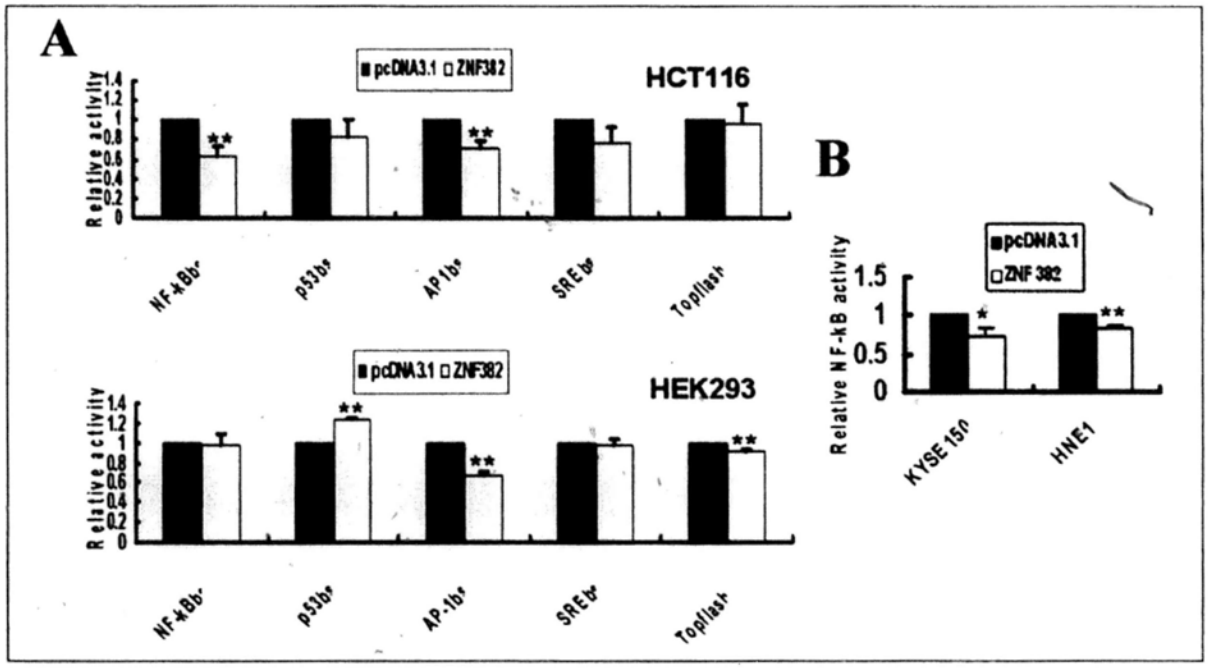


Figure 19. ZNF382 mediates several signaling pathways. (A) Effects of ZNF382 on several signaling pathways in HCT116 and HEK293 cells were assessed by dual-luciferase reporter assays, $*p<0.05$, $**p<0.01$. (B) ZNF382 significantly inhibited NF-κB pathway reporter activities in KYSE150 and HNE1 cell lines, $*p<0.05$, $**p<0.01$.

4.2.7 ZNF382 inhibits clonogenicity, proliferation and induces apoptosis of tumor cells

To assess the functions of *ZNF382* in tumor cells, Flag-*ZNF382*-expressing plasmid was transfected into HONE1, KYSE510 and HCT116 cell lines with completely methylated/silenced *ZNF382*. Ectopic expression of ZNF382 dramatically reduced the colony formation efficiency of these cells (down to 10.2%-42.9%, $^{**}p<0.01$) (Figure 20). The inhibition of ZNF382 on cell proliferation was further determined by flow cytometry with DDAO-SE staining. Forty-eight hours post-transfection, the fluorescence intensity in *ZNF382*-transfected HCT116 cells was higher than that in control vector-transfected cells after GFP sorting, which provided direct evidence that ZNF382 could inhibit tumor cell proliferation (Figure 21).

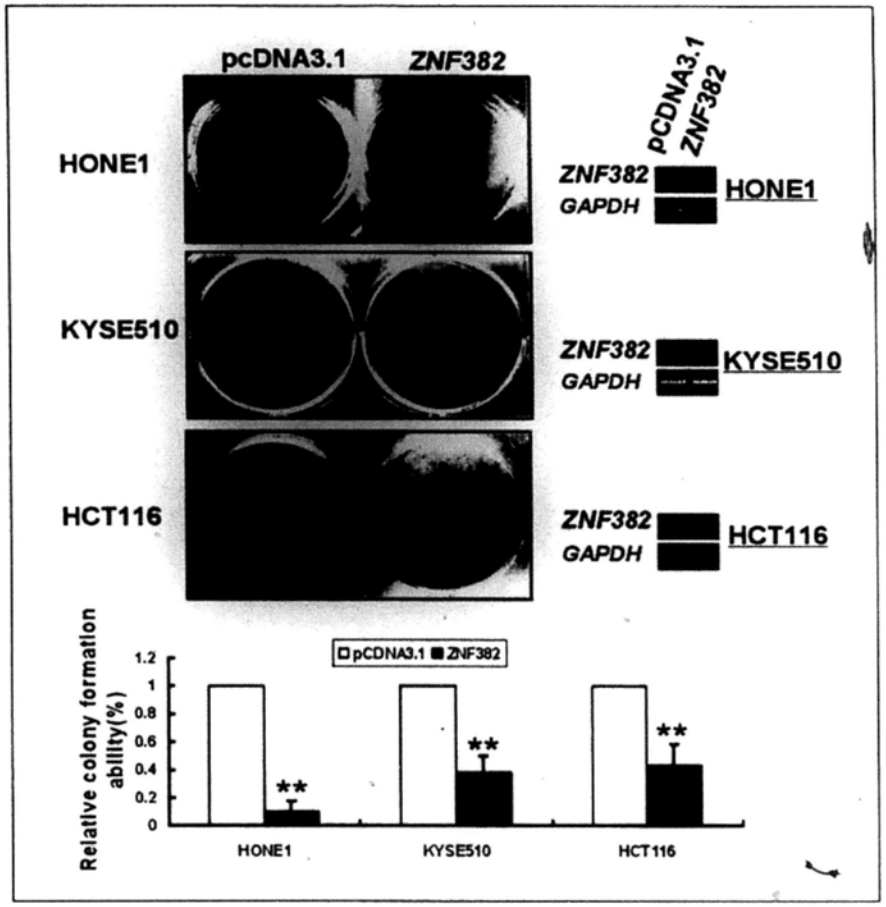


Figure 20. *ZNF382* inhibits clonogenicity ability of tumor cell lines. Left panel: representative colony formation assay with monolayer culture. Right panel: ectopic *ZNF382* expression in tumor cells was confirmed by RT-PCR. Bottom panel: quantitative analysis of colony formation ability in *ZNF382*-transfected cells. The number of G418-resistant colonies (>50 cells) in each vector-transfected cells was set to 100, $**p<0.01$.

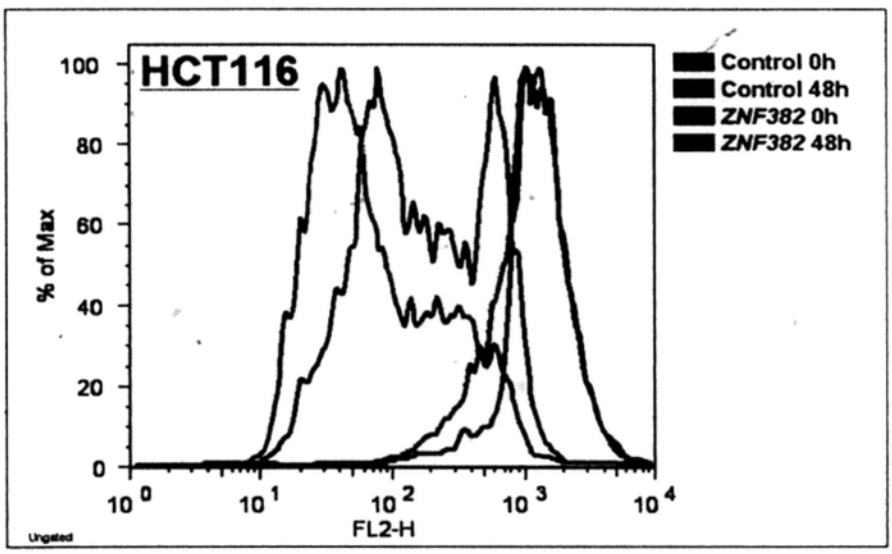


Figure 21. ZNF382 inhibits cellular proliferation. ZNF382 inhibits cell proliferation in HCT116 cells by DDAO-SE assay. The fluorescence intensities of *ZNF382*- and control vector-transfected HCT116 cells at different time points were indicated by different colors.

To explore the mechanism of the tumor suppression by ZNF382, we performed apoptosis assay using flow cytometry with Annexin V-PE and 7-AAD double staining. pIRES-ZsGreen1, which could translate the gene of interest and ZsGreen1 from a single bicistronic mRNA, was used to sort transfected positive cells. We determined the percentage of Annexin V (+/-) and 7-AAD (+/-) cells for GFP-positive cells. In HCT116 cells, ectopic ZNF382 expression resulted in a significant increase of apoptotic cells as compared with control (Figure 22A). Apoptotic induction was further confirmed by DAPI staining at individual cell level. pIRES-ZsGreen1-*ZNF382* transfected cells (GFP positive) showed obvious condensed or fragmented nuclei - a remarkable cell apoptotic feature, but not in vector control transfected cells (Figure 22C). Evidence of apoptosis was also detected by Western blot. Ectopic expression of ZNF382 could significantly increase the cleavage of poly (ADP-ribose) polymerase (PARP) in HCT116 and HNE1 cells, compared to control (Figure 22B). Taken together, these results support that ZNF382 functions as a tumor suppressor, through inducing apoptosis and inhibiting cell proliferation of tumor cells.

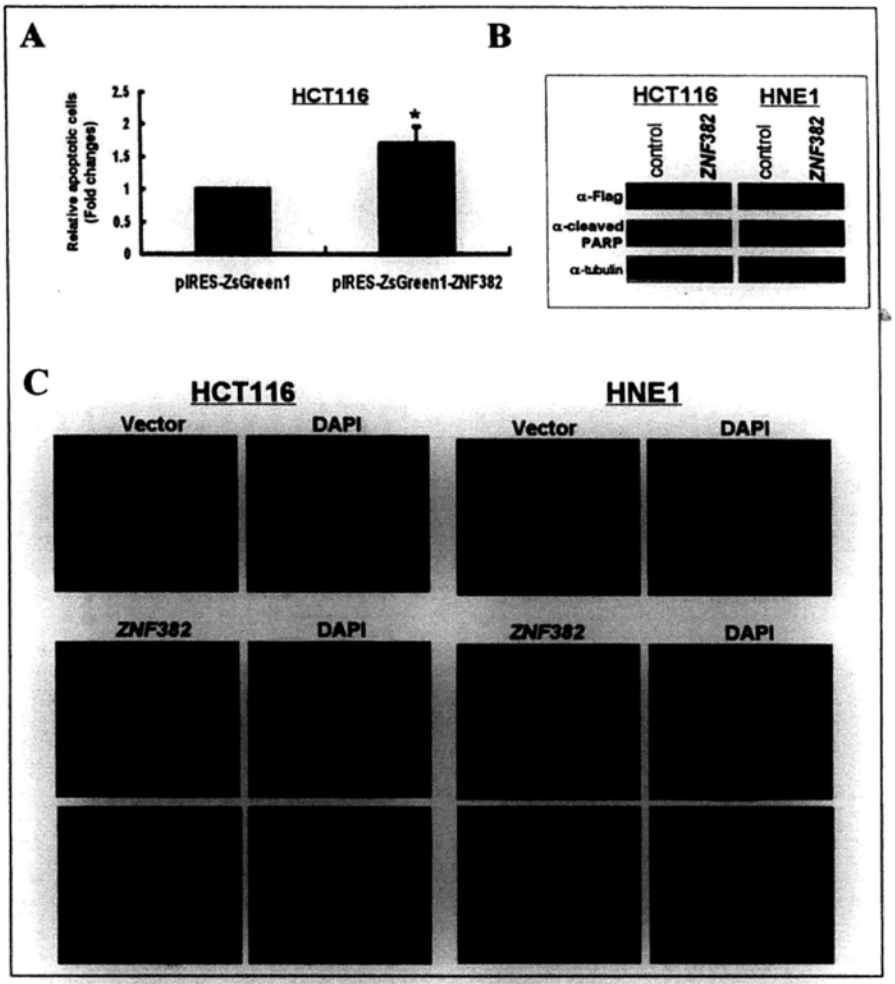


Figure 22. ZNF382 induces tumor cell apoptosis. (A) Relative proportions of apoptotic cells in *ZNF382*- and control vector-transfected HCT116 cells as analyzed by flow cytometry after stained with Annexin V and 7-AAD. (B) Western blot of apoptotic indicator, cleaved PARP, in *ZNF382*-transfected HCT116 and HNE1 cells. (C) ZNF382 causes the apoptosis-associated cell nuclear changes. Cells were stained with DAPI and photographed under a fluorescence microscope at 48 hours after transfection. The transfection positive cells were indicated by red arrows.

4.2.8 ZNF382 represses the expression of multiple oncogenes including NF-κB pathway upstream effectors through heterochromatin silencing

As ZNF382 is a repressor, we screened for its potential target genes by examining the expression changes of 34 major oncogenes which have been reported to be involved in proliferation/transformation/apoptosis inhibition and signaling pathways, after ectopic ZNF382 expression in HCT116. Real-time PCR analysis revealed that, as compared with vector control transfected cells, ZNF382 expression significantly

downregulated the expression of several oncogenes at the mRNA level, including STAT3, MYC, ID1, IKBKE, STAT5B, MITF, HMGA2, and CDK6, while the expression of other oncogenes like ID2 and CCND1 was not affected (Figure 23A). Downregulation of STAT3 and STAT5B was further confirmed by Western blot (Figure 23B). Some of these oncogenes were known to be NF-κB pathway upstream effectors such as STAT3, STAT5B, IKBKE and ID1 (Lee et al., 2009; Han et al., 2009; Boehm et al., 2007; Ling et al., 2003). Thus, ZNF382 could inhibit NF-κB pathway through suppressing its upstream effectors.

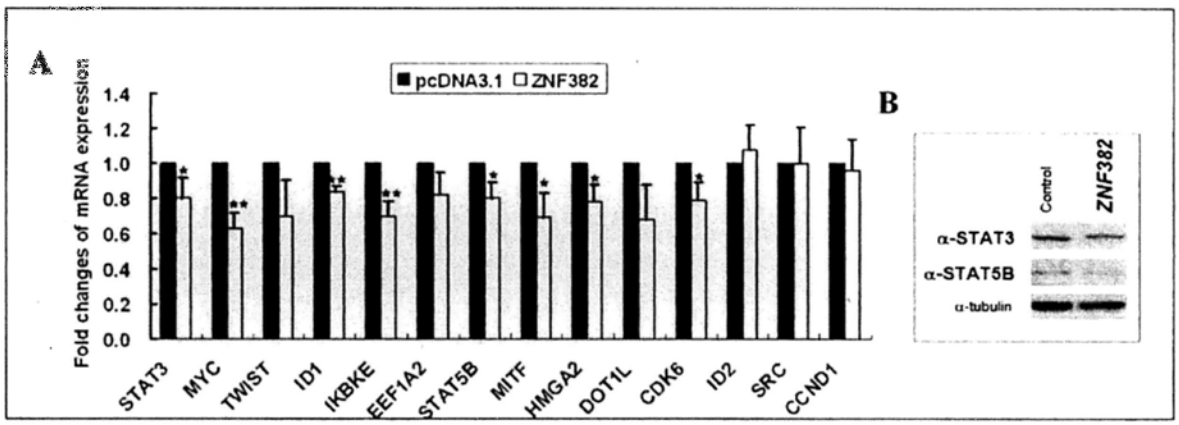


Figure 23. ZNF382 represses multiple oncogenes. (A) Suppression of multiple oncogenes by *ZNF382* expression, evaluated by real-time PCR in HCT116 cells. All the downregulated genes are shown. *: $p<0.05$, **: $p<0.01$. (B) Ectopic *ZNF382* expression downregulated the protein levels of STAT3 and STAT5B in HCT116 cells.

To explore the possible mechanism of transcriptional repression mediated by ZNF382, we checked the involvement of heterochromatin silencing as previous study revealed that KS1 is located in the nucleus and interacts with KAP1 (Gebelein et al., 1998; Gebelein and URRUTIA, 2001), while KAP1 is known to form a complex with heterochromatin proteins (HP) to exert gene silencing (Ryan et al., 1999). Indirect-immunofluorescence staining for Flag-tagged ZNF382 in HCT116 and

COS7 cells clearly showed that ZNF382 is a nuclear protein (Figure 18), and also co-localized with HP1 (Figure 24A). The interaction between ZNF382 and HP1 was further confirmed by co-IP assay (Figure 24B). It is well known that H3K4me3 (trimethylation of histone H3 at the lysine 4 residue) is a marker of transcriptionally active chromatin whereas H3K9me3 modification is a marker of inactive chromatin (Best and Carey, 2010). Our ChIP assays further showed that ectopic expression of ZNF382 could significantly decrease the levels of H3K4me3 on the promoters of its target oncogenes - STAT3 and c-MYC (Figure 25). Taken together, our results suggested that ZNF382 is functionally involved in heterochromatin-mediated gene silencing, including the suppression of multiple oncogenes.

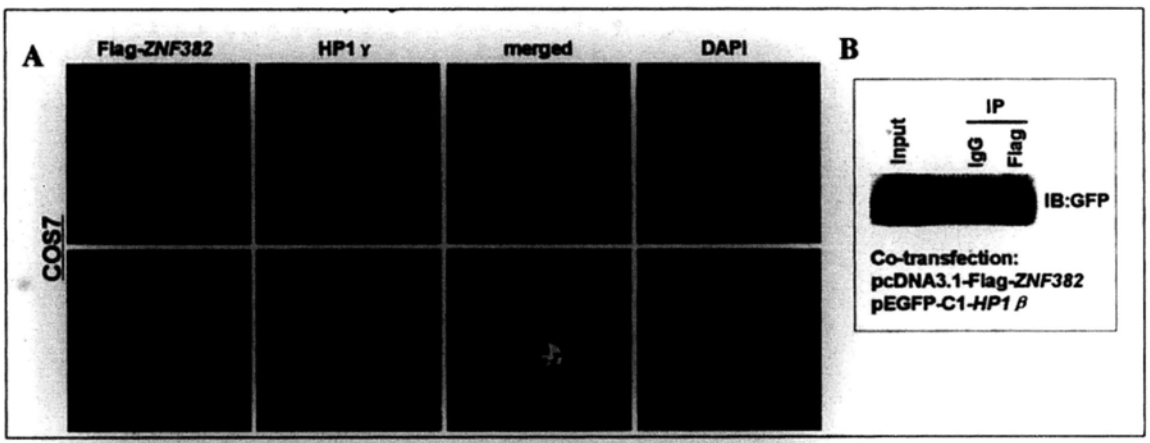


Figure 24. ZNF382 interacts with heterochromatin protein. (A) co-localization of RFP-HP1γ and Flag-ZNF382 (detected by FITC-conjugated antibody) in COS7 cells. (B) Co-immunoprecipitation showed the physical interaction between ZNF382 and HP1β.

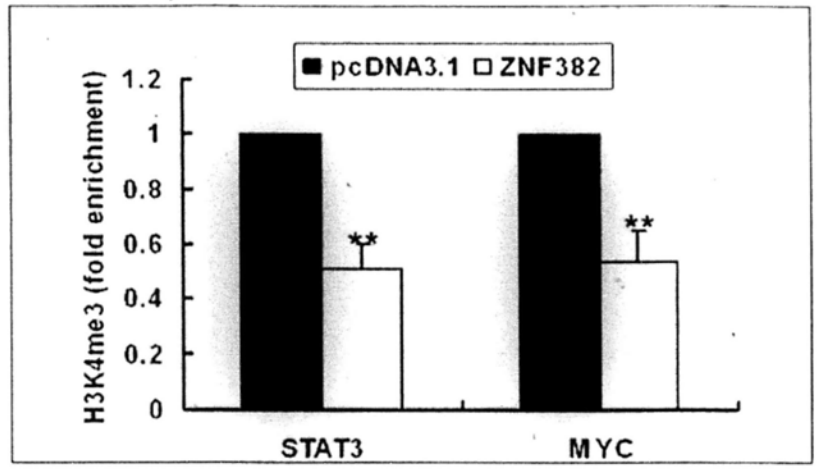


Figure 25. ZNF382 decreases H3K4me3 levels on the promoters of STAT3 and c-MYC by ChIP assay. **: $p<0.01$.

4.2.9 Discussion

In this report, I found that *ZNF382*, a KRAB- ZNF we identified previously, is frequently downregulated by promoter CpG methylation in multiple tumors. I further found that ectopic expression of ZNF382 in silenced carcinoma cells dramatically inhibits their colony formation through inhibiting cell proliferation and inducing apoptosis. I also showed that ZNF382 inhibited NF-κB and AP-1 signaling and suppressed the expression of multiple oncogenes likely through heterochromatin silencing. Thus, ZNF382 functions as a broad tumor suppressor for human cancers.

KRAB-ZNFs play important roles in various epigenetic regulations. During the early mouse development, KRAB domain triggers *de novo* promoter methylation (Wiznerowicz et al., 2007). Zfp57, a zinc finger protein, maintains both maternal and paternal imprints in mouse (Li et al., 2008). KRAB domain binds to KAP1 which further recruits HP1, SETDB1 (H3K9 methyltransferase) and NuRD (histone deacetylase) complexes, while the zinc finger domain specifically binds to nucleation

sites, forming heterochromatin for gene silencing (Schultz et al., 2002). Defects in chromatin related activities, such as chromatin assembly and remodeling, may cause tumorigenesis (Coral Wynter et al., 2007). The disruption of DNA binding proteins (KRAB-ZNFs) may thus cause failure in heterochromatin formation and contribute to tumorigenesis. I found that ZNF382 co-localizes and interacts with heterochromatin protein HP1, together with the previous report of the co-localization of ZNF382 rat homologene-KS1 with KAP1 (Gebelein and URRUTIA, 2001), indicating that ZNF382 is indeed involved in heterochromatin formation and silencing. Thus, ZNF382 might directly bind to target gene promoters as a repressor (Gebelein and URRUTIA, 2001), or form heterochromatin on target gene promoters together with KAP1 and HP1, leading to transcriptional repression of target genes.The epigenetic disruption of ZNF382 would cause the aberrant expression of normally repressed genes mediated by heterochromatin.

Indeed, I found that ZNF382 could significantly repress the RNA levels of multiple oncogenes involved in neoplastic transformation, apoptosis, cell cycle and proliferation, including MYC, STAT3, ID1, IKBKE, STAT5B, MITF, HMGA2, and CDK6 etc. I further verified that the downregulation of STAT3 and MYC by ZNF382 was mainly mediated through heterochromatin silencing. Among these targets, MYC, MITF and CDK6 are involved in cell cycle regulation and apoptosis (Meyer and Penn, 2008; Dynek et al., 2008; Steinman, 2002). STAT3, MITF and HMGA2 are involved in neoplastic transformation (Joo et al., 2004; Berlingieri et al., 2002; Di et

al., 2008). Some targets are NF-κB upstream regulatory factors, such as STAT3, STAT5B, ID1 and IKBKE. STAT3 and STAT5B are required for maintaining NF-κB activity (Lee et al., 2009; Han et al., 2009). ID1 promotes cell survival through regulating NF-κB activity in prostate and breast cancers (Ling et al., 2003; Kim et al., 2008a); IKBKE is an oncogene controlling the activity of NF-κB in cell proliferation and malignant transformation (Boehm et al., 2007). In agreement, ZNF382 inhibited the oncogenic NF-κB signaling pathway, as well as the AP-1 signaling pathway.

NF-κB is a dimeric transcriptional factor, involved in regulating cell proliferation, apoptosis, angiogenesis, and cell invasion (Basseres and Baldwin, 2006). NF-κB is highly activated in many cancers, accompanied by the upregulation of its downstream oncoproteins. Thus, NF-κB pathway is considered as a cell survival and anti-apoptotic signaling pathway (Basseres and Baldwin, 2006). Some TSGs exert their tumor suppressive functions through inhibiting NF-κB pathway, such as CYLD and CHFR (Hellerbrand et al., 2007; Kashima et al., 2009). NF-κB also cross-talks with AP-1 signaling pathway (Fujioka et al., 2004). AP-1 is another dimeric factor regulating multiple cellular processes including proliferation, apoptosis, and differentiation. Activated AP-1 induces apoptosis for certain tumors or specific stages of tumorigenesis, but also promote cell survival for other tumor types (Eferl and Wagner, 2003). Several TSGs can inhibit the cell proliferation of colon cancer through inhibiting AP-1 activity such as PDCD4 and HINT1 (Wang et al., 2008; Wang et al., 2007). *KS1,* the rat homolog of human *ZNF382,* antagonizes Ras, Gα12

or Gα13 -induced neoplastic transformation, but does not induce apoptosis (Gebelein et al., 1998). As NF-κB and AP-1 are both downstream targets of Ras, Gα12 or Gα13 (Basseres and Baldwin, 2006; Eferl and Wagner, 2003; Ki et al., 2007; Parnell et al., 2002), ZNF382 could antagonize Ras oncogene-induced transformation by inhibiting the downstream events such as NF-κB and AP-1 signaling.

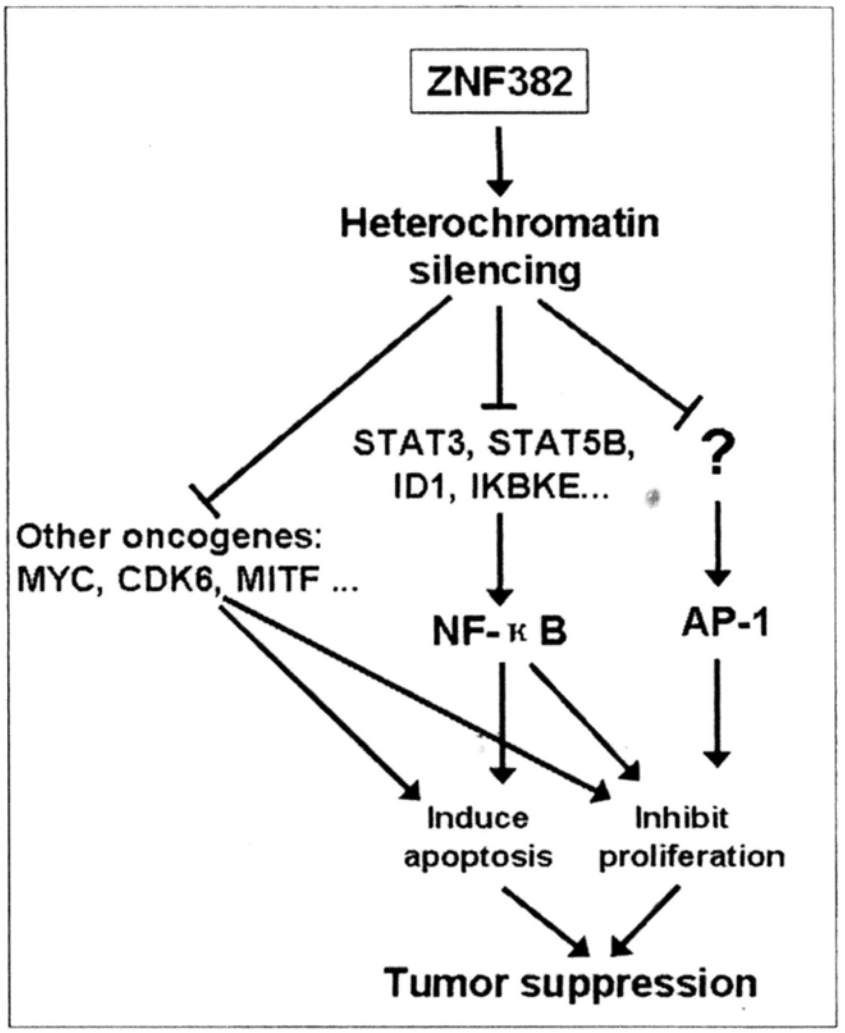


Figure 26. A possible mechanism model of the tumor suppression by *ZNF382*. ZNF382 suppresses the expression of MYC, CDK6 and MITF which control apoptosis and proliferation. ZNF382 also suppresses the expression of STAT3, STAT5B, ID1 and IKBKE, and further inhibits NF-κB and AP-1 signalings.

In conclusion, I found that *ZNF382* is a functional TSG, inducing apoptosis, inhibiting cell proliferation and suppressing multiple oncogenes. The

anti-tumorigenic effect of *ZNF382* may act through both NF-κB and AP-1 signaling pathways (Figure 26), while epigenetic silencing of *ZNF382* would activate these cancer signaling pathways during tumorigenesis. In addition, the high frequency and tumor-specific methylation of *ZNF382* in NPC and esophageal carcinomas indicated that it could be used as a potential epigenetic biomarker for their molecular diagnosis.

4.3 Homologous ZNFs- ZNF545 and ZFP30 are functional tumor suppressors with frequent epigenetic inactivation in multiple carcinomas

Through genome-wide screening, I identified *ZNF545* and *ZFP30* as novel TSGs which are frequently downregulated in multiple malignancies through promoter DNA methylation. Both *ZNF545* and *ZFP30* contain a KRAB domain and 13 C2H2 zinc fingers with 76% similarity at protein sequences (Figure 27). I found that both ZNF545 and ZFP30 are transcriptional repressors which could significantly inhibit tumor cell clonogenicity. More functional studies on ZNF545 revealed that ZNF545 could inhibit cell proliferation and induce apoptosis, possibly through repressing NF-κB and AP-1 signaling. These two proteins were found to be localized mainly within nucleoli by indirect immunostaining. Functional and structural changes of nucleoli will lead to abnormal ribosome biogenesis, affect the balance of protein translation, and thereby contribute to cancer development. In agreement, I further found that ZNF545 inhibits the activity of rDNA promoter, decreases the translation efficiency and cell size. It might exert the tumor suppressor function through regulating ribosome biogenesis or downregulating the transcription of target genes. The data demonstrated that *ZNF545* and *ZFP30* are functional TSGs frequently methylated in multiple carcinomas, and could be valuable to develop as epigenetic biomarkers for cancer diagnosis.

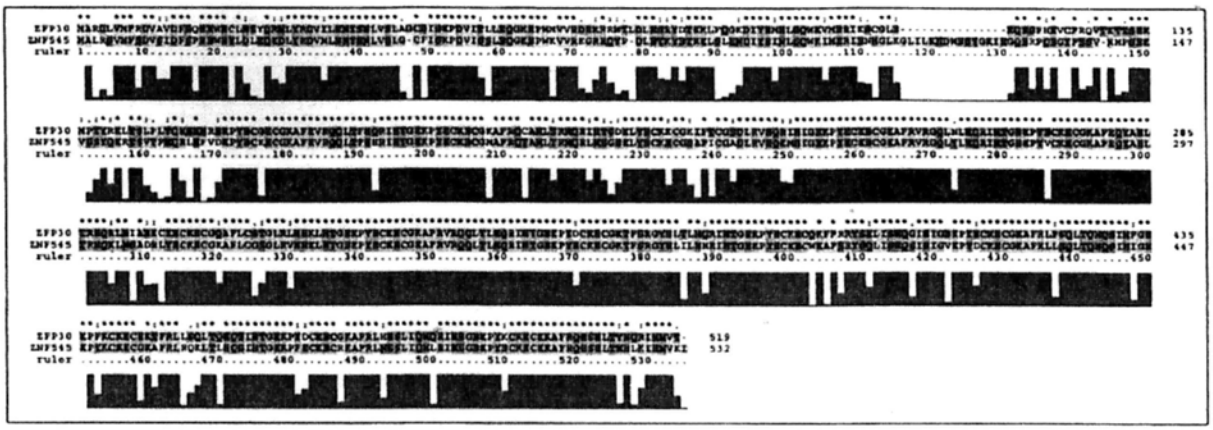


Figure 27. Protein sequences alignment of *ZNF545* and *ZFP30*. These two proteins share 76% similarity at protein level.

4.3.1 Identification of *ZNF545* and *ZFP30* as downregulated genes in multiple tumors but broadly expressed in normal tissues

I examined the expression profiles of *ZNF545* and *ZFP30* in human normal adult and fetal tissues by semi-quantitative RT-PCR. These two genes widely expressed in normal tissues with varying expression levels (Figure 28B). In contrast, *ZNF545* and *ZFP30* were frequently silenced or downregulated in multiple carcinoma cell lines of nasopharyngeal, esophageal, colon and gastric cancers. *ZNF545* but not *ZFP30* was also frequently silenced in lung, breast and cervical cancer cell lines. Both genes silencing were infrequently in liver, renal and prostate cancer cell lines (Figure 29A). The expression pattern of *ZNF545* was further confirmed by real-time PCR in some representative cell lines (Figure 29B). These two genes were also highly expressed in nine immortalized epithelial cell lines (NP69, NE1, NE3, NE083, Het-1A, HEK293, RHEK-1, HMEC and HMEpC) suggesting that the transcriptional silencing of *ZNF545* and *ZFP30* is tumor specific. As *ZNF545* and *ZFP30* are novel genes, we thus determined their TSSs by 5'-RACE with human testis RNA. The full length

sequences were submitted to NCBI GenBank (Accession NO: *ZNF545*: GU388429, *ZFP30*: GU388430).

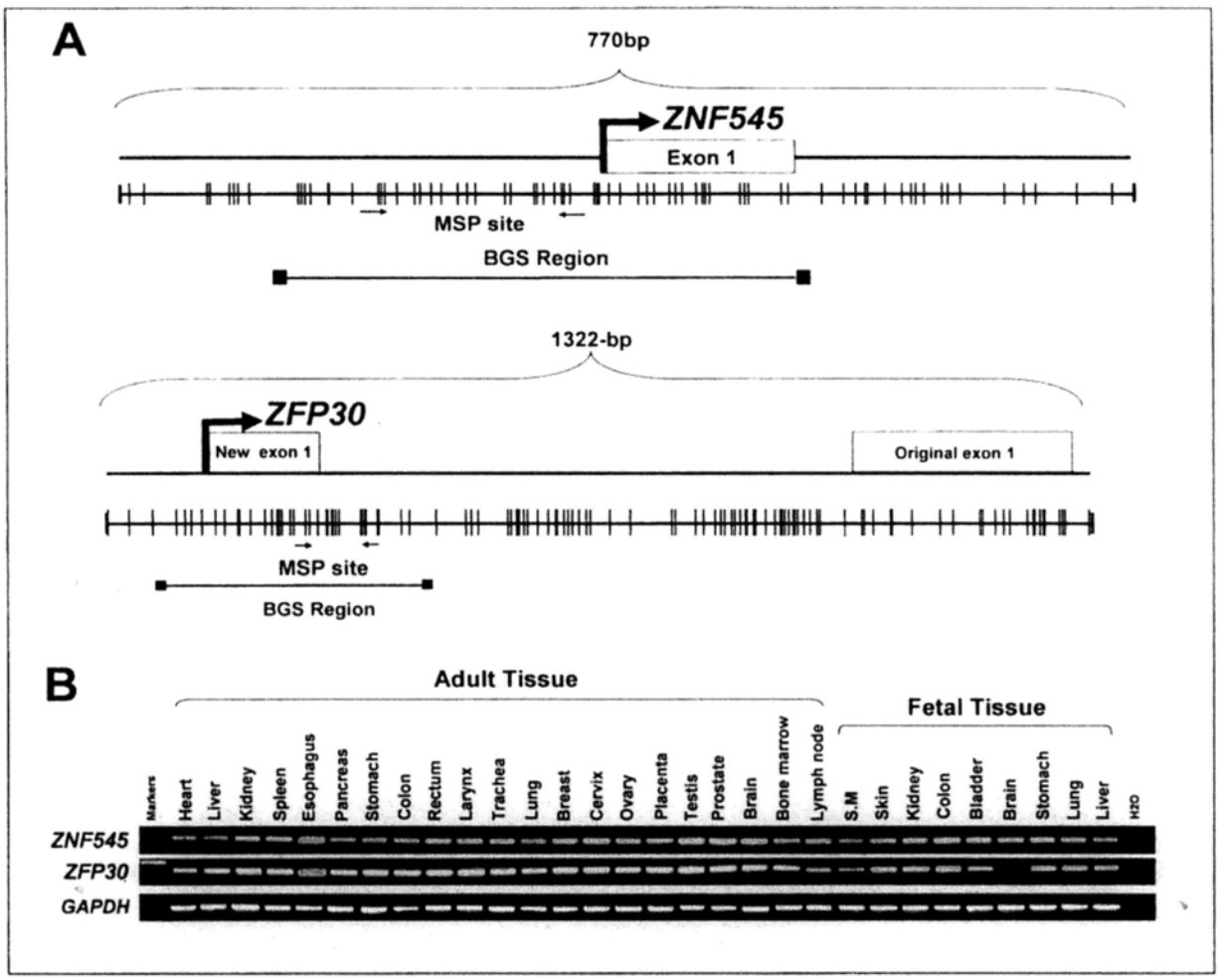


Figure 28. CpG island structures and expression profile in human normal tissues of *ZNF545* and *ZFP30*. (A) Schematic structures of *ZNF545* and *ZFP30* CGI. The TSS was indicated by a curved arrow. The CGI, MSP and BGS regions analyzed were indicated. (B) Expression profiles of *ZNF545* and *ZFP30* in human normal adult and fetal tissues by semi-quantitative RT-PCR. *GAPDH* was used as an internal control.

4.3.2 Frequent silencing of *ZNF545* and *ZFP30* by promoter methylation in multiple carcinoma cell lines

CpG site analysis revealed that the promoters of *ZNF545* and *ZFP30* contain typical CGI (Figure 28A) (Qiu et al., 2004). MSP was used to determine the promoter methylation status. The MSP primers were tested for not amplifying any unbisulfited

DNA. It was found that *ZNF545* and *ZFP30* promoter are frequently methylated in tumor cell lines with silenced or reduced expressions but only methylated in one immortalized epithelial cell line (Figure 29A). Detailed BGS analysis further confirmed the MSP results (Figure 30). Thus, the transcriptional silencing in multiple carcinoma cell lines is well correlated with the promoter methylation.

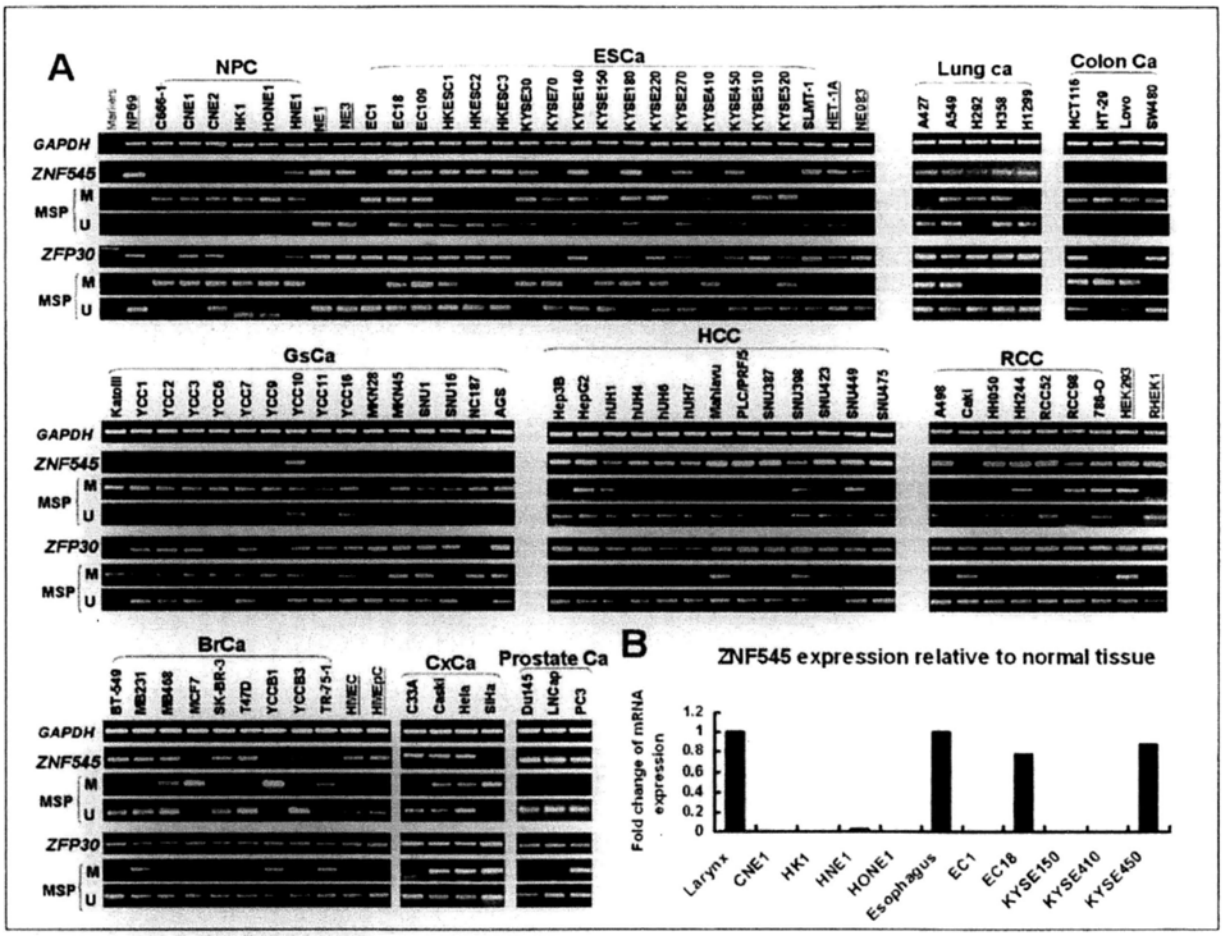


Figure 29. Expression profile and methylation analysis of *ZNF545* and *ZFP30*. (A) Representative analyses of *ZNF545* and *ZFP30* expression and promoter methylation in multiple tumor cell lines and immortalized normal cell lines (underlined). (B) Real-time PCR analysis of *ZNF545* expression in several representative cell lines. The expression level of *ZNF545* in NPC cell lines was normalized to that in normal larynx, while expression in esophageal carcinoma cell lines was compared to that in normal esophagus, respectively.

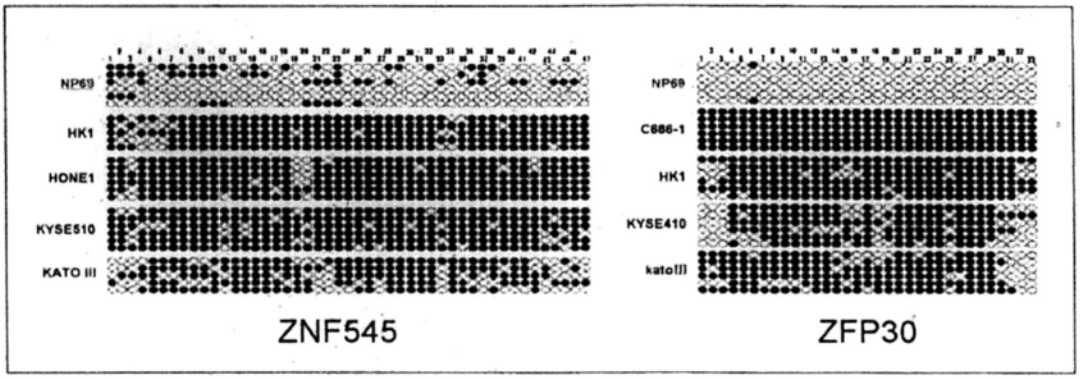


Figure 30. BGS analysis of *ZNF545* and *ZFP30*. High-resolution methylation analysis of the *ZNF545* and *ZFP30* promoters by BGS in normal (NP69) and carcinoma cell lines.

4.3.3 Pharmacologic and genetic demethylation reactivates the expressions of *ZNF545* and *ZFP30*

To determine whether DNA methylation directly mediates *ZNF545* and *ZFP30* silencing, several carcinoma cell lines with methylated promoters and silenced *ZNF545* and *ZFP30* were treated with DNA methyltransferase inhibitor Aza, or combined with histone deacetylase inhibitor TSA. Results indicated that both gene expressions were dramatically increased after the drug treatment (Figure 31A). *ZNF545* expression was also found to be reactivated in a completely methylated colorectal cancer cell line HCT116 by genetic demethylation through double knock-out of both DNMT1 and DNMT3B (DKO cell line)(Rhee et al., 2002), but not in the single KO of DNMT1 or DNMT3B alone (1KO or 3BKO cell line) (Figure 31B). Further high-resolution methylation analysis by BGS confirmed the demethylation of *ZNF545* (Figure 31C). These results demonstrated that CpG methylation of *ZNF545* and *ZFP30* promoters directly mediates their transcriptional silencing in multiple carcinoma cell lines.

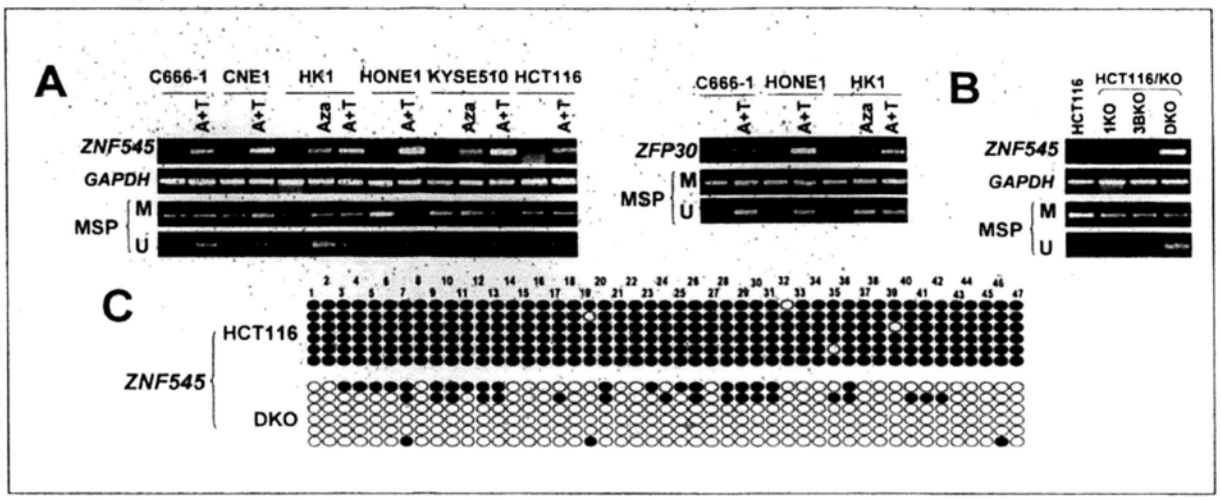


Figure 31. Pharmacologic and genetic demethylation reactivates the expressions of *ZNF545* and *ZFP30*. (A) Demethylation with Aza plus TSA activated *ZNF545* (left) and *ZFP30* (right) expression in tumor cell lines. (B) Genetic knockout of *DNMT1* and *DNMT3B* restored the mRNA level of *ZNF545*. Demethylation (U) of *ZNF545* was detected in HCT116-DKO cell line. (C) Detailed BGS analysis confirmed the demethylation of *ZNF545* promoter in HCT116-DKO cell line.

4.3.4 Frequent *ZNF545* methylation in multiple primary carcinomas

I next investigated *ZNF545* methylation in primary tumors, including nasopharyngeal, esophageal, breast, gastric and colon tumors, together with their corresponding normal epithelial tissues as controls. *ZNF545* methylation was detected in 33/39 (85%) but none in normal nasopharyngeal tissues (0/6). *ZNF545* methylation was also frequently detected in 21/31 (68%) of esophageal carcinomas, but rare in paired surgical marginal esophageal tissues from patients with esophageal carcinoma, which probably due to the appearance of premalignant lesions or the presence of small number of tumors cells disseminated into the non-tumorous region. In normal esophageal epithelial tissues from healthy individuals, virtually no methylation was found except for NEE1 showing very weak methylation (Figure 32A&B). I also examined *ZNF545* promoter methylation in 5/22 (23%) breast, 11/13 (85%) gastric and 10/11 (91%) colon tumors (Figure 32B). These results clearly

demonstrated that *ZNF545* methylation is tumor-specific, and its methylation frequencies in primary tumors are similar to those in the corresponding cancer cell lines.

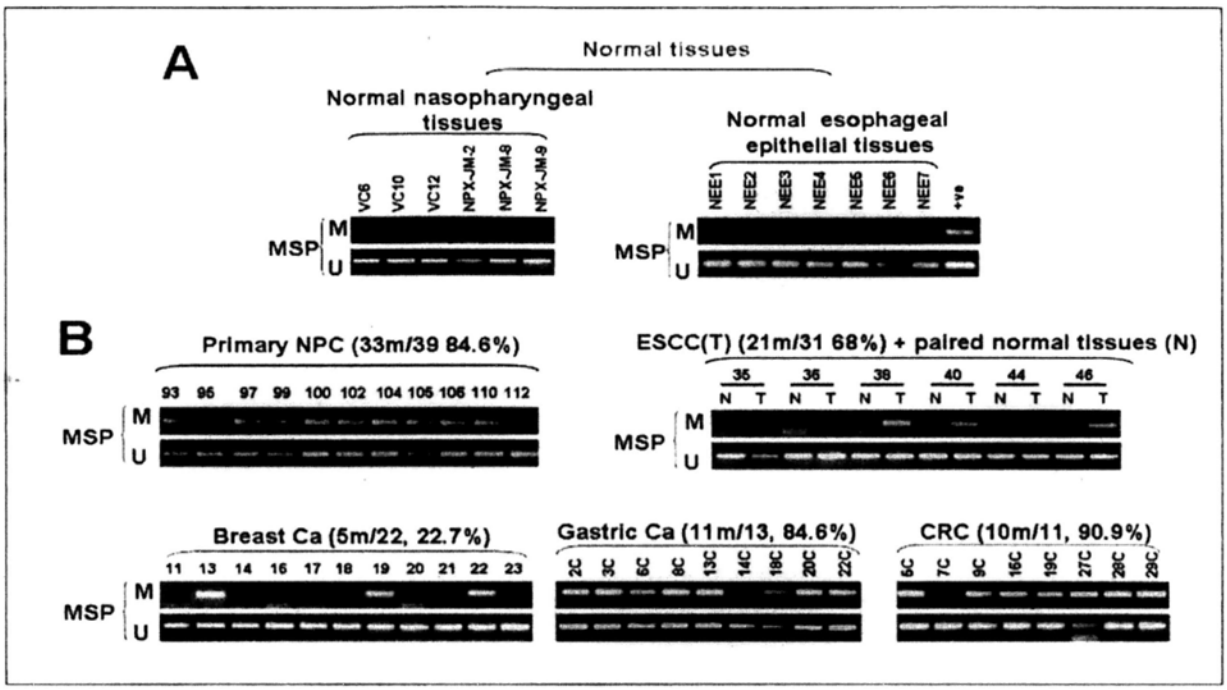


Figure 32. Representative analyses of *ZNF545* methylation in normal tissues and primary tumors. (A) Normal nasopharyngeal and esophageal tissues from healthy individuals. (B) Primary NPC, esophageal carcinoma (T) and their surgical marginal tissues (N), breast, gastric and colon cancers. M: methylated; U: unmethylated.

4.3.5 ZNF545 and ZFP30 are nuclear and nucleolar proteins

The subcellular localization provides useful information for gene functions. COS7, HONE1 and KYSE150 were transfected with pcDNA3.1(+)-Flag-*ZNF545* or pcDNA3.1(+)-Flag-*ZFP30*, respectively. Twenty-four hours after transfection, cells were fixed by methanol and immunostained with Flag-M2 antibody, visualized with fluorescence microscope after DAPI staining for nucleus. Both ZNF545 and ZFP30 are located in cell nucleus and mainly accumulated in the nucleoli, indicating that

these two proteins might function as transcriptional factors and thus play important roles in nucleoli functions such as ribosome biogenesis (Figure 33A).

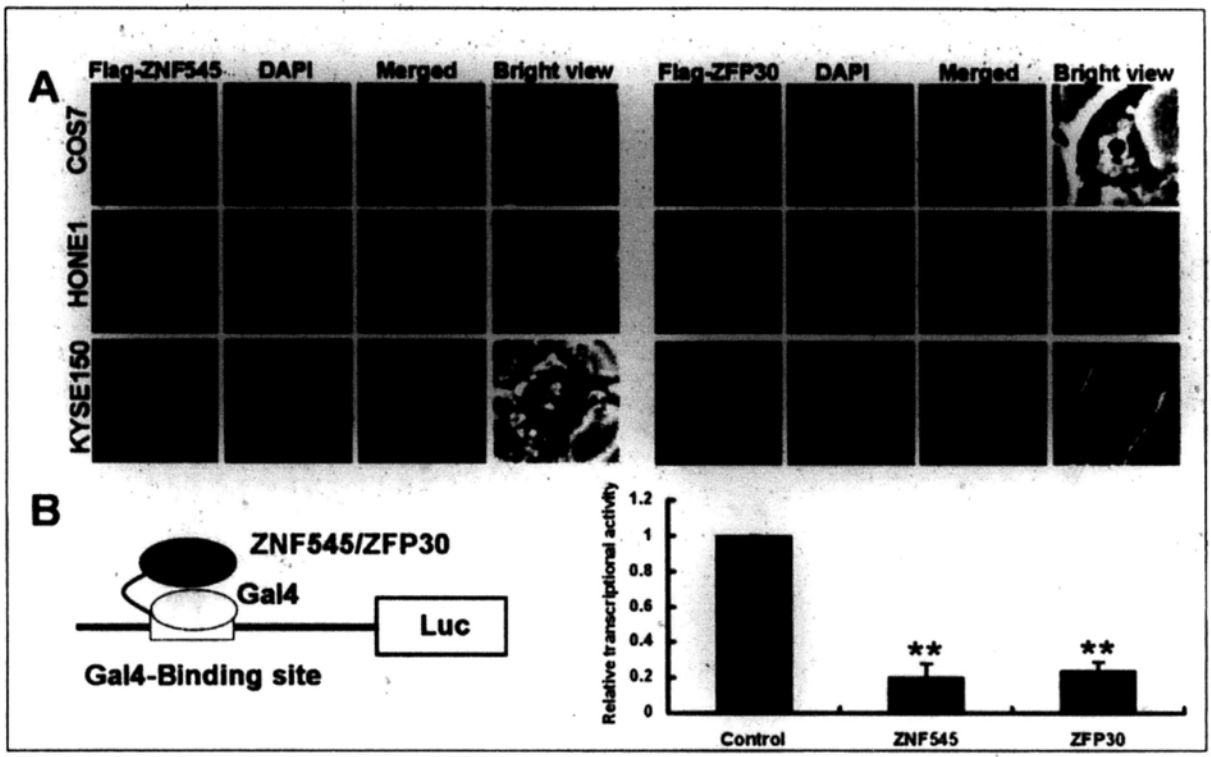


Figure 33. ZNF545 and ZFP30 are nucleolar proteins and transcriptional repressors. (A) ZNF545 and ZFP30 are located in the nucleus and nucleolus by indirect immunostaining using anti-Flag M2 antibody in transfected COS7, HONE1 and KYSE150 cells. (B) ZNF545 and ZFP30 repress the Gal4 reporter transcriptional activity by 4~ 5 folds as compared to the empty vector by dual-luciferase assay.

4.3.6 ZNF545 and ZFP30 are transcriptional repressors and functional TSGs

Sequence analysis of *ZNF545* and *ZFP30* suggests that these proteins may function as transcriptional factors. In order to investigate the transcriptional activity of ZNF545 and ZFP30, a GAL4-based transcription reporter system was used here. Results showed that ZNF545 and ZFP30 could significantly repress the transcriptional activity about 4-5 folds compared to the empty vector, suggesting that they may play direct roles in transcriptional repression (Figure 33B).

To further elucidate the cellular functions of these two transcriptional repressors in tumor cells, I first examined the effects of ZNF545 and ZFP30 on clonogenicity in several carcinoma cell lines. pcDNA3.1(+)-ZNF545 or ZFP30 and a control vector were transfected into the completely silenced cell lines, respectively. For ZNF545, colony formation assay was performed in HONE1, KYSE510 and HCT116 cell lines; while for ZFP30, HONE1 and KYSE410 cells were applied. The ectopic expression of ZNF545 or ZFP30 could dramatically reduce the colony formation efficiency of these cell lines in monolayer culture (Figure 34A&B), suggesting that ZNF545 and ZFP30 can function as TSGs.

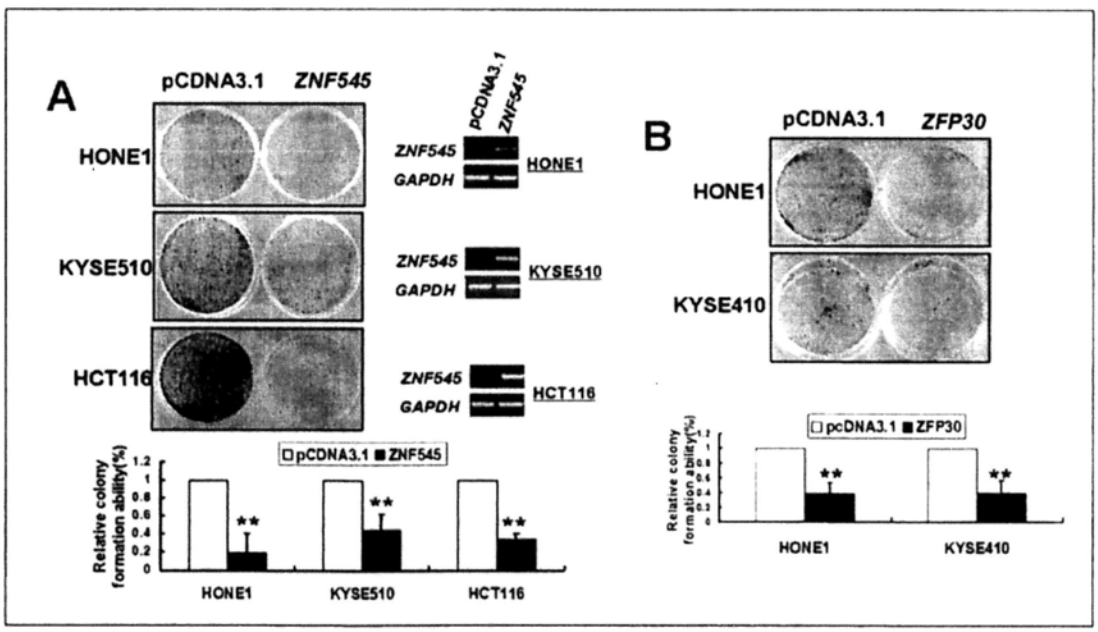


Figure 34. ZNF545 and ZFP30 inhibit clonogenicity of tumor cells. (A) Left panel: representative colony formation assay with monolayer culture of ZNF545. Right panel: expression of ZNF545 in tumor cell lines used for colony formation was analyzed by RT-PCR. Bottom panel: quantitative analysis of colony numbers. (B) Upper panel: representative colony formation assay with monolayer culture of ZFP30. Bottom panel: quantitative analysis of colony numbers. The numbers of G418-resistant colonies in each vector-transfected cell lines were set to 100%, $^{**}p<0.01$.

4.3.7 ZNF545 inhibits proliferation, induces apoptosis and mediates oncogenic pathways

The tumor suppressor function of ZNF545 was further determined by cell proliferation assay (DDAO-SE staining). pIRES-ZsGreen1 was used to sort transfected positive cells with flow cytometry. Forty-eight hours post-labeling, the fluorescence intensity in *ZNF545* transfected HCT116 cells was higher than that in empty vector control cells after GFP sorting by flow cytometry (Figure 35), thus provided direct evidence that ZNF545 could inhibit tumor cell proliferation.

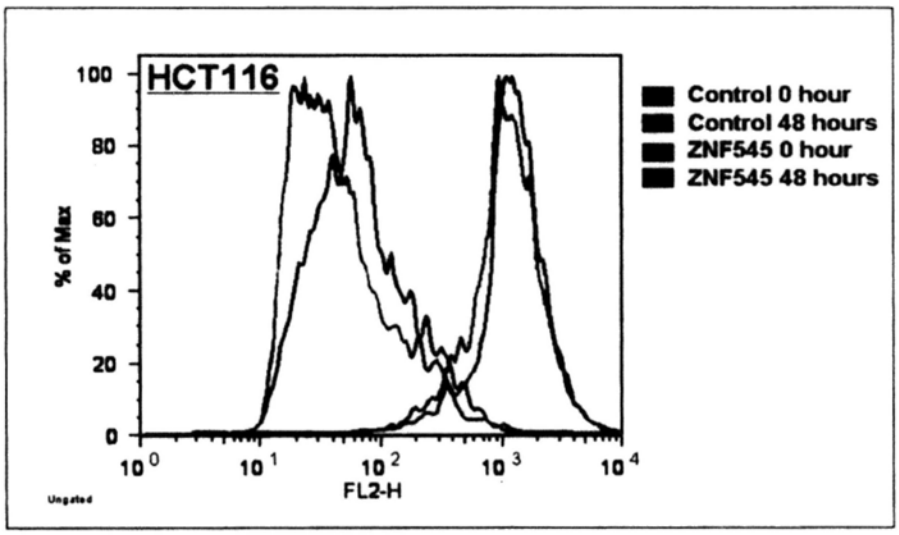


Figure 35. ZNF545 inhibits cellular proliferation. ZNF545 inhibits cell proliferation in HCT116 cells analyzed by DDAO-SE assay.

Apoptosis analysis showed that ectopic ZNF545 expression resulted in a significant increase of apoptotic cells in transfected HCT116 cells as compared with control cells using flow cytometry with Annexin V-PE and 7-AAD double staining (Figure 36A). Apoptotic induction was further confirmed by DAPI staining at individual cell

level. pIRES-ZsGreen1-*ZNF545* transfected cells (GFP positive) showed obvious condensed or fragmented nuclei - a remarkable cell apoptotic feature, but seldom in vector control transfected cells (Figure 36C). The proapoptotic ability of ZNF545 was further confirmed by Western blot. Ectopic expression of ZNF545 could significantly increase the cleavage of PARP in HCT116 and HNE1 cells, compared to vector control transfected cells (Figure 36B). Taken together, these results strongly support that ZNF545 functions as a TSG through inhibiting proliferation and inducing apoptosis of tumor cells.

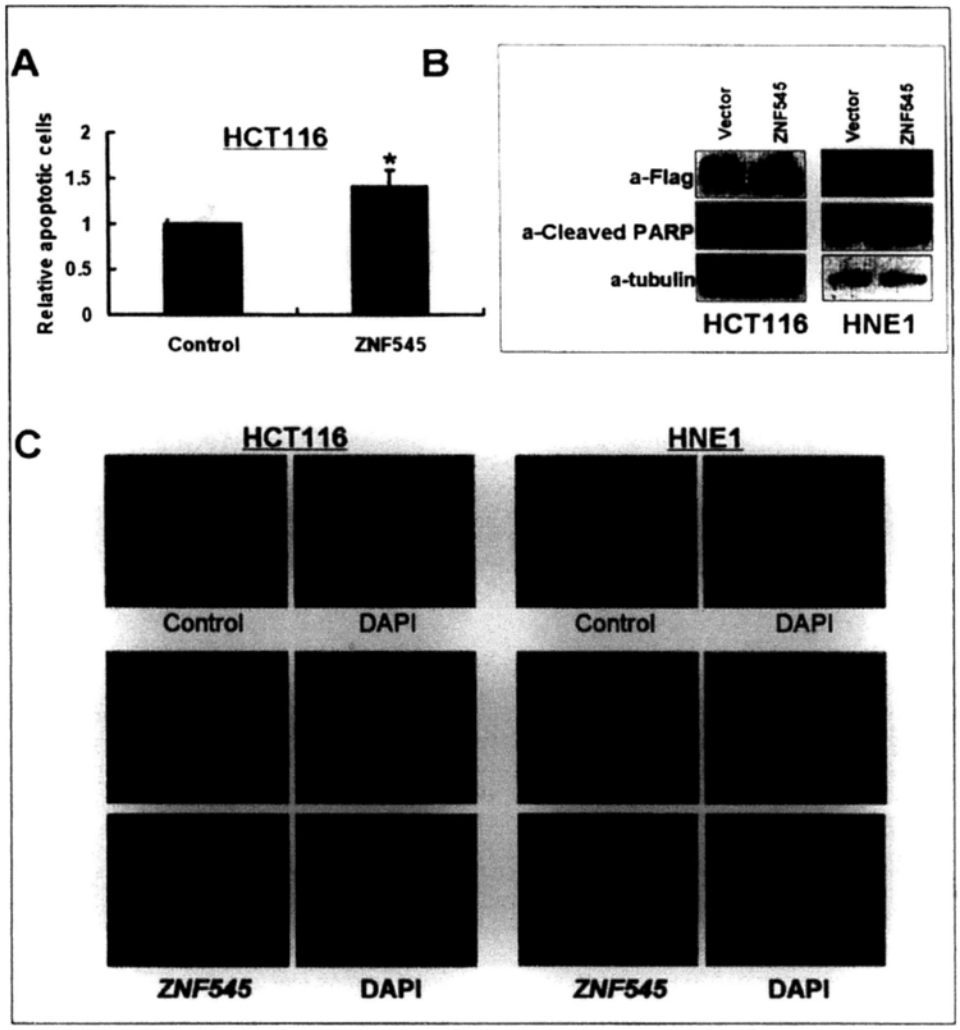


Figure 36. ZNF545 induces apoptosis in cancer cell lines. (A) Relative proportions of apoptotic cells in ZNF545- and control vector-transfected HCT116 cells were analyzed by flow cytometry after Annexin V and 7-AAD staining. (B) Western blot of apoptotic indicator, cleaved PARP, in ZNF545-transfected HCT116 and HNE1 cells. (C) ZNF545 causes the apoptosis-associated cell nuclear changes. Cells were stained with DAPI and photographed under a fluorescence microscope at 48 hours after transfection. The transfection positive cells were indicated by red arrows.

To explore the mechanism of the tumor suppression by ZNF545, I screened several cancer pathways by luciferase reporter assays. I found ZNF545 could significantly suppress AP-1 reporter activities in both HCT116 cancer cells and an immortalized epithelial normal cell line HEK293. And ZNF545 can also inhibit NF-κB activity in HCT116 cells (Figure 37A&B). AP-1 and NF-κB signalings are important in cell proliferation, survival, apoptosis and malignant transformation (Karin, 2006; Eferl and Wagner, 2003). Thus, ZNF545 might induce apoptosis and inhibit cell

proliferation through suppressing both NF-κB and AP-1 signaling pathways.

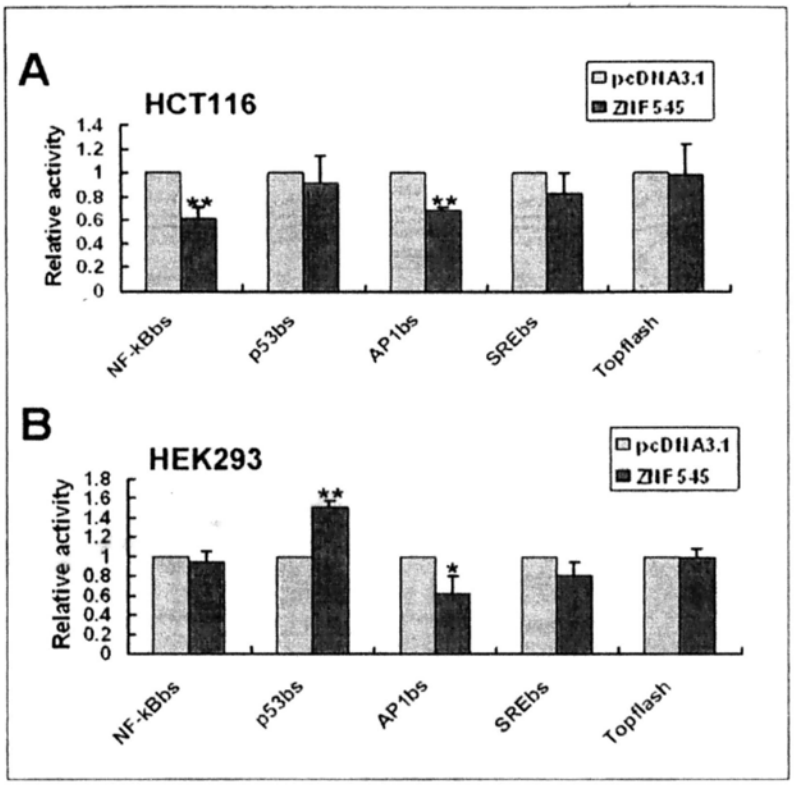


Figure 37. ZNF545 mediates several signaling pathways. Effects of ZNF545 on several signaling pathways in HCT116 and HEK293 cells was assessed by dual-luciferase reporter assays, *p<0.05, **p<0.01.

4.3.8 ZNF545 decreases rDNA promoter activity, protein translation efficiency and cell size

Since several nucleolar proteins can repress rRNA transcription and protein translation, such as JHDM1A and JHDM1B (Tanaka et al., 2010; Frescas et al., 2007), we thus investigated whether ZNF545 is involved in such activities. A human rDNA promoter reporter- pHrD-IRES-Luc was used here (gift from Prof. Kalpana Ghoshal, Ohio State University) (Ghoshal et al., 2004b). We found that ZNF545 significantly inhibits the activity of rDNA promoter (Figure 38A). We further examined the effect of ZNF545 on protein translation, and

pIRES-ZsGreen1-ZNF545 construct which could translate both ZsGreen1 and ZNF545 proteins in one cell was used here. The fluorescent ZsGreen1 protein was used as a marker to indicate the protein translation efficiency. I found that the fluorescence intensity in pIRES-ZsGreen1-ZNF545 transfected cells was lower than that in pIRES-ZsGreen1 control vector transfected HCT116 cells after analyzing by flow cytometry (Figure 38B). The results were further confirmed by direct fluorescent microscope imaging (Figure 38C). It is known that the protein translation efficiency is also associated with cell size. We found that the cell size of ZNF545 transfected positive cells was smaller than that of controls through analyzing by flow cytometry at day 4 after transfection (Figure 38D). Thus, our results strongly indicated that ZNF545 could function as TSG through inhibiting ribosome biogenesis.

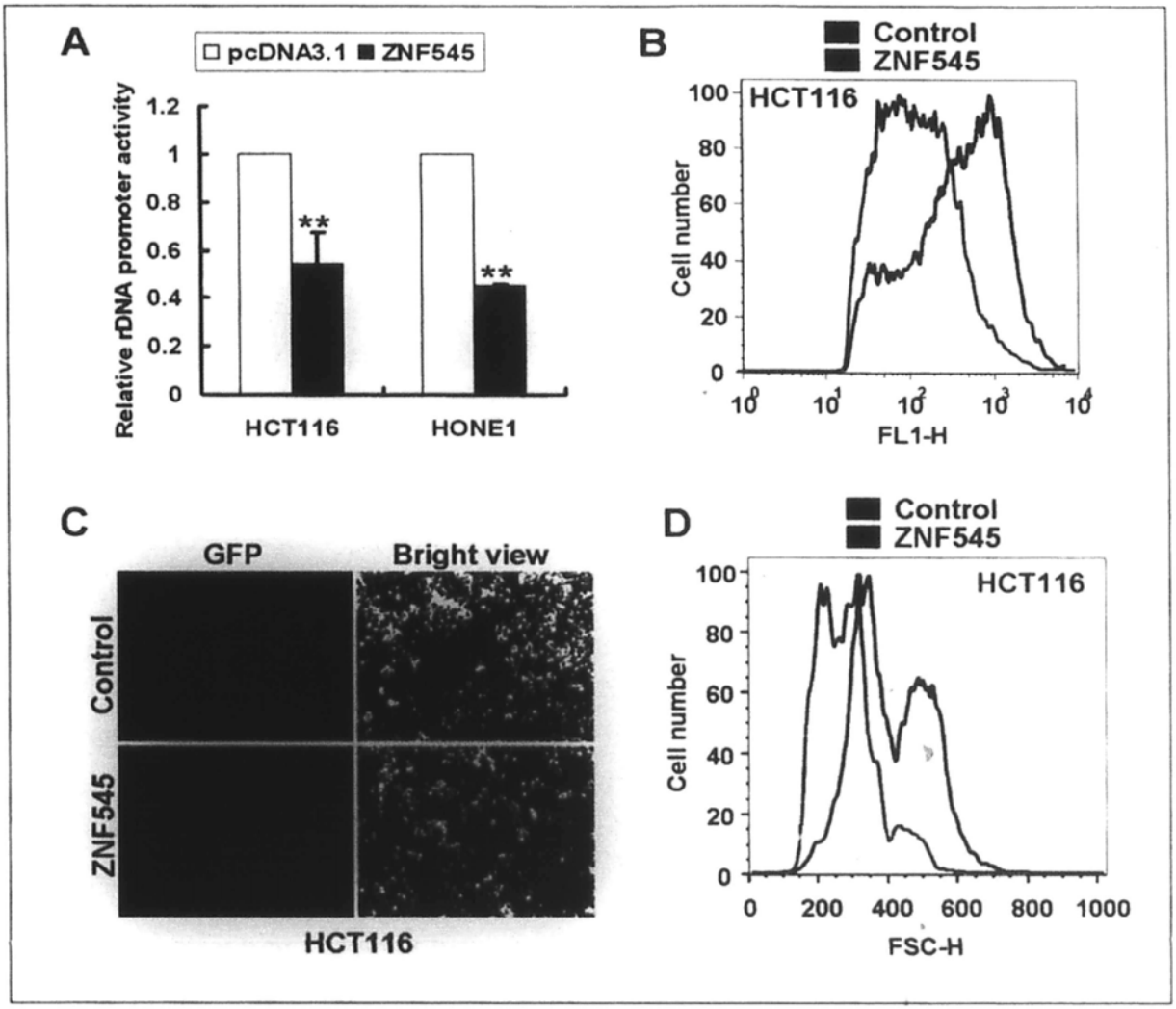


Figure 38. ZNF545 inhibits rDNA promoter activity and protein translation. (A) ZNF545 inhibits the rDNA promoter activity using the pHrD-IRES-Luc rDNA promoter reporter in HCT116 and HONE1 cells, $^{**}p<0.01$. (B&C) pIRES-ZsGreen1-ZNF545 or control vector was transfected into HCT116 cells, and the ZsGreen1 fluorescent intensity was analyzed by flow cytometry (B) and fluorescence microscope (C). (D) Cells were transfected with pIRES-ZsGreen1-ZNF545 or control vector, and cell size of the positive transfected HCT116 cells was determined by flow cytometry.

4.3.9 Discussion

Epigenetic and genetic silencing of TSG is an important mechanism underlying tumorigenesis (Jones and Baylin, 2002). The aberrant hypermethylation of TSG promoter CpG islands is frequently involved in tumorigenesis. Identification of novel candidate TSGs silenced by promoter methylation will help for better understanding the epigenetic mechanism of carcinogenesis. Many TSGs silenced by promoter

methylation were identified. Here, through genome-wide screening, I identified two novel candidates TSGs-*ZNF545* and *ZFP30* that were downregulated by promoter methylation in tumors. It was found that ZNF545 and ZFP30 could inhibit the tumor cell clonogenicity. Further studies revealed that ZNF545 could also inhibit tumor cell proliferation, induce apoptosis and inhibit NF-κB and AP-1 pathways. Both ZNF545 and ZFP30 proteins were found to be nuclear protein and mainly localized in nucleolus and function as transcriptional repressor indicating that they may play important roles in regulating the activity of nucleoli. Further functional studies revealed that ZNF545 represses the rDNA promoter activity and protein translation, as well reduces cell size, thus suggesting that this novel TSG could inhibit ribosome biogenesis.

The relationship between the nucleoli and cancer has been studied for many years. The nucleolus is not only the place of ribosome biogenesis, but also involved in several important cellular processes including cell cycle control, stress responses and coordination of biogenesis of other classes of functional RNPs through sequestration key cell cycle and transcriptional factors (Ruggero and Pandolfi, 2003; Boisvert et al., 2007). Hypertrophic and irregularly shaped nucleoli are the characteristics of malignant cells (Montanaro et al., 2008). It is well known that the rate of cellular proliferation and growth is proportional to the rate of protein synthesis. The protein synthesis by nucleoli is highly regulated. Alterations of ribosome biogenesis lead to human diseases, such as dyskeratosis congenital syndrome (due to *DKC1* mutations),

Diamond-Blackfan anaemia (caused by *RPS19* or *RPS24* mutations), and also increasing susceptibilities to cancers (Dokal, 2000; Draptchinskaia et al., 1999; Gazda et al., 2006). Some proto-oncogenes and tumor suppressors can directly regulate ribosome production or the initiation of protein translation, or both (Ruggero and Pandolfi, 2003). The proto-oncogene *myc* regulates the ribosome proteins targets, and modulates the ribosome biogenesis directly (Boon et al., 2001; Menssen and Hermeking, 2002). PHF8 associates with hypomethylated rRNA genes and exerts a positive effect on rDNA transcription (Feng et al., 2010). TP53 and RB negatively regulate the activities of PolI and Pol III to suppress protein synthesis, while mutations that inactivate the tumor-suppressive activity of RB/or p53 will result in aberrant upregulation of the essential components of protein synthesis machinery and increase ribosome biogenesis, further lead to the enhanced mRNA translation rates (Ruggero and Pandolfi, 2003). JHDM1A and JHDM1B directly bind to rDNA promoter and repress the transcription of rRNA genes through epigenetic mechanisms (Tanaka et al., 2010; Frescas et al., 2007).

Some zinc finger proteins such as ZFP37, ZNF274 or JAZ also locate in nucleolus (Payen et al., 1998; Yano et al., 2000; Yang et al., 2006). JAZ binds to RNA sequence and induces cell cycle arrest and apoptosis through p53 pathway (Yang et al., 1999; Yang et al., 2006). The nucleoli related protein can be downregulated by DNA methylation. It is known that TSGs, p14ARF and p19ARF which are located in nucleoli, are silenced by DNA methylation in tumors (Farmer et al., 2008; Badal et

al., 2008). The expression level of JHDM1B is also decreased in brain tumor (Frescas et al., 2007). Hypomethylation is also found in ribosome proteins in hepatocellular carcinomas (Ghoshal et al., 2004a). Here, we identified ZNF545 as a novel transcriptional factor which localized in nucleoli and silenced by DNA methylation. The results demonstrated that the ectopic expression of ZNF545 in methylated cells inhibits cell proliferation, reduces the activity of rDNA promoter and decreases protein translation efficiency, indeed indicating ZNF545 is involved in the nucleoli related activities to regulate cell proliferation. The reduced rRNA synthesis was also observed in HCT116 DKO cell which loss the DNA methyltranferase DNMT1 and DNMT3b (Gagnon-Kugler et al., 2009). It is further support the role of ZNF545 in ribosome biogenesis due to the reactivation of ZNF545 in DKO cell.

Increasing evidences showed that nucleolus is related to tumor cell apoptosis (Horky et al., 2002; Paik et al., 2010). The inhibition of rRNA synthesis in cancer cell lines is known to cause nucleolar stress which results in disintegration of nucleoli (Martin et al., 1990). In this study, I found that ZNF545 exerts proapoptotic abilities, probably due to its roles on ribosome biogenesis or other mechanisms. Through screening several signalling pathways with dual-luciferase assay, I revealed that ZNF545 can significantly suppress both NF-κB and AP-1 reporter activities in HCT116 tumor cells but only inhibit AP-1 pathway in HEK293 which is similar to the effects of ZNF382, another known TSG (Cheng et al., 2010b). Both NF-κB and

AP-1 signalings are important in cell proliferation, survival, apoptosis and malignant transformation (Karin, 2006; Eferl and Wagner, 2003). NF-κB is a dimeric transcriptional factor which is highly activated in multiple tumors (Basseres and Baldwin, 2006). NF-κB is also related to nucleolar functions due to RelA, a subunit of NF-κB complex, can sequester in the nucleoli in response to the proapoptotic NF-κB stimuli and mediate apoptosis (Stark and Dunlop, 2005). Activated AP-1 is considered as a double-edged sword in tumorigenesis. It can induce apoptosis for certain tumors or specific stages of tumorigenesis, while promote cell survival for other tumor types. TSGs PDCD4 and HINT1 inhibit cell proliferation through inhibiting AP-1 activity in colorectal cancer cells (Wang et al., 2008; Wang et al., 2007). Our findings showed that ZNF545 regulates proliferation and induces apoptosis through NF-κB and AP-1 pathways. Further studies are still needed to focus on the detail mechanisms of how ZNF545 regulates rDNA promoter activity and oncogenic pathways- NF-κB and AP-1.

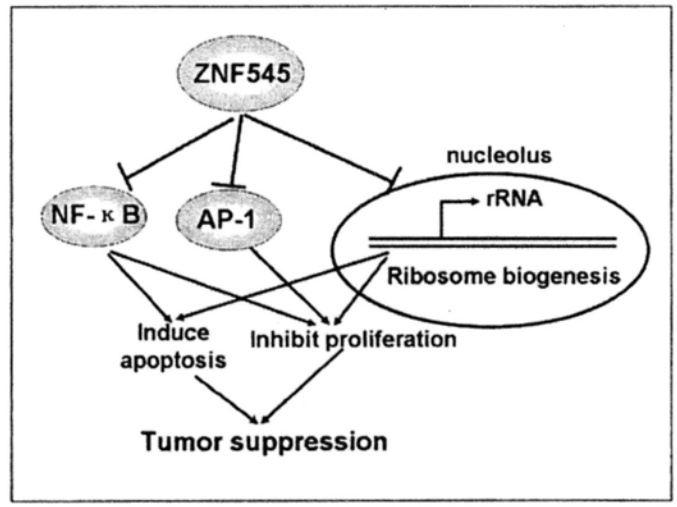


Figure 39. A possible mechanism model of tumor suppression by ZNF545. ZNF545 inhibits NF-κB and AP-1 signaling pathways. Simultaneously, ZNF545 suppresses rRNA transcription and ribosome biogenesis. All can lead to tumor suppression.

In conclusion, ZNF545 and ZFP30 are found to be functional TSGs inhibiting tumor cell clonogenicity. ZNF545 inhibits cell proliferation, induces apoptosis and inhibits NF-κB and AP-1 signaling pathways. Both ZNF545 and ZFP30 function as transcriptional repressors and located in nucleoli indicating their anti-tumorigenic effects may act through nucleoli related activities such as ribosome biogenesis. ZNF545 shows inhibition potential on rRNA gene transcription which results in decreased protein translation efficiency and smaller cell size (Figure 39). The discovery of these two novel tumor suppressors gives more insights on nucleoli and carcinogenesis. In addition, the high frequency and tumor-specific methylation of *ZNF545* in nasopharyngeal, esophageal, gastric, colon and breast carcinomas indicates that it could be further developed as a potential epigenetic biomarker for cancer diagnosis。

4.4 Epigenetic disruption of two novel KRAB-zinc finger tumor suppressors in multiple tumors through hypermethylation of a common bidirectional promoter.

Here, I identified two novel candidate TSGs: *ZNFT1* and *ZNFT2* which are regulated by a common bidirectional promoter and have similar expression patterns in tumor cell lines. These two ZNFs were frequently silenced in multiple carcinoma cell lines through hypermethylation of their bidirectional promoter. Pharmacologic and genetic demethylation restored the expression of these two genes. The tumor specific downregulation of *ZNFT1* and *ZNFT2* indicates that these two genes might play important roles in carcinogenesis, and the frequent promoter methylation in cancer cell lines suggesting that they might be used as potential epigenetic biomarkers for cancer diagnosis.

4.4.1 *ZNFT1* and *ZNFT2* promoter functions bidirectionally

Through screening of ZNFs on chromosome 19, I identified *ZNFT1* and *ZNFT2* as novel candidate TSGs. These two proteins localize on chromosome 19q12.13 in a head to head fashion and share a bidirectional promoter (Figure 40A). Both of them contain a KRAB domain with different number of C2H2 zinc finger domains (*ZNFT1*: 18 C2H2 zinc fingers, *ZNFT2*: 11 C2H2 zinc fingers). In order to check the bidirectional nature of *ZNFT1* and *ZNFT2* promoter, a 2022bp promoter region between *ZNFT1* and *ZNFT2* TSS was cloned into a pGL3-basic luciferase reporter vector in two orientations. This promoter showed bidirectional transcriptional

activity in dual-luciferase assay. The promoter activity of *ZNFT2* was lower than that of *ZNFT1* which might be caused by posttranscriptional mechanisms (Figure 40B) (Shu et al., 2006).

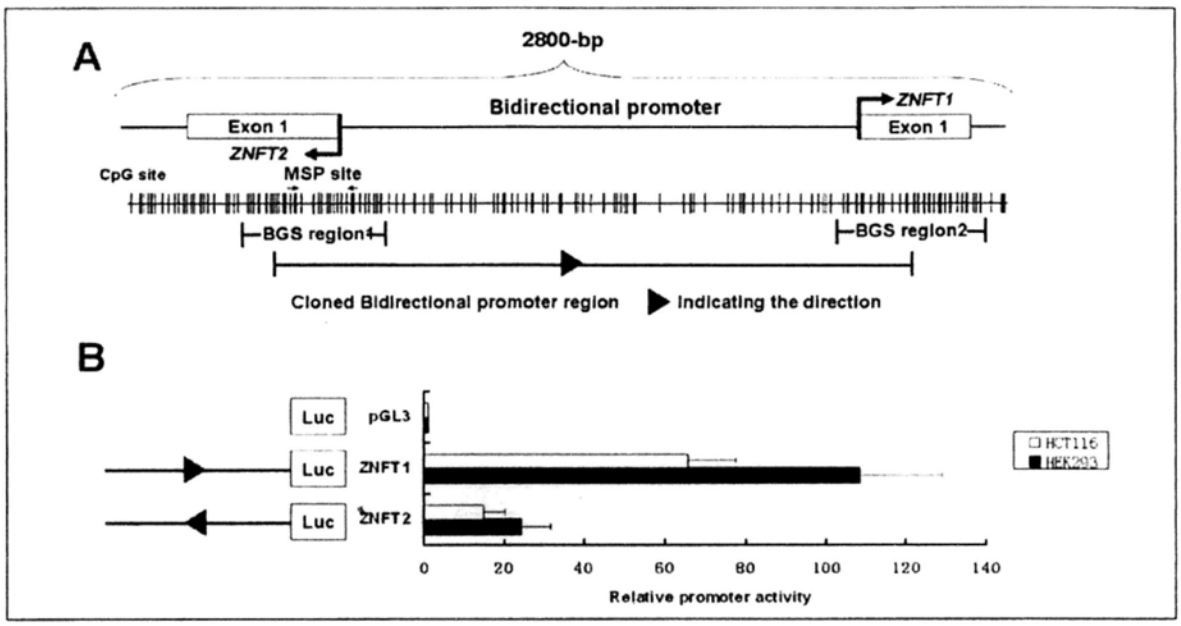


Figure 40. *ZNFT1* and *ZNFT2* promoter functions bidirectionally. (A) The promoter of *ZNFT1* and *ZNFT2* is a bidirectional promoter. The TSS is indicated by a curved arrow. The CGI, MSP and BGS regions analyzed are indicated. (B) The luciferase assay indicates that the promoter of *ZNFT1* and *ZNFT2* functions bidirectionally.

4.4.2 *ZNFT1* and *ZNFT2* were frequently downregulated in carcinoma cell lines

Since the bidirectional promoter controls the expression of both downstream genes, the expression patterns of ZNFT1 and ZNFT2 were examined together. The broad expressions of these two genes were detected by semi-quantitative RT-PCR in a panel of human normal adult and fetal tissues (Figure 41A). Then, the expressions of *ZNFT1* and *ZNFT2* were checked in a serial of tumor cell lines including nasophegeal, esophageal, colon, cervix, gastric, liver, lung and breast cancers. These two genes were silenced or downregulated in several cell lines of nasophegeal,

esophageal, colon, cervix, and gastric cancers, but normally expressed in liver, lung, and breast cancers (Figure 41B&C). The similar expression pattern of these two genes suggested that the bidirectional promoter regulates the expression of these two genes together.

4.4.3 Frequent silencing of *ZNFT1* and *ZNFT2* by promoter methylation in multiple carcinoma cell lines

ZNFT1 and *ZNFT2* bidirectional promoter contains a typical CGI, indicating these two genes may be regulated by an epigenetic mechanism. MSP was thus applied to detect the promoter methylation status of *ZNFT1* and *ZNFT2*. MSP primers were first validated for the specificities and not amplifying any unbisulfite DNA. MSP results showed that the promoter of *ZNFT1* and *ZNFT2* was methylated in reduced or silenced cell lines but not in immortalized normal epithelial cell lines (Figure 41B). Detailed BGS analysis further confirmed the MSP results (Figure 42). Thus, transcriptional silencing of *ZNFT1* and *ZNFT2* in multiple carcinoma cell lines was well correlated with promoter methylation.

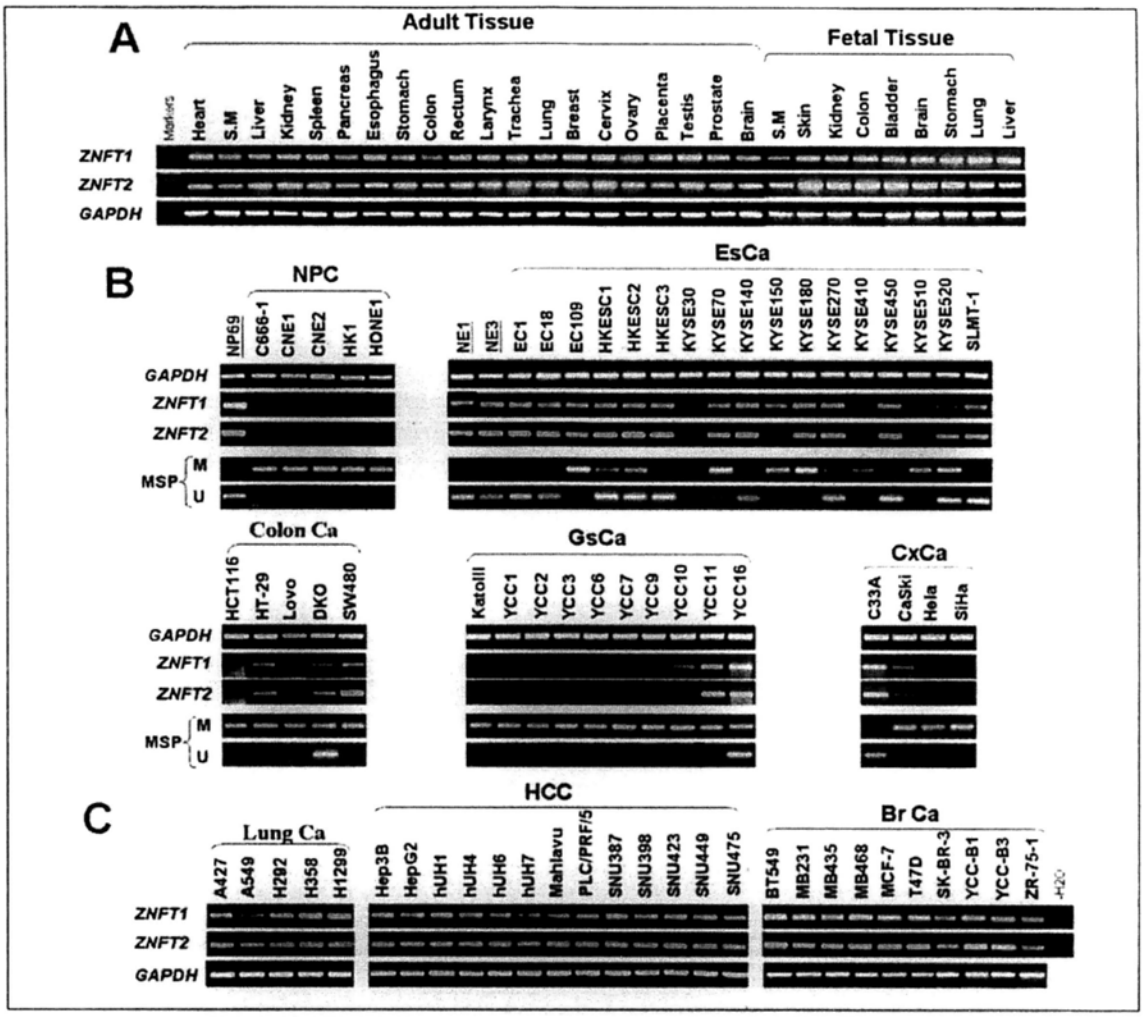


Figure 41. The expression patterns of *ZNFT1* and *ZNFT2* in normal tissues and cancer cell lines. Normal tissues (A) and cancer cell lines (B&C). The expressions of *ZNFT1* and *ZNFT2* were downregulated in NPC, ESCC, colon, gastric, and cervical cancers by promoter methylation but normally expressed in lung, liver and breast cancers.

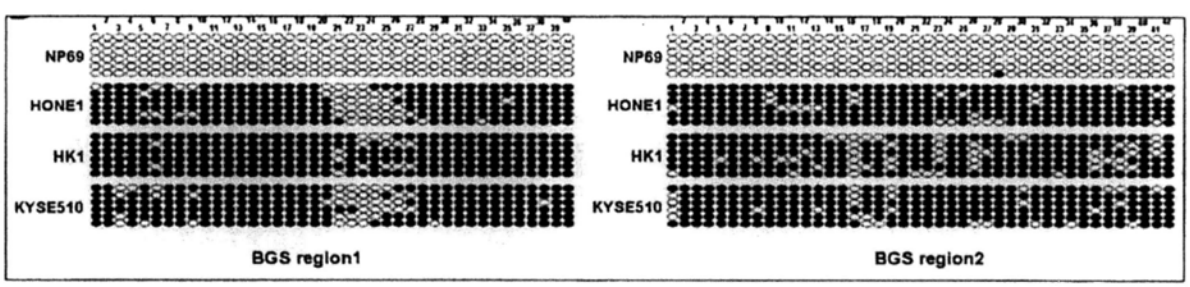


Figure 42. BGS analysis of *ZNFT1* and *ZNFT2*. High-resolution methylation analysis of *ZNFT1* and *ZNFT2* promoter by BGS in normal (NP69) and carcinoma cell lines (HONE1, HK1 and KYSE510).

4.4.4 Pharmacologic and genetic demethylation activates *ZNFT1* and *ZNFT2* expressions

In order to further determine whether promoter methylation directly mediates *ZNFT1* and *ZNFT2* silencing, several carcinoma cell lines (HONE1, HNE1, C666-1, HK1, KYSE510 and HCT116) were treated with DNA methyltransferase inhibitor Aza, combined with or without histone deacetylase inhibitor TSA. The expressions of *ZNFT1* and *ZNFT2* were dramatically restored after drug treatment (Figure 43A). *ZNFT1* and *ZNFT2* were also activated in the completely methylated colorectal cancer cell line HCT116 by genetic demethylation through double knock-out of both DNMT1 and DNMT3B (DKO cell line)(Rhee et al., 2002), but not single KO of DNMT1 or DNMT3B alone (1KO or 3BKO cell line) (Figure 43B). Further high-resolution methylation analysis by BGS confirmed the demethylation of *ZNFT1* and *ZNFT2* promoter (Figure 43C). Thus, these results demonstrated that CpG methylation of the bidirectional promoter directly silenced *ZNFT1* and *ZNFT2* expressions in multiple carcinoma cell lines.

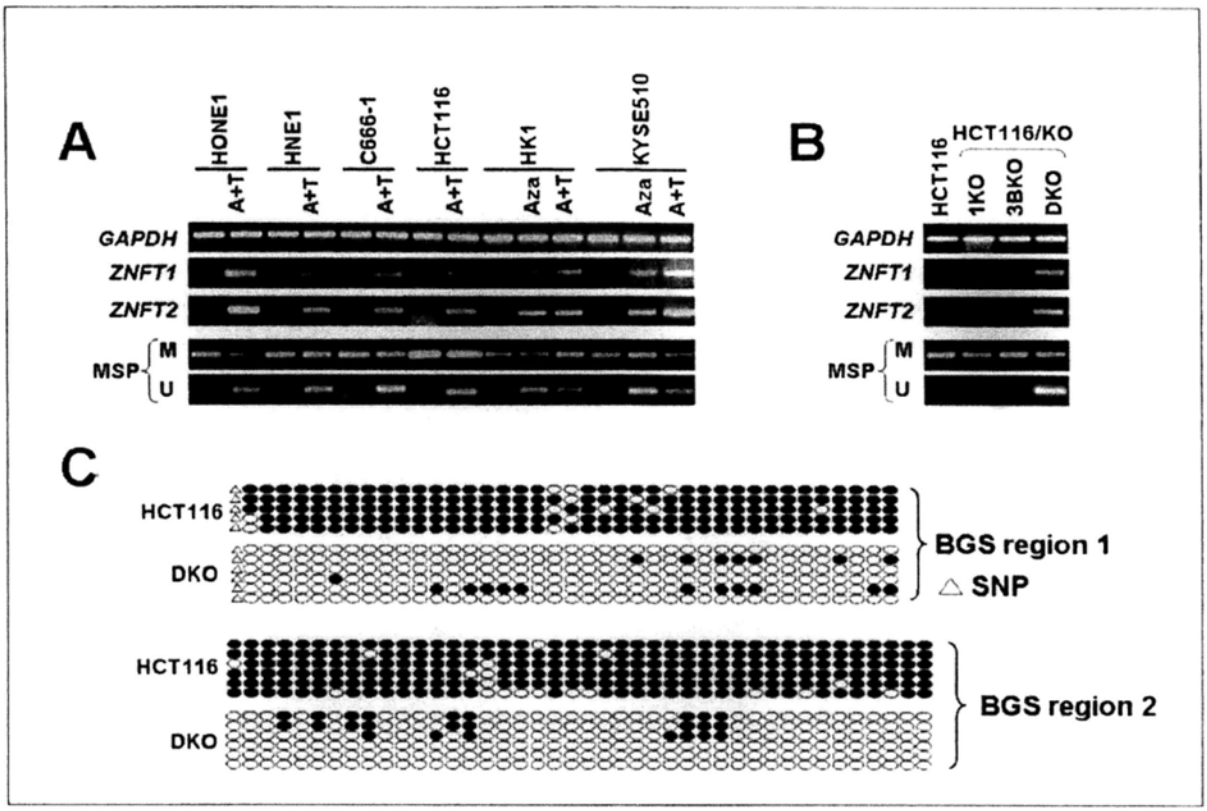


Figure 43. Pharmacologic and genetic demethylation reactivates the expressions of *ZNFT1* and *ZNFT2*. (A) Demethylation with Aza plus TSA activated the expressions of *ZNFT1* and *ZNFT2* in tumor cell lines. (B) Genetic knockout of *DNMT1* and *DNMT3B* restored the expressions of *ZNFT1* and *ZNFT2*. Demethylation (U) of *ZNFT1* and *ZNFT2* promoter was detected in HCT116-DKO cell line. (C) Detailed BGS analysis confirmed the demethylation of bidirectional promoter in HCT116-DKO cell line.

4.4.5 Discussion

Bidirectional promoter is defined as two unidirectional promoters sharing the same genomic sequence (Adachi and Lieber, 2002). About 11% of human genes were arranged in this way, and mostly, their TSSs were separated by less than 1000 base pairs (Trinklein et al., 2004). The abundance of bidirectional promoter was also found in other mammalian, plant, even in yeast genomes (Yang et al., 2008; Dhadi et al., 2009; Neil et al., 2009). It has been reported that the GC contents are higher in bidirectional promoters and most of the bidirectional promoters lack the TATA box (Adachi and Lieber, 2002; Trinklein et al., 2004; Takai and Jones, 2004).

Bidirectional promoter provides strong resistance to invasion through transposable elements (Takai and Jones, 2004), which may be one of the reasons that many important genes are located in this way especially for genes involved in DNA repair (Adachi and Lieber, 2002; Trinklein et al., 2004).

Methylation of promoter CpG can cause the loss of TSG functions and is frequently occurred in tumor development and progression (Knudson, 2001; Jones and Baylin, 2002). It is known that the transcriptions of many bidirectional pairs are co-expressed (Trinklein et al., 2004) while the hypermethylation of bidirectional promoter causes the transcriptional silencing of the genes in both directions (Shu et al., 2006). Several bidirectional pairs in genome are tumor-specific methylated. Methylation of WNT9A/CD558500 and CTDSPL/BC040563 promoters frequently occurs in primary colon cancers and acute lymphoid leukemia (ALL) (Shu et al., 2006). Two proapoptotic genes MAPK10/JNK3 and PTPN13/FAP-1 were silenced in multiple lymphomas and carcinomas through the DNA methylation of bidirectional promoter (Ying et al., 2006a). In breast cancer, LRRC49 and THAP10 genes were frequently silenced by the common bidirectional promoter hypermethylation (De Souza et al., 2008). Hypermethylation of the bidirectional promoter region of COL4A5/COL4A6 causes the expression losing of α5(IV)/α6(IV) chains and the remodelling of the epithelial BM during cancer cell invasion (Ikeda et al., 2006). PARK2 and PACRG are also epigenetically silenced by hypermethylation of their common promoter in acute lymphoblastic leukaemia (Agirre et al., 2006).

Here, I identified a pair of ZNFs regulated by a bidirectional promoter and silenced in multiple carcinoma cell lines. The transcriptional silencing could be reversed by pharmacologic and genetic demethylation. The discovery of bidirectional silenced genes will provide more insights in genome evolution. The bidirectional organization also indicates that these two genes may play critical roles in tumorigenesis such as DNA repair. The tumor specific silencing of these two genes could be used as epigenetic biomarkers for cancer molecular diagnosis.

Chapter 5: Conclusions

The biochemical functions of KRAB-ZNFs are thought to be critical to their cellular roles, including cell differentiation, cell proliferation, apoptosis, and neoplastic transformation (URRUTIA, 2003). Some ZNFs function as oncogenic proteins such as *ZNF217* and *ZNF639* (Cowger et al., 2007; Imoto et al., 2003), while others act as tumor suppressors, including *OAZ*, *ZNF23*, *ZAC*, *ST18*, *ZNF652*, *ZIPK*, *KS1* and *JAZ* et al (Hata et al., 2000; Huang et al., 2007; Kamikihara et al., 2005; Jandrig et al., 2004; Kumar et al., 2006; Bi et al., 2009; Gebelein et al., 1998; Yang et al., 2006). Aberrant inactivation of negative mediators of cell proliferation (including TSGs) through promoter methylation is frequently involved in tumorigenesis (Coleman and Tsongalis, 2006; Baylin and Ohm, 2006). Identification of candidate TSGs silenced by promoter methylation will uncover the epigenetic mechanism of carcinogenesis and also identify novel epigenetic tumor biomarkers for early tumor detection (Belinsky, 2004). Up to date, only few ZNFs, such as *ZAC* and *ST18*, have been reported as tumor suppressors and down-regulated through DNA methylation (Kamikihara et al., 2005; Jandrig et al., 2004), indicating that more epigenetically silenced ZNFs are yet to be identified.

In this study, through screening on chromosome 19, I identified several candidate KRAB-ZNFs tumor suppressors. Five genes were chosen for further studies (*ZNF382*, *ZNF545*, *ZFP30*, *ZNFT1* and *ZNFT2*). Compared with its broad expression in normal tissues and normal cell lines, I found *ZNF382* was silenced or

downregulated in multiple carcinoma cell lines due to promoter CpG methylation, which could be reversed by pharmacologic or genetic demethylation. *ZNF382* was also frequently methylated in multiple primary tumors (nasopharyngeal, esophageal, colon, gastric, and breast cancers). Ectopic expression of *ZNF382* in silenced tumor cells significantly inhibited their clonogenicity, proliferation and induced apoptosis. We further found that ZNF382 inhibited NF-κB and AP-1 signaling and downregulated the expression of multiple oncogenes including *MYC*, *MITF*, *HMGA2*, *CDK6*, as well as NF-κB upstream factors *STAT3*, *STAT5B*, *ID1* and *IKBKE*, most likely through heterochromatin silencing. ZNF382 could suppress tumorigenesis through heterochromatin-mediated silencing, as ZNF382 was co-localized and interacted with heterochromatin protein HP1 and thus further changed the chromatin modifications of ZNF382 target oncogenes. Our data demonstrated that ZNF382 is a functional tumor suppressor frequently methylated in multiple carcinomas, which could be an attractive tumor biomarker.

ZNF545 and *ZFP30* are downregulated in multiple tumor cell lines in contrast to their broad expressions in normal tissues. It was found that transcriptional silencing of *ZNF545* and *ZFP30* could be reversed by demethylation, indicating a direct epigenetic silencing mechanism. *ZNF545* also frequently methylated in primary tumors. ZNF545 and ZFP30 were found to be transcriptional repressors and could inhibit the colony formation in silenced cancer cell lines. More function studies on ZNF545 revealed that ZNF545 also inhibits cell proliferation, induces apoptosis and

suprresses NF-κB and AP-1 pathways which play important roles in tumorigenesis. I further found that ZNF545 and ZFP30 have similar subcellular localization. They were located in the nucleus and mainly concentrated in the nucleoli. I proposed that ZNF545 and ZFP30 could inhibit tumorigenesis through regulating nucleolus functions such as ribosome biogenesis. In agreement, I found that ZNF545 inhibited the activity of rDNA promoter, decreased the translation efficiency and cell size. It might exert the tumor suppressor function through regulating ribosome biogenesis or downregulating the transcription of target genes. The tumor specific-silencing of *ZNF545* and *ZFP30* could serve as an epigenetic diagnosis biomarker for common cancers.

ZNFT1 and *ZNFT2* were broadly expressed in normal tissues, but silenced or down-regulated in nasopharyngeal, esophageal, colon, stomach and cervix cancer. The transcriptional silencing of *ZNFT1* and *ZNFT2* could be reversed by pharmacologic demethylation and genetic demethylation. Their bidirectional promoter was confirmed by luciferase assay. Here, I identified two novel candidate TSGs whose downregulation was directly mediated by DNA methylation of the bidirectional promoter, and their frequent methylation may be further developed as epigenetic biomarkers for cancer detection. Further functional studies will be performed on these two proteins.

In conclusion, several candidate zinc finger tumor suppressors were indentified in

this study. The study of ZNFs will help for better understanding of TFs and tumorigenesis. The tumor specific methylation of novel tumor suppressors could be as potential epigenetic biomarkers for cancer early detection.

Chapter 6: Future Studies

In this study, through RT-PCR screening of ZNFs on chromosome 19, several KRAB-ZNFs were identified as novel epigenetically silenced functional TSGs. Functional studies revealed that some ZNFs inhibit tumor cell clonogenicity, proliferation and induced apoptosis. Further investigations on their tumor suppressive mechanisms uncovered that some ZNFs mediate different signaling pathways and regulate cellular activities through various mechanisms such as heterochromatin silencing or inhibiting ribosome biogenesis. However, there are still some aspects remaining to be explored.

1. Further functional characterization of the candidate TSGs

In this study, five candidate ZNFs were chosen for further functional studies. ZNF382 inhibits several oncogenes through heterochromatin silencing. ZNF545 and ZFP30 are functional tumor suppressors and mainly located in nucleoli. More functional studies revealed that ZNF545 inhibits ribosome biogenesis. For the better understanding the mechanisms of their tumor suppressive functions, more studies will be performed on these genes. For instances, *in vivo* studies to define the tumor suppressor functions of these functional TSGs; *in vitro* studies such as ChIP-chip to investigate the transcriptional regulation networks of ZNFs. Also, in-deep investigations will be performed for ZNF545 in ribosome biogenesis. I will investigate how ZNF545 regulates rDNA promoter activity. In addition, the functional studies will be performed on ZNFT1 and ZNFT2.

2. Clinical significance of epigenetic silenced zinc finger TSGs

With the increasing reports about frequent epigenetic silencing of TSGs in multiple tumors, epigenetic aberration is quite important and even precedes genetic alteration in cancers. The well developed technologies (such as methylation-specific PCR) can specifically and sensitively detect hypermethylated promoter alleles. In this study, we have identified several candidate TSGs frequently silenced or downregulated by promoter methylation in tumors. Further studies will be focused on the clinical applications that whether the tumor specific downregulation of those TSGs is an early event in the development of cancer and whether it is possible to develop a serial of specific TSGs methylation profiling as diagnostic biomarkers or therapeutic targets for a certain cancer.

3. Further screening of other ZNFs

There are about 700 ZNFs in human genome and only one third zinc fingers localize on chromosome 19. According to the expression profiles of ZNFs in this study, some ZNFs localized on other chromosomes might have similar functions with epigenetic modification properties. The expression profiles (normal tissues and carcinoma cell lines) and functional studies of other zinc fingers with CpG island will be worth for further exploring.

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