THE INTERPLAY BETWEEN RUNX3 AND TEAD-YAP COMPLEX IN GASTRIC CANCER

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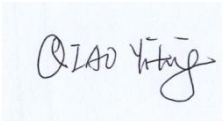
me love and encouragement to help me go through ups and downs.

To all of them, I dedicate this thesis.

Declaration

I hereby declare that the research and work described in this dissertation is my original work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person, except where due acknowledgement has been made in the text. Some of the work described in Chapter 5 was performed by Dr. Lin Suling and Chen Ye and I assess my overall contribution to the work described in this thesis to be 90%.



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Summary

RUNX3 (Runt-related transcription factor 3) is a well-documented tumour suppressor, whose inactivation, either due to promoter methylation or protein mislocalization, is frequently observed in gastric cancer. The mechanisms by which RUNX3 exerts its tumour suppressor activities are not fully understood yet. TEAD-YAP complex is a transcriptional factor regulating cell proliferation and migration. In normal quiescent adult cells, the activity of TEAD-YAP complex is restricted at a low level by Hippo pathway as well as other mechanisms, while the hyper-activation of TEAD-YAP complex is frequently observed in liver cancer and breast cancer.

In this study, we proved that the TEAD-YAP complex also conferred strong oncogenic activities in gastric epithelial cells. The higher expression of TEAD-YAP in tumour tissues significantly correlated with poorer overall survival time in a gastric cancer cohort. Strikingly, RUNX3 physically interacted TEAD (TEA domain family members) proteins through its Runt domain. This interaction markedly reduced the DNA binding ability of TEAD, which attenuated the downstream signalling of TEAD-YAP complex.

Our findings identify RUNX3 as a novel negative regulator of TEAD-YAP complex. Our discoveries also reveal that a significant aspect of RUNX3's tumour suppressor function is via its negative regulation of TEAD-YAP oncogenic complex in gastric cancinogenesis.

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List of Abbreviations

APC	adenomatous polyposis coli
Bim	Bcl-2 homology domain-only factor
CBF	core binding factor
CBP	CREB-binding protein
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
CO2	Carbon dioxide
CTGF	connective tissue growth factor
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
DRG	Dorsal Root Ganglion
ECM	extracellular matrix
EMSA	electrophoretic mobility shift assay
EMT	epithelial-mesenchymal transition
esiRNA	Endoribonuclease-prepared siRNAs
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde-3-phophate dehydrogenase
GFP	green fluorescent protein
H2O	Dihydrogen monoxide
HDAC	histone deacetylases
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IgCα	immunoglobulin constant alpha
IP	immunoprecipitation
LB	Luria-Bertani
Lgr5	leucine-rich repeat-containing G-protein coupled receptor 5
LOH	loss of heterozygosity
MgCl2	Magnesium chloride
NP-40	Tergitol-type NP-40
PBS	Phosphate-Buffered Saline
PBST	Phosphate-Buffered Saline-Tween20
PCR	polymerase Chain Reaction
PEBP2	Polyoma virus enhancer-binding protein2
PVDF	polyvinylidene difluoride
Py	polyoma virus
RNA	Ribonucleic acid
RNAi	RNA interference
RPMI	Roswell Park Memorial Institute
RT	Reverse Transcript
RUNX	Runt-related
SDS-	sodium dodecyl sulfate polyacrylamide gel

PAGE	electrophoresis
shRNA	short hairpin RNA
siRNA	small interfering RNA
SMAD	small mothers against decapentaplegic
TCF	T cell factor
TEAD	TEA domain family member
TGF-β	Transforming Growth Factor-β
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
Wnt	wingless-type MMTV integration site
wt	wild-type
YAP	Yes-associated protein
ZO	zonula occludens

A	ampere
cm	centimeter
g	gram
L	liter
min	minute
ml	millilitre
mM	millimolar
pMol	picomole
U	unit
V	Volt
µg	microgram
µL	microlitre
µM	micromolar
µm	micrometer

Chapter 1

Introduction

1.1 A brief introduction about gastric cancer

Cancer is the leading cause of death in developed countries and the second leading cause of death in developing countries (Ferlay et al, 2010). Among all kinds of cancers worldwide, gastric cancer ranks fourth in incidence (after lung, breast and colorectal) and second in mortality (after lung cancer) (Jemal et al, 2011). Eastern Asia has the highest incidence of gastric cancer, reflecting the role of dietary patterns and prevalence of *Helicobacter pylori* (*H.pylori*) infection in the progression of disease (Parkin, 2006).

According to Laurén classification system, histologically there are two major types of gastric cancer: (1) intestinal type gastric cancer, which is characterized by malignant epithelial cells that show cohesiveness and glandular differentiation infiltrating the stroma; (2) diffuse type gastric cancer, which is composed of discohesive cells that infiltrate the stroma individually or in small groups. A small percentage of gastric adenocarcinomas are mixed, presenting features of both types (Lauren, 1965).

Gastric cancer is a multifactorial disease caused by a variety of infectious, environmental, and host-related factors. A small percentage of gastric cancer can be attributed to familial cancer syndromes, including hereditary diffuse gastric cancer, which is caused by mutations of *CDH1* gene (encoding a cell adhesion protein E-cadherin), and Lynch syndrome, which is caused by mutations of *MLH1, MSH2, MSH6, PMS2, or EPCAM* (encoding proteins involved in DNA repair) (Caldas et al, 1999).

However, most gastric cancer cases are sporadic and influenced by infectious factors and environmental factors. *H.pylori* infection has been

recognized as a type I carcinogen by International Agency for Research on Cancer since 1994, due to its strong correlation with gastric cancer (Vogiatzi et al, 2007). Environmental and lifestyle factors including tobacco smoking, high salt intake and meat consumption, increase the risk of developing gastric cancer, while an adequate intake of fresh fruits and vegetables decreases risk of gastric cancer (Gonzalez & Agudo, 2012).

Currently, some genome-wide association studies (GWAS) have been conducted to identify more genetic factors related to sporadic gastric cancer. Single Nucleotide Polymorphism (SNP) of Prostate stem cell antigen (*PSCA*) gene and Mucin 1 (*MUC1*) gene show positive correlation with diffuse type gastric cancer (Saeki et al, 2013; Study Group of Millennium Genome Project for et al, 2008). A recent exome sequencing project using gastric cancer specimen identified *TP53*, *PIK3CA*, *ARID1A* as well as *FAT4* as frequently mutated genes (Zang et al, 2012). More in-depth research is needed to reveal the underlying relationship between mutated genes and gastric cancer.

The focus of this thesis, RUNX3 and TEAD-YAP complex, are both closely related to the progression of gastric cancer, either as a tumour suppressor or an oncogene. The biological functions and importance of them would be further explained in following chapters.

1.2 A brief introduction of Runt-Related Transcription Factor family

1.2.1 Runt-Related Transcription Factor family (RUNX family)

Runt (*Drosophila* homolog of *RUNX* genes) was first discovered as one of the genes required for the establishment of segmented body pattern of the *Drosophila* embryo (Gergen & Butler, 1988). The pattern of *runt* transcript accumulation undergoes a series of changes during the early stages of *Drosophila* embryogenesis, suggesting its critical role in development.

Later, *RUNX1* gene (named as *AML1* at the time of discovery) was identified as a part of the fusion gene generated by t(8;21) chromosome translocation frequently observed in acute myeloid leukemia (AML), indicating that RUNX1 is involved in the neoplasitc transformation of AML carrying this translocation (Miyoshi et al, 1991). Further studies have proven the correctness of this hypothesis, which would be explained in details in Chapter 1.2.2.

During the studies of polyomavirus in mouse embryonic cells, many observations were obtained showing the existence of pylyomavirus enhancer binding protein, which bound to the polyomavirus enhancer (Kryszke et al, 1987; Piette & Yaniv, 1987). Further analysis showed that pylyomavirus enhancer binding protein consisted of two subunits: α subunit, which bound to DNA, and β subunit, which enhanced the DNA binding ability of α subunit. Later, RUNX2 was isolated as α subunit of pylyomavirus enhancer binding protein 2 (PEBP2; also called PEA2) (Ogawa et al, 1993b). The sequence and function of β subunit of PEBP2 (now known as CBFβ) were also described during this period (Ogawa et al, 1993a; Wang et al, 1993). Shortly after RUNX2's discovery, the third member of RUNX family, *RUNX3* gene, was cloned by independent groups

(Bae et al, 1995; Levanon et al, 1994). Even since then, a new voyage had begun, searching for a better understanding about the structure as well as functions of RUNX proteins.

RUNX proteins are highly evolutionarily conserved from unicellular Capsaspora owczarzaki to mammals, indicating these proteins' importance in metazoan as well as their single-celled ancestors (Sebe-Pedros et al, 2011). There are three mammalian runt-related genes: *RUNX1*, *RUNX2* and *RUNX3*, which play distinct roles during normal development or diseases.

The RUNX family members show a high degree of structural and sequence homology within the Runt domain and VWRPY domain at the C-terminal end. The diagrammatic representation of these domains is shown in **Figure** 1.1A, and the comparison of amino acid sequences of RUNX proteins across various species is shown in **Figure** 1.1B. Such a high level of conservation reflects the importance of these domains for the proper function of RUNX family.

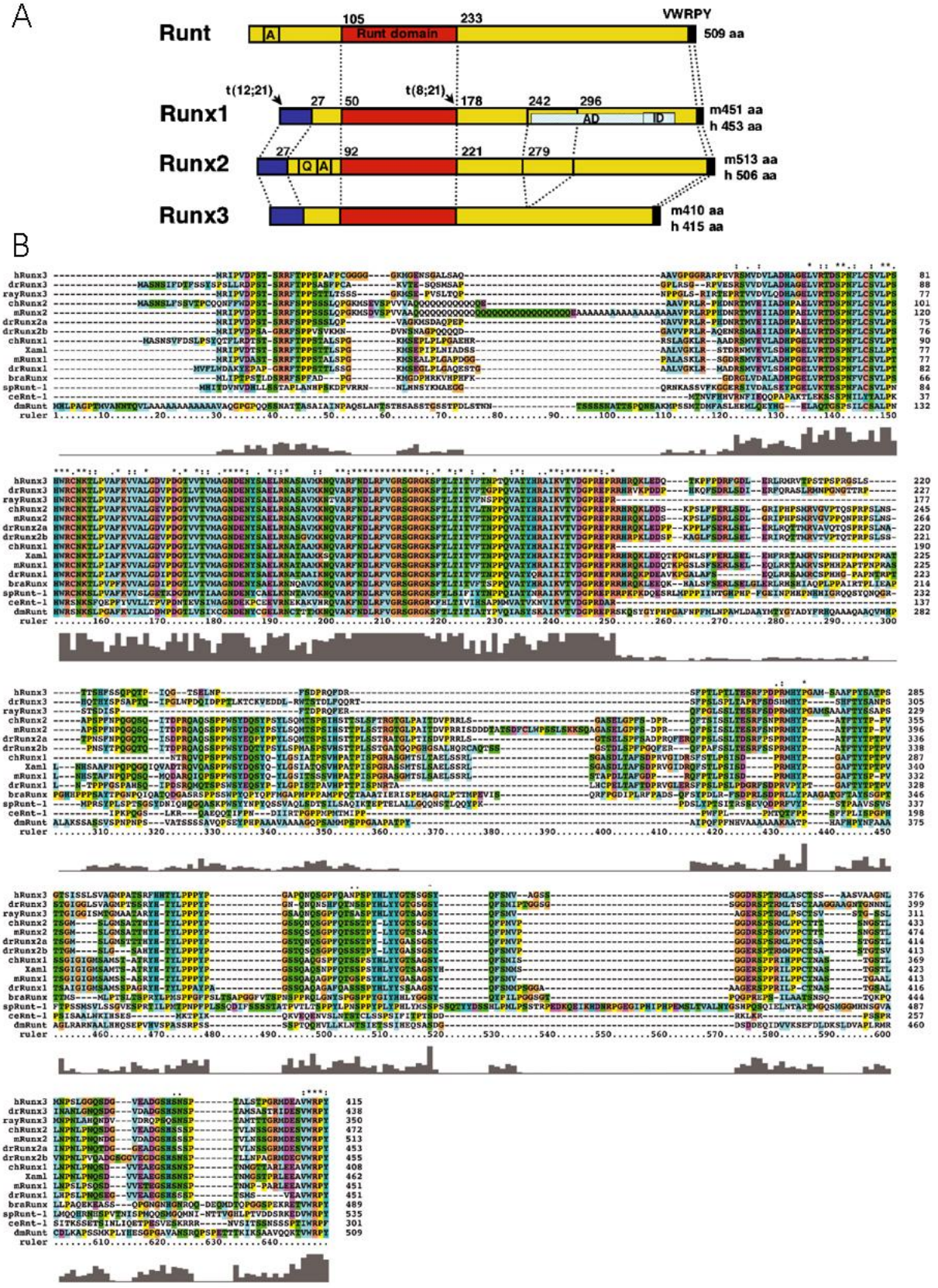


Figure 1.1 Runt domain and VWRPY domains are highly conserved in Runt-Related Transcription Factor family. (A) A diagrammatic representation of RUNX1, RUNX2 and RUNX3 together with Drosophila Runt. Conserved Runt domain (red) and VWRPY sequence (black) at the C-terminus of the proteins

are indicated. (B) Comparison of amino-acid sequences of the RUNX proteins of various species. The gray bars below sequences indicated the level of conservation of each amino acid residue. The Runt domain and VWRPY are highly conserved among species. (Ito, 2004)

The Runt domain confers sequence-specific DNA binding and dimerization with non DNA-binding partner, core-binding factor β (CBFβ), which can enhance the DNA binding affinity, compared with Runt domain alone (Kamachi et al, 1990). The three-dimensional crystal structure of the Runt domain, heterodimerized with the 134-amino acid region of CBFβ and bound to DNA, is shown in **Figure** 1.2. The consensus sequence for DNA binding of RUNX family is either 5'-ACCpuCA-3' or in the reverse orientation, 5'-TG(T/C)GGT-3'. However, the sequence 5'-ACCACA-3' appears more frequently in RUNX target promoters than other sequences which are also in agreement with the consensus (Otto et al, 2003). **Figure** 1.3 summarizes all possible RUNX binding sites (Ito, 2008). Heterodimerization of CBFβ with RUNX proteins can also prevent the ubiquitination and degradation of RUNX proteins. Due to CBFβ's essential functions, *Cbfβ* knockout mice manifest a phenotype which is very similar to that of *Runx* deficiency, showing a failure of definitive hemopoiesis, at a very early age of embryogenesis (Wang et al, 1996).

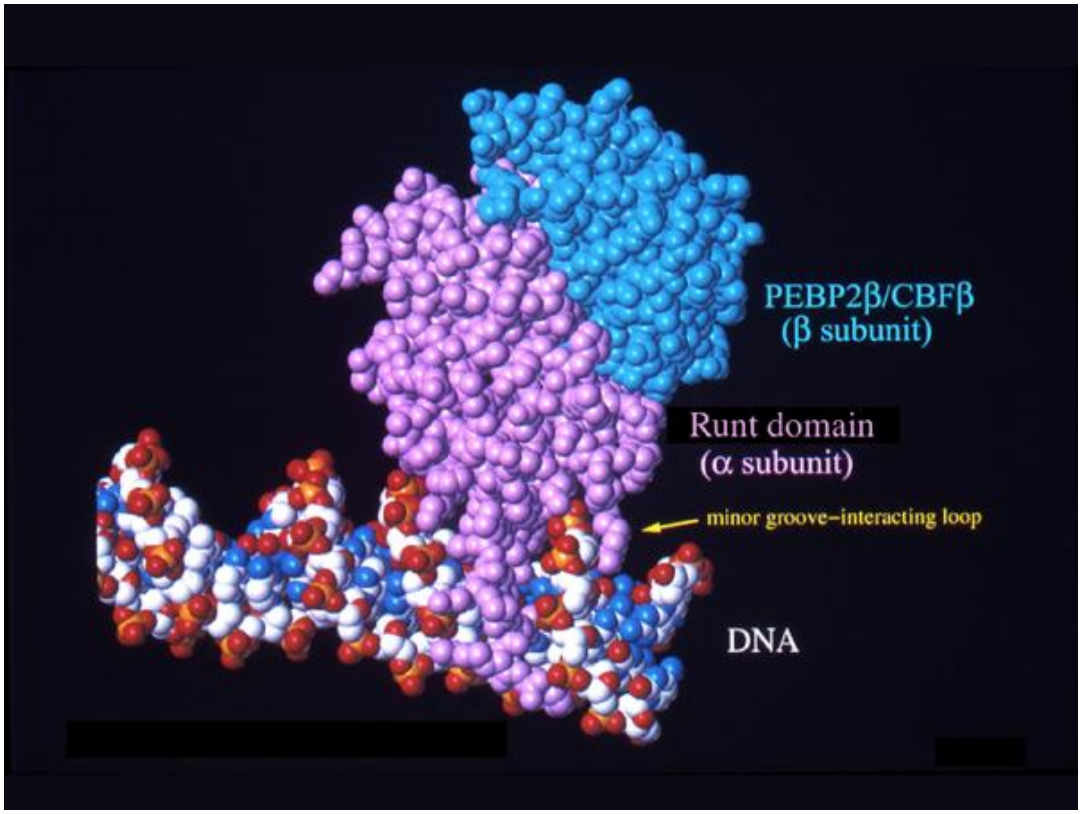


Figure 1.2 Crystal structure of the Runt domain heterdimerized with CBFβ bound to DNA. (Ito, 2004)

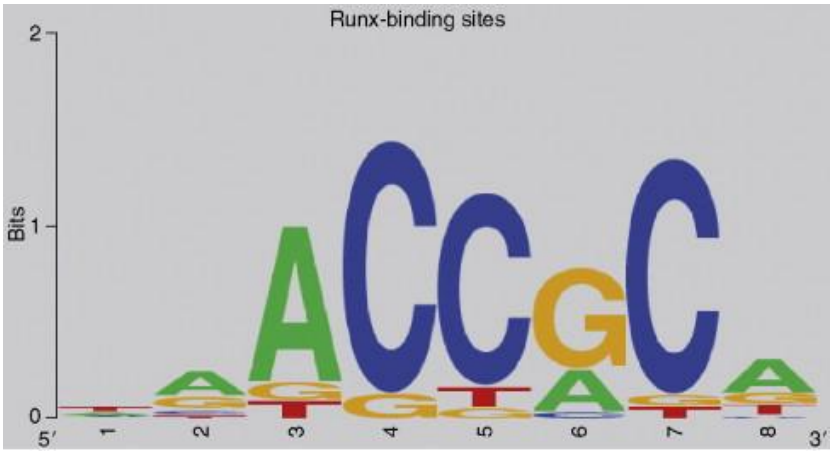


Figure 1.3 Preferred RUNX binding sequences. (Ito, 2008)

The VWRPY motif, which is located at the carboxyl-terminal end, modulates RUNX proteins' transcriptional activity by recruiting transducin-like enhancer (TLE)/Groucho co-repressor (Aronson et al, 1997). This interaction is crucial for the epigenetic silencing of CD4 by RUNX3 in $CD8^+$ T cells, and $Runx3^{VWRPY-/-}$ mice also exhibit failure in retaining spontaneous maturation of dendritic cells (Yarmus et al, 2006).

RUNX proteins are context-dependent transcription regulators, which can either activate or repress gene expression by cooperating with specific transcription factors or cofactors in specific tissue types (Wheeler et al, 2000). RUNX1's opposite roles on $p21^{Waf1/Cip1}$ promoter in different conditions serves as a typical example to illustrate this point. Specifically, RUNX1 can interact with Sin3A co-repressor at residues 208–237, repressing $p21^{Waf1/Cip1}$ promoter in NIH3T3 cells (Lutterbach et al, 2000). Meanwhile, RUNX1 is also able to form a complex with p53 upon adriamycin (ADR) exposure, and stimulates the transcription of p53 target genes such as $p21^{Waf1/Cip1}$, *BAX*, *NOXA*, and *PUMA* in HCT116 and U2OS cells (Wu et al, 2013).

RUNX proteins function as transcriptional activators in multiple ways. For example, RUNX1 can cooperate with Myb, C/EBPα, P300/CBP and P/CAF, forming a multimeric complex and activating *MPO* (Myeloperoxidase) gene expression though the recruitment of the basal transcription factors and chromatin acetylation during myeloid cell differentiation (Kitabayashi et al, 1998). Moreover, RUNX1 and Ets-1 can mutually stimulate each other's DNA binding activity and transactivation function by masking the autoinhibitory domain of each other through protein-protein interaction, and cooperate

synergically for the transactivation of the T cell receptor (TCR) beta chain enhancer (Kim et al, 1999b).

On the other hand, RUNX proteins also function as transcriptional repressors in multiple ways. For example, in mutant *Runx1*$^{\Delta V/\Delta V}$*; Runx3*$^{\Delta V/\Delta V}$ mice in which the VWRPY motifs are removed from both the Runx1 and Runx3 proteins, Full CD4 de-repression of mature $CD8^+$ T cells is observed, while ThPOK (T-Helper-Inducing POZ/Krueppel-Like Factor) is only partially de-repressed, indicating that RUNX proteins suppress CD4 expression in a VWRPY dependent manner, while they suppress ThPOK expression in both VWRPY dependent and independent manners (Seo et al, 2012). Till now, several VWRPY independent mechanisms of gene repression have been discovered. Firstly, RUNX1 can recruit Sin3A co-repressor to repress $p21^{Waf1/Cip1}$ promoter in NIH3T3 cells (Lutterbach et al, 2000). Secondly, RUNX1 and RUNX3 can associate with SUV39H1, a histone methyltransferase involved in gene silencing (Durst & Hiebert, 2004).

Since RUNX proteins have complicated roles as versatile gene expression regulators in the determination of cell fate during development, their deregulation often leads to serious disease like cancer (Blyth et al, 2005). **Table** 1.1 is a summary about each RUNX protein's essential functions, knockout mice's phenotype and roles in tumorigenesis, which would be further explained in later chapters.

Gene	Proposed essential function	Mouse (−/−) phenotype	Tumorigenesis
RUNX1	Definitive hematopoiesis	Embryonic lethal. Absence of definitive hematopoiesis.	Hemizygocity in humans predisposes to Familial platelet disorder with predisposition to acute myelogenous leukemia (FPD/AML). Frequent target of chromosome translocation and mutations in acute myeloid leukemia. Mutation hotspot in breast cancer.
RUNX2	Osteoblast differentiation Skeletal morphogenesis	Dies at birth from respiratory failure.	Transgenic Runx2 overexpression predisposes to T-cell lymphomas. Elevated expression in osteosarcoma tumours. Expressed by metastatic cancer cells to promote their homing to the bone.
RUNX3	Cell lineage decisions in thymopoiesis. Regulation of neurotrophin receptors' expression. TGF-beta-mediated dendritic cell function and tumour suppressor pathway. Development of gastrointestinal tract, lung epithelial cells.	Dies soon after birth. Severe motor discoordination caused by axon extension defects. Hyperplastic gastric epithelium. Neoplasm in lung and mammary gland.	Frequently inactivated in human gastric cancer, colon cancer, lung cancer and breast cancer by promoter methylation, Loss of heterozygosity or mis-localization. Possess an oncogenic role in ovarian cancer as well as head and neck cancer.

Table 1.1 Functions, knockout mice's phenotype and roles in oncogenesis of Runt-related transcription factors. [Modified from (Lund & van Lohuizen, 2002)].

1.2.2 RUNX1 and RUNX3's roles in hematopoiesis and leukemia

As indicated in **Table** 1.1, both RUNX1 and RUNX3 play a role in cell differentiation during hematopoiesis, which would be further explained in this section.

RUNX1 is the master regulator of definitive hematopoiesis in mammals (Okuda et al, 1996). Homozygous deletion of *Runx1* would result in a complete lack of definitive hematopoiesis, indicating that RUNX1 is indispensable for definitive hematopoiesis (Okuda et al, 1996; Wang et al, 1996). Further research suggested that RUNX1 is critical for the emergence of the first hematopoietic stem cells (HSCs) from hemogenic endothelium in the embryonic aorta-gonad-mesonephros (AGM) region (North et al, 1999; Yokomizo et al, 2001). Conditional knockout of *Runx1* in adult mice would lead to hematopoietic stem cell exhaustion after an initial short term expansion of HSCs with limited self-renewal capacity, possibly due to the compromised HSC-niche interactions (Ichikawa et al, 2004; Jacob et al, 2010). HSC with long term self-renewal capacity would exit quiescence in responding to disrupted HSC-niche associations, and become short term HSCs, causing the stem cell exhaustion (Wang et al, 2010). These observations show the importance of RUNX1 for maintaining HSCs in quiescence through a niche-related mechanism, whose deficiency triggers a pre-leukemic state by increasing the number of short term HSCs as a cell pool for further oncogenic alterations (Growney et al, 2005; Putz et al, 2006).

Apart from its role in hematopoietic stem cells, RUNX1 is also essential for the differentiation of T and B cells in adult hematopoiesis (Ichikawa et al, 2004).

RUNX3 is also involved in this process, playing both redundant and non-redundant roles with RUNX1. During the development of T lymphocytes, First, RUNX1 is required for the repression of CD4 in $CD4^-CD8^-$ double negative immature thymocytes through direct binding to two RUNX-binding motifs in the CD4 silencer (Taniuchi et al, 2002). Then in $CD4^-CD8^+$ thymocytes, RUNX3 also suppresses CD4 expression in a similar manner (Grueter et al, 2005). During the development of B cells, Runx1 is essential at two stages: (1) to promote survival and development of progenitors specified to the B-cell lineage; (2) to enable the developmental transition through the pre-B stage triggered by the pre-B-cell antigen receptor (pre-BCR) (Niebuhr et al, 2013). RUNX3 has a role in B-cell development at a later stage than RUNX1 (de Bruijn & Speck, 2004). RUNX3 mediates TGFβ activation of the germline Igα promoter in advance of IgA class switching in cooperation with the Smad family of transcription factors (Shi & Stavnezer, 1998). Furthermore, in naive B cells, RUNX3 suppresses the expression of RUNX1 after activation or Epstein–Barr virus (EBV) infection (Spender et al, 2005), by binding specifically to conserved RUNX binding sites in *RUNX1*'s P1 promoter in a VWRPY dependent manner (Brady et al, 2009).

Apparently, RUNX1 is involved in multiple levels of adult hematopoiesis from the maintenance of quiescent HSCs to defining proper differentiation programs towards a full range of hematopoietic lineages. Given such pivotal roles in mammalian hematopoiesis, it is not surprising to note that RUNX1 is one of the most frequently disrupted genes in human leukemias (Look, 1997). Loss of RUNX1's function due to chromosomal translocations or point mutations is common in various types of leukemias such as acute myelogenous

leukemia, myelodysplastic syndrome, chronic myelogenous leukemia and childhood acute lymphoblastic leukemia (Golub et al, 1995; Mitani et al, 1994; Nucifora et al, 1993).

RUNX1-ETO caused by t(8;21) translocation fuses the DNA-binding domain of RUNX1 to the transcriptional co-repressor ETO protein (Gao et al, 1991). It is one of the most common molecular abnormalities in acute myelogenous leukaemia (AML), with a frequency of approximately 12% (Peterson & Zhang, 2004). AML1-ETO negatively regulates AML1 target genes including *IL-3*, *c-fos*, *TCRβ*, immunoglobulin α, and $p14^{ARF}$ (Frank et al, 1995; Linggi et al, 2002; Meyers et al, 1995). RUNX1-ETO can also activate the expression of genes including M-CSF receptor, *BCL2* and G-CSF receptor (Klampfer et al, 1996; Petrovick et al, 1998; Shimizu et al, 2000). The effects of this fusion protein are highly context-dependent, depending on the RUNX1 DNA binding site in the regulatory element of its target gene or the presence of other transcription regulators interacting with it (Peterson & Zhang, 2004). However, this t(8;21) translocation is a leukemia-initiating event, and fusion gene sequences can be found long before the onset of leukaemia in the blood from newborn children (Wiemels et al, 2002). The requirement of secondary genetic alterations for the full induction of developed AML in t(8;21) patients reflects the complexity of pathogenic mechanisms of AML (Yuan et al, 2001).

Another fusion protein TEL-RUNX1 (also known as ETV6-RUNX1) is observed in 20% of pediatric B-cell acute lymphoblastic leukaemia's (ALL). It is caused by t(12;21) translocation, juxtaposing the *RUNX1* gene on chromosome 21 with the *TEL/ETV6* gene on chromosome 12 (Shurtleff et al,

1995). TEL-AML1 increases the self-renewal capacity of myeloid progenitors and impairs differentiation of the B-cell compartment, resulting in the accumulation of both multipotent and B-cell progenitors (Fischer et al, 2005). TEL-RUNX1 fusion protein represses RUNX1 targets like Complement Component (3b/4b) Receptor 1 (*CR1*) as well as genes which are not usually regulated by RUNX1 (Kim et al, 1999a). Several mechanisms have been proposed, including the recruitment of nuclear hormone co-repressor (N-Cor) and histone deacetylase (mSin3A), heterodimerization with TEL or sequestering p300 into the cytoplasm (Lee et al, 2004). By multiple means, TEL-RUNX1 interferes with RUNX1's key regulatory functions and globally re-shapes the biological process of lymphocytes (Fischer et al, 2005).

Taken together, RUNX1 is a key regulator of both embryonic and adult hematopoiesis where its disruption is strongly linked to leukemogenesis, while RUNX3 also plays a part in the cell lineage decisions in thymopoiesis.

1.2.3 RUNX2 in skeleton development and cancer metastasis

RUNX2 plays critical functions for the bone formation in mammals (Chen et al, 2012). *Runx2*$^{-/-}$ mice show impaired osteoblasts maturation and osteogenesis, causing a complete lack of bone formation, so these mice would die soon after birth due to severe respiratory defects possibly caused by the absence of a proper rib cage (Komori et al, 1997; Otto et al, 1997).

RUNX2 regulates the expression of some bone-specific genes such as osteocalcin and alkaline phosphatise during osteoblast differentiation from

mesenchymal precursor cells (Ducy et al, 1999). *Runx2*$^{+/-}$ mice display skeletal abnormalities resembling that of the human congenital skeletal disorder called cleidocranial dysplasia, and mutations of *RUNX2* have been identified in patients suffering from this disease (Tessa et al, 2003; Xuan et al, 2008).

RUNX2 is also able to promote the metastasis and bone-homing of tumour cells in breast and prostate tumours (Akech et al, 2010; Das et al, 2009; Javed et al, 2005; Lim et al, 2010; Pratap et al, 2008). RUNX2 can induce epithelial-mesenchymal transition (EMT) by sitimulating SNAI2 expression in a Wnt and transforming growth factor β (TGFβ) dependent manner (Chimge et al, 2011). Reduction of RUNX2 by RNAi in MDA-MB-231 breast cancer cell line significantly reduces cell motility, with only marginal effects on cell growth (Leong et al, 2010). A transcriptome profiling in prostate cancer cells with induced RUNX2 expression showed that RUNX2 up-regulated the expression of *SDF-1* (The stromal cell-derived factor 1), *CXCR7* (C-X-C chemokine receptor type 7) and *BSP* (Bone sialoprotein), which promoted homing and attachment to bone (Baniwal et al, 2010). At the same time, RUNX2 up-regulated osteoclast activators like *CSF2* (Colony Stimulating Factor 2) and *SPHK1* (Sphingosine Kinase 1), to alter the bone microenvironment in response to prostate cancer metastasis to further fuel the growth tumour cells (Baniwal et al, 2010).

Our increasing knowledge about RUNX2's role in bone formation as well as cancer metastasis reflects the versatility of RUNX proteins in various biological processes.

1.2.4 RUNX3' role in the development of dorsal root ganglion neurons

RUNX3 is highly expressed in the dorsal root ganglion neurons (DRG), spleen and thymus. Its expression is relatively lower in epithelia in various organs likes lung, liver and gastrointestinal tract (Inoue et al, 2007; Ito et al, 2009; Ito et al, 2008; Levanon et al, 2002; Li et al, 2002; Taniuchi et al, 2002; Woolf et al, 2003).

In the developing DRGs of mice embryo, Runx3 is first detected at E10.5 in numerous neurons, and it is co-expressed with the proprioceptive markers including tyrosine kinase receptor C (TrKC) and parvalbumin (PV). *Runx3*-deficient mice develop severe limb ataxia and abnormal posture. Different views still exist about the cause of this phenotype. Inoue, *et al* showed that *Runx3*$^{-/-}$ mice maintained normal number of TrkC positive DRG proprioceptive neurons throughout development, but these DRG neurons failed to form target-specific axon pathfinding in the spinal cord (Inoue et al, 2003). However, Levanon, *et al* suggested that the number of DRG proprioceptive neurons decreased and they could not survive long enough to extend their axons toward target cells in the absence of Runx3, resulting in a lack of connectivity and ataxia (Levanon et al, 2002).

Apart from the different opinions about RUNX3's role in the survival of DRG proprioceptive neurons, a common conclusion is that RUNX3 has a critical role in the proper development of dorsal root ganglion neurons.

1.2.5 RUNX3's role in cancer development

Except in the embryonic dorsal root ganglion neurons, RUNX3's expression in other epithelia is generally lower than that in spleen and thymus. But RUNX3 is still playing an irreplaceable role in the epithelia of various tissues.

Runx3$^{-/-}$ mice displayed hyperplasia of epithelium in gastrointestinal tract, mammary glands and lung. Further analysis performed in newborn mouse stomach revealed that the glandular stomach displayed excessive cell proliferation and inhibition of apoptosis induced by TGF-β in the epithelial cells, suggesting the importance of Runx3 in the TGF- β signalling (Li et al, 2002). A small number of these mice can survive to adulthood, and these mice exhibit a significant elevation of Trefoil Factor 2 (TFF2) level in mucin 6 (Muc6)-expressing cells, coupled with a lack of chief cells, presenting a remarkably similar phenotype to SPEM (Spasmolytic polypeptide-expressing metaplasia), a precancerous state of the stomach. At the same time, the *Runx3*$^{-/-}$ gastric epithelium exhibits an intestinal phenotype, marked by the expression of intestine-specific transcription factor Cdx2, whose expression is triggered by β-catenin/TCF, which is hyperactivated due to loss of Runx3 (Ito et al, 2011).

About 20% of *Runx3*$^{+/-}$ female mice develop mammary tumours at the age of 15 months. The expression of estrogen receptor α (ERα) is significantly enhanced in these tumours. Further studies revealed that RUNX3 could vigorously reduce the transcriptional activity of ERα by inducing the proteasome-mediated degradation of ERα (Huang et al, 2012). This mechanism suggests that RUNX3 functions as a "gate-keeper" to prevent the onset of breast

cancer by controlling the mammary epithelial cells' response to circulating estrogens through restricting the cellular level of ERα (Chen, 2012).

Runx3$^{-/-}$ mouse embryos begin to develop lung hyperplasia at E17.5. The proliferation marker Ki67 is more abundant in the *Runx3*$^{-/-}$ lungs (84%) than in wild-type lungs (53%), possibly because Bim expression is reduced when Runx3 is deficient. 85% of *Runx3*$^{+/-}$ mice develop spontaneous lung adenomas at 18 months of age, while only 3% develop lung adenocarcinomas (Lee et al, 2010). *K-Ras* mutation might be associated with the progression from adenoma to malignant carcinoma. In another study using targeted inactivation of *Runx3* in mouse lung, oncogenic *K-Ras* can markedly shorten the latency of adenocarcinoma formation caused by loss of *Runx3*, due to the impairment of oncogenic *K-Ras* induced p14^{ARF}-p53 pathway when Runx3 is knocked out (Lee et al, 2013).

The tumour suppressor activities of Runx3 observed during the analysis of *Runx3* knockout mice are further supported by human clinical data. In human, silencing of *RUNX3* is observed in about 80% of primary gastric cancer specimen as well as gastric cancer cell lines, due to promoter hypermethylation, hemizygous deletions or protein mislocalization into cytoplasm (Ito et al, 2008; Ito et al, 2005). Down-regulation of RUNX3 could be frequently observed even from the precancerous intestinal metaplasia (IM) in stomach, which would provide favourable conditions for the progression from adenomas to malignant adenocarcinomas (Li et al, 2002).

Loss of RUNX3 function is observed in nearly all human breast cancer samples, due to promoter hypermethylation and mislocalization (Lau et al, 2006;

Subramaniam et al, 2009). This event takes place from the ductal carcinoma in situ (DCIS) stage, supporting the theory that RUNX3 functions as a "gate-keeper" to prevent the onset of breast cancer by controlling the mammary epithelial cells' response to circulating estrogens through restricting the cellular level of ERα (Chen, 2012).

In human lung cancer, inactivation of RUNX3 is observed in 70% of the adenocarcinoma (ADC) samples, while the frequency is only 20% in squamous cell carcinoma (SCC) samples, indicating that RUNX3 might have different roles in ADC and SCC (Omar et al, 2012).

Taken together, data from both mice and human strongly argue that RUNX3, whose disruption appears to be a key event in early gastrointestinal carcinogenesis, breast cancer and lung cancer, functions as a tumour suppressor in various kinds of solid tumours. But there are still some exceptions. RUNX3 exhibits oncogenic activity in ovarian cancer as well as head and neck cancer, whose mechanism is not thoroughly understood yet (Kudo et al, 2011; Lee et al, 2011a).

1.3 RUNX3's involvement in major signalling pathways

As it is shown in Chapter 1.2.5, conclusive evidence generated in clinical specimen as well as mouse model shows that RUNX3 functions as a tumour suppressor in various kinds of cancers. Till now, an enormous amount of research effort has been devoted into the study about the mechanisms how RUNX3 achieves such an important function.

Great progress has been made in understanding RUNX3's versatile roles in several major signalling pathways, including TGF-β/SMAD signalling pathway, Wnt pathway way, K-RAS-p14^{ARF}-p53 pathway, which would be further explained in details in this chapter.

1.3.1 RUNX3 in the TGF-β/SMAD signalling pathway

Transforming growth factor-β (TGF-β) is a family of multifunctional cytokines that regulate cell growth, differentiation, apoptosis, matrix accumulation and motility (Blobe et al, 2000; Derynck & Feng, 1997; Massague, 2012). It is a potent cell growth inhibitor for hematopoietic cells, endothelial cells and epithelial cells, while it stimulates the growth of mesenchymal cell (Derynck et al, 2001). TGF-β superfamily mainly consists of TGF-βs, activins and bone morphogenetic proteins (BMPs). Binding of these TGF-β ligands causes the formation of type I or type II receptor heterodimeric complex, leading to the activation of downstream effectors of the SMAD family. SMAD2 and SMAD3 belong to R-SMADs transducing the TGF-β/activin-like signals while SMAD1, SMAD5 and SMAD8 belong to R-SMADs mediating BMP-like signals (Miyazono et al, 2004). These receptor-activated SMADs (R-SMADs) get phosphorylated and associate with SMAD4 (Co-SMAD) and translocate to

cell nucleus to regulate the transcription of target genes together with other transcription factors (Feng & Derynck, 2005; Massague et al, 2005).

TGF-β family members have both tumour suppressive and oncogenic activities (Massague, 2012). According to the current paradigm, the tumour suppressive functions of TGF-β dominate in normal tissues or in early stages of cancer. However, as the cancer advances, changes of TGF-β expression or cellular responses tip the balance in favour of its oncogenic activities, which is supported by the observation that activated TGF-β signalling promotes cancer progression and metastasis via epithelial-mesenchymal transition (EMT), angiogenesis and immune-suppression (Derynck et al, 2001; Wakefield & Roberts, 2002).

All three RUNX proteins have been shown to physically interact with R-SMADs (Hanai et al, 1999). As we have mentioned in chapter 1.2.2, RUNX3 plays an important role in B cell maturation by interacting with SMAD2 and SMAD3 to cooperatively activate *germline immunoglobulin constant α* (*IgCα*) promoter to direct class switching to IgA in B lymphocytes (Hanai et al, 1999; Pardali et al, 2000). In the gastrointestinal tract, where TGF-β pathway mainly functions as a tumour suppressor pathway, RUNX3 cooperates with R-SMADs to activate the transcription of the negative regulators of cell cycle, $p21^{WAF/Cip1}$ and proapopototic gene, *BIM* (Chi et al, 2005; Ito, 2008; Mishra et al, 2005; Yano et al, 2006). Consistent with this, $Runx3^{-/-}$, $p53^{-/-}$ GIF cells are highly resistant to TGF-β1 induced growth arrest and apoptosis and they also exhibit spontaneous epithelial-mesenchymal transition (EMT) due to dysregulation of the TGF-β pathway (Li et al, 2002; Voon et al, 2012). In the skeletal system,

where RUNX2 is the dominant RUNX protein, impaired RUNX2-SMAD interaction due to *RUNX2* mutation may contribute the cleidocranial dysplasia, since the physical interaction between RUNX2 and BMP-specific SMAD1 or SMAD5 synergistically activates osteoblast-specific gene expression in pluripotent mesenchymal precursor cells (Zhang et al, 2000).

Based on these evidences, RUNX proteins can form complexes with specific R-SMADs to control transcription in a context dependent manner, functioning as nuclear effectors of TGF-β signalling pathway. Modulation of the TGF-β pathway partially contributes to the tumour suppressor functions of RUNX3.

1.3.2 RUNX3 attenuates the oncogenic Wnt signalling pathway

The canonical Wnt pathway is critical for cell fate determination during embryogenesis and it orchestrates self-renewal in various tissues (Clevers, 2006). Wnt signalling would functionally deactivate glycogen synthase kinase-β (GSK3β) and promote the stabilisation of cytoplasmic β-catenin, resulting in the translocating of unphosphorylated β-catenin into cell nucleus to stimulate the transcription of Wnt target genes by interacting with T-cell factor (TCF) or lymphoid enhancer-binding factor (LEF) (Bienz & Clevers, 2000). In *Tcf-4*$^{-/-}$ mice, the proliferative stem cell compartment is entirely absent in small intestines, suggesting that Wnt/TCF4/β-catenin pathway is necessary for the maintenance of crypt stem/progenitor cells in intestinal epithelium (Korinek et al, 1998). Further study revealed that Wnt target gene, leucine-rich repeat-containing G-protein coupled receptor 5 (*Lgr5*) could mark the crypt base

columnar (CBC) cells, which could give rise to all lineages of intestinal epithelium and maintain the epithelia self-renewal over a long period of time (Barker et al, 2007).

The dysregulation of Wnt signalling is closely correlated with gastrointestinal cancers (Clevers, 2006). Inactivation of adenomatous polyposis coli (APC) complex or oncogenic mutations of β-catenin would lead to aberrant stabilisation and nuclear accumulation of β-catenin, finally causing cancer (Bienz & Clevers, 2000; Kinzler & Vogelstein, 1996). For example, in $Apc^{min/+}$ transgenic mice, which carry a heterozygous mutation of *Apc* tumour suppressor gene, spontaneous intestinal adenomas are repeatedly observed (Moser et al, 1990).

RUNX3 is able to antagonize Wnt signalling through a direct interaction with TCF4/β-catenin complex. This ternary complex has a significantly impaired DNA binding ability, resulting in a reduction of TCF4/β-catenin's transcriptional activity. Supportive to this mechanism, target genes of β-catenin/Tcf4, like *CD44*, cyclin D1, *c-Myc*, conduction and *EphB2*, are all up-regulated in intestinal epithelial cells of $Runx3^{-/-}$ mice, in which small adenomas develop at a frequency comparable to that of $Apc^{Min/+}$ mice in small intestine at around 65 weeks of age. In human colorectal cancer specimen, inactivation of RUNX3, either by loss of expression or mislocalization, is frequently concomitant with accumulation of β-Catenin, which would lead to ligand-independent and unconstrained Wnt pathway signalling that favours the progression of tumours (Ito et al, 2008).

Similar observations can be obtained in stomach. β-catenin/TCFs' occupancy on *Cdx2* promoter and *c-Myc* promoter is significantly higher in gastric epithelial cells when *Runx3* is knocked out, causing an intestinal phenotype as well as an increased proliferation capacity in *Runx3*$^{-/-}$ gastric epithelium. R122C mutation within the Runt domain of RUNX3 is previously identified in a gastric cancer patient (Li et al, 2002). This $RUNX3_{R122C}$ mutant, which exhibits weaker binding with β-catenin/TCF than wild-type RUNX3, can not effectively reduce Cdx2 and c-Myc expression, indicating that hyper-activated Wnt signalling, due to mutation of RUNX3, might contribute to the progression of tumour for this patient (Ito et al, 2011).

The research performed in both intestine as well as stomach highlights the importance of RUNX3 as a negative regulator for oncogenic Wnt pathway, which partially explains the strong tumour suppressor activity of RUNX3 we observed in clinical specimen as well as in mouse models.

1.3.3 RUNX3 mediates K-Ras^{G12D} dependent ARF-p53 activation

p53 is the tumor suppressor which is most frequently mutated in human cancers (Hollstein et al, 1991). Wild-type p53 functions as a tetrameric transcription factor that regulates cell growth, inhibiting the cell cycle in some circumstances and promoting apoptosis in others, through the activation or repression of key target genes, such as *CDKN1A*, *CDC25C* and *BAX* (Lohr et al, 2003; Miyashita & Reed, 1995; Riley et al, 2008; St Clair et al, 2004). It has also been suggested that p53 promotes apoptosis and inhibits autophagy in the cytoplasm through mechanisms that do not involve transcription (Green &

Kroemer, 2009). Generally speaking, p53 is highly versatile, functioning in various cellular process including senescence, metabolism, autophagy, angiogenesis and DNA repair, and this explains why p53 is frequently mutated in human cancer (Dameron et al, 1994; Nigro et al, 1989; Serrano et al, 1997; Smith et al, 1995; Tasdemir et al, 2008; Vousden & Ryan, 2009).

It is a common observation that excessive expression of genes that normally induce mitogenic signals, such as *MYC* or *RAS*, can activate $p14^{ARF}$, which stabilizes p53 (Palmero et al, 1998). The stabilized p53 would trigger the expression of $p21^{WAF/CIP}$ and proapoptotic genes, resulting in cell cycle arrest, premature senescence and apoptosis. This process is sometimes referred as ARF-p53-p21 pathway, which is an important cell defense against oncogenic transformation (Serrano et al, 1997). Experiments conducted in transgenic mice carrying murine $K\text{-}Ras4b^{G12D}$ showed that deficiency in either the *p53* gene or the *Ink4A/Arf* locus would accelerate the formation of tumours which exhibited more malignant features (Fisher et al, 2001). However, the mechanism how Ras activates ARF has not be fully elucidated.

Recent study in lung adenocarcinoma (ADC) revealed that the expression of RUNX3 has a strong protective effect against $K\text{-}Ras^{G12D}$ (Lee et al, 2013). Expression of $K\text{-}Ras^{G12D}$ would cause the development of nonmucinous lung adenoma (AD) and adenocarcinoma (ADC) in mice, but the combination of *Runx3* inactivation and $K\text{-}Ras^{G12D}$ activation leads to dramatically more serious lung adenocarcinoma of both mucinous and non-mucinous types, indicating that the combination of *Runx3* inactivation and $K\text{-}Ras^{G12D}$ activation is critical for the progression from adenoma to adenocarcinoma. Consistent with the mouse

model, in human lung adenocarcinoma samples, the rate of RUNX3 inactivation, either due to reduced expression or mislocalization, is much higher in samples carrying K-Ras or EGFR mutations, compared with the rate in samples with wild-type K-Ras and EGFR (Lee et al, 2013).

Further studies carried out in human cell lines cultured *in vitro* revealed that acetylated RUNX3 physically interacts with Bromodomain-Containing Protein 2 (BRD2), a transcriptional regulator mediating chromatin remodeling. The RUNX3-BRD2 complex could trigger the persistent expression of ARF and $p21^{WAF/CIP}$, and this interaction is stabilized by the expression of $K\text{-}Ras^{G12D}$, which exactly explains how oncogenic K-Ras triggers the ARF-p53-p21 pathway (Lee et al, 2013).

This novel discovery about RUNX3's fundamental role in the initiation of oncogene induced senescence supports the long-term observation that RUNX3 is frequently silenced in various kinds of cancer. RUNX3 mediates the first line protection against oncogenic transformation, whose malfunction paves the way for further disease progression (Lee et al, 2013).

1.4 Introduction of TEAD-YAP complex

1.4.1 The structure of TEAD-YAP complex

TEA domain (TEAD) proteins are a family of transcription factors sharing a common the TEA DNA binding domain, which recognizes and binds to a specific sequence (5'-CATTCC-3') (Xiao et al, 1991). Mammals express four TEAD proteins, named TEAD1, TEAD2, TEAD3 and TEAD4, which are highly conserved (Yasunami et al, 1996; Yockey et al, 1996). At least one TEAD gene is expressed in most adult tissues, while in some tissues, such as lung, all four TEAD proteins are abundantly expressed. Even though TEAD proteins are highly conserved in TEA domain, each TEAD protein exhibits different expression patterns with respect to tissue and developmental stage specificity, suggesting that each protein has a unique function (Jacquemin et al, 1998; Kaneko & DePamphilis, 1998; Yasunami et al, 1996).

The full transcriptional activity of TEAD proteins requires the binding to other transcriptional coactivators, due to the lack of intrinsic transcription activation domain (Jiang & Eberhardt, 1996; Xiao et al, 1991). Among all the possible transcriptional coactivators which interact with TEAD proteins, the most putative and general coactivator which can trigger the transcriptional activity of all four TEAD proteins, is identified as Yes-associated-protein (YAP), a protein whose carboxyl-terminus contains an acidic transcriptional activation domain (Vassilev et al, 2001; Yagi et al, 1999; Zhao et al, 2008). The three-dimensional structure of the hYAP (residues 50–171)–hTEAD1 (residues 194–411) and mYAP (residues 39-92)-mTEAD4 (residues 210-427) complexes have been solved (Chen et al, 2010; Li et al, 2010). According to the protein

crystallography data shown in **Figure** 1.4, the amino terminal of hYAP wraps around the globular structure of hTEAD1's carboxyl terminal via three highly conserved interfaces, among which the interface 3, including hYAP residues 86–100, is most critical for complex formation (Li et al, 2010).

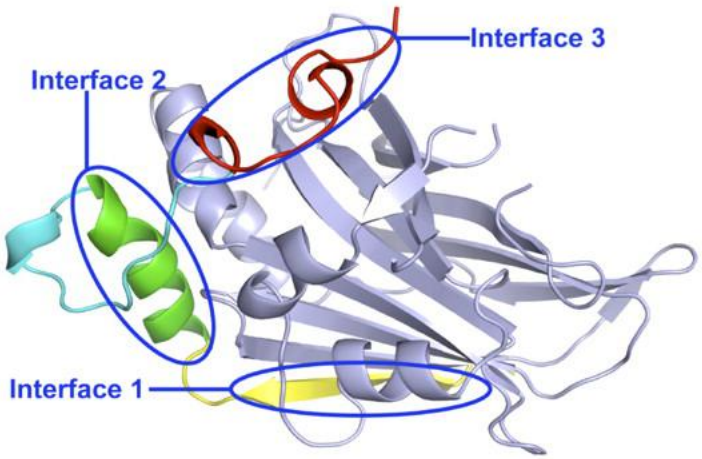


Figure 1.4 Overall interaction between YAP and TEAD shown as a ribbon representation. TEAD is shown in light blue, and different YAP elements are shown in yellow, green, cyan, and red. Secondary structural elements are labelled. (Li et al, 2010)

1.4.2 TEAD-YAP complex and Hippo pathway

TEAD proteins are mainly concentrated in cell nucleus, while YAP is able to shuttle between cytoplasm and nucleus, and the activity of TEAD is limited by the level of nuclear YAP, which is under the precise control of Hippo pathway as well as other pathways (Huang et al, 2005; Mohler et al, 1999; Zhao et al, 2007).

In mammals, the core components of Hippo pathway consists of Mammalian STE20-Like Protein Kinase 1/2 (Mst1/2), Salvador Homolog 1 (Sav1), large tumour suppressor kinase 1/2 (Lats1/2) and MOB kinase activator 1 (Mob1) (Chow et al, 2010; Hao et al, 2008; Lee et al, 2008; Praskova et al, 2008; Zhou et al, 2009b). Mst1/2-Sav1 complex phosphorylates Lats1/2-Mob1 complex, and the phosphorylated Lats1/2-Mob1 complex would become active and phosphorylates YAP at multiple sites, including Serine^{127} and Serine^{381} (Zhao et al, 2010a). The phosphorylated YAP^{S127} would be recognized by the 14-3-3 proteins, and get sequestered in cytoplasm (Zhao et al, 2007). The phosphorylated YAP^{S381} would recruit CK1δ/ε to initiate the ubiquitination of YAP by SCF$^{\beta\text{-TRCP}}$ E3 ubiquitin ligase, resulting in the proteasomal degradation of ubiquitinated YAP (Zhao et al, 2010b). Hippo pathway is highly potent in the controlling of nuclear YAP level since it regulates YAP in both spatial and temporal manners (Zhao et al, 2010a).

The core components of Hippo pathway are conserved from *Drosophila* to Mammals. MST's *drosophila* homologue is Hpo. SAV's *drosophila* homologue is still called as Sav. LATS's *drosophila* homologue is Wts/Lats. *Drosophila* Yorkie is homologous to mammaliam YAP (Yes-Association-Protein) (Dong et

al, 2007). Hippo pathways in both *Drosophila* and Mammals are summarized in **Figure** 1.5.

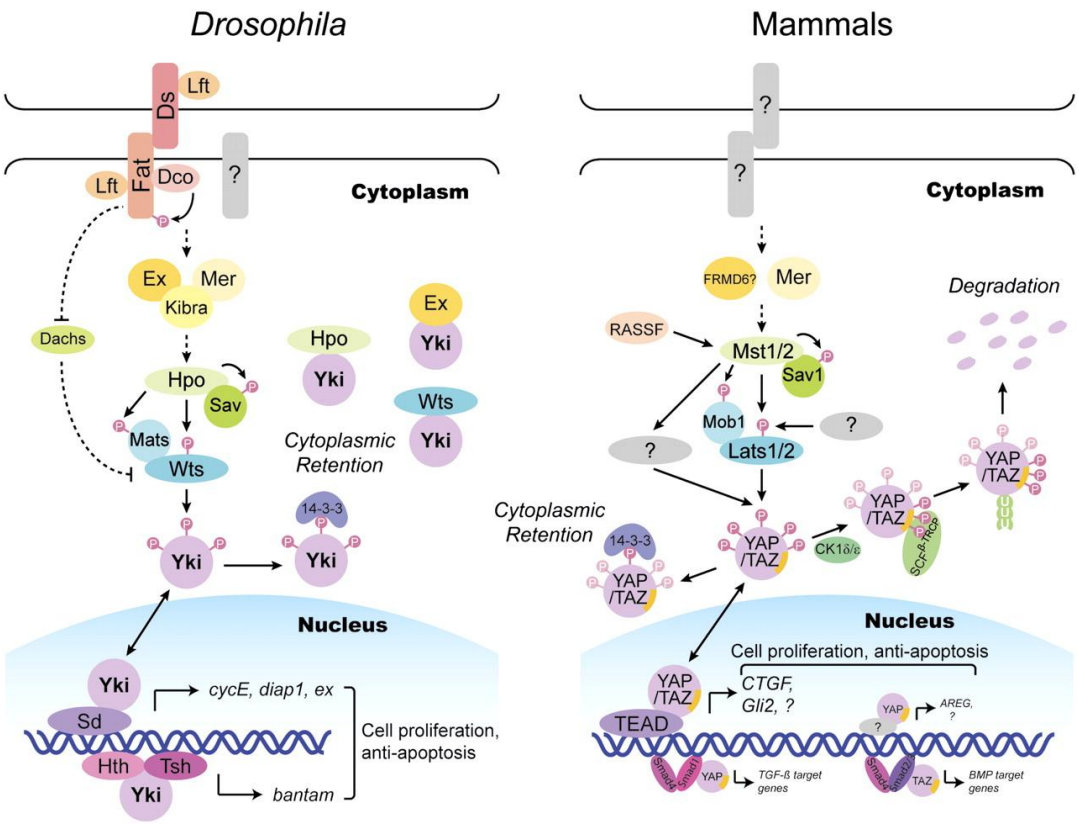


Figure 1.5 Models of the Hippo pathway in *Drosophila* and mammals. (Zhao et al, 2010a)

Various upstream signals can regulate the activity of Hippo pathway, resulting in the change of TEAD-YAP activity. Cell membrane protein neurofibromin 2 (NF2), adherens junction protein E-Cadherin (CDH1), α-catenin and β-catenin, as well as cell skeleton protein Fibrous Actin (F-Actin) are all able to enhance the activity of Hippo pathway, indicating that Hippo pathway is closely related with cell-cell contact and cell morphology (Kim et al,

2011; Konsavage et al, 2012; Wada et al, 2011; Yokoyama et al, 2008). Serum-borne lysophosphatidic acid (LPA) and sphingosine 1-phosphophate (S1P) can strongly inhibit the activity of Hippo pathway through G12/13-coupled receptors, leading to robust activation of TEAD-YAP transcriptional activity, while the stimulation of Gs-coupled receptors by glucagon or epinephrine activates Hippo pathway, causing the reduction of TEAD-YAP activity (Cai & Xu, 2013; Yu et al, 2012a). The complicated regulation of Hippo pathway by different kinds of G protein coupled receptors (GPCRs) emphasizes the importance of extracellular diffusible signals in Hippo pathway.

The activity of YAP can also be regulated by other means apart from canonical Hippo pathway. For example, Wnt pathway has an influence on YAP expression at the transcription level (Konsavage et al, 2012; Konsavage & Yochum, 2013). Angiomotin (AMOT) family proteins, which mainly localize on cell membrane and have a strong affinity to YAP, can restrict YAP on tight junction and enhance YAP phosphorylation (Chan et al, 2011; Zhao et al, 2011). Another cell membrane protein called zona occludens-2 protein (ZO-2) is able to bind with YAP and to improve YAP's translocation to the nucleus (Oka et al, 2010; Oka et al, 2012). Recently, a transcriptional cofactor called VGLL4, which contains a TEAD-interacting domain (TDU domain) while lacks any transcriptional activation domain, is discovered to be able to interact with TEAD in the same manner of YAP, competing with YAP and reducing the transcriptional activity of TEAD proteins (Jiao et al, 2014; Pobbati & Hong, 2013; Zhang et al, 2014).

In summary, YAP activity is under strict control of Hippo pathway as well as other regulatory partners through both phosphorylation and physical interactions, resulting in the efficient silencing of YAP activity in most quiescent adult cells.

1.4.3 The role of TEAD-YAP complex / Hippo pathway in organ size control and carcinogenesis

The crucial role of TEAD-YAP complex in organ size control was first discovered using *Drosophila* as model (Justice et al, 1995; Xu et al, 1995). Firstly, scientists discovered several cell proliferation suppressors, named hippo (hpo), salvador (sav), and warts (wts also called Lats), whose inactivation would result in massive expansion of cell number (Harvey et al, 2003; Jia et al, 2003; Pantalacci et al, 2003; Tapon et al, 2002; Udan et al, 2003; Wu et al, 2003). Studies from several groups all showed that these genes functioned in a common pathway in which Hpo, facilitated by Sav, phosphorylated Wts (Lai et al, 2005; Shimizu et al, 2008; Wei et al, 2007; Wu et al, 2003). This pathway was later named as Hippo pathway since the major phenotype after deleting any elements of this pathway was a significantly enlarged worm (Edgar, 2006). Later, yorkie (yki), the *Drosophila* homologue of YAP, was identified as the elusive target of the Wts/Lats tumor suppressor, and overexpression of yki phenocopied loss-of-function mutations of hpo, sav, or wts (Dong et al, 2007; Oka et al, 2010).

In mammals, the study about the role Hippo pathway in organ size control was mainly done using mouse liver as model (Avruch et al, 2011). Liver was an organ with remarkable regenerative capacity, and the change of liver size within

a certain range can be tolerated by mice (Yokoyama et al, 1953). Double knockout of both *Mst1* and *Mst2* in liver would cause liver enlargement, tumour formation and oval cell accumulation (Lu et al, 2010; Song et al, 2010; Zhou et al, 2009a). Double knockout of *Sav1* in liver would lead to liver enlargement and single formation of tumours probably from oval cell origin (Lee et al, 2008; Lu et al, 2010). YAP overexpression in liver would cause enlarged liver size, cancer development and expansion of progenitor cells, while knockout of *Yap* in liver led to slightly enlarged liver with injury and fibrosis, loss of biliary epithelial cells and increased hepatocyte turnover (Camargo et al, 2007; Dong et al, 2007). A summary of the phenotype after Yap hyperactivation by deleting different components of Hippo pathway is shown in **Table**1.2.

Gene	Mice	liver defects	liver tumourigenesis
Sav1	Albumin-Cre $Sav^{c/c}$	Increased liver size. Specific proliferation and expansion of oval cells.	Liver tumours with a mixed (HCC/CC) phenotype at 13-14 months of age.
Mst1,2	Albumin-Cre $Mst^{-/-}$; $Mst2^{c/-}$	Liver enlargement at 1 month of age, with increased hepatocyte proliferation	Dysplasia at 1 month.
	Albumin-Cre $Mst1,2^{c/c}$	Increased liver size from 1 month of age, due to hepatocyte proliferation. Accumulation of oval cells at 2 months of age.	HCCs begin to develop around 4 months of age.
	Albumin-Cre $Mst^{-/-}$; $Mst2^{c/c}$	Massive liver overgrowth by 3 months of age	Mixed-type (HCC/CC) tumours and HCCs by 3 months of age.
YAP	Double transgenic LAP1/tTA-YAPS127A	Hepatocyte proliferation and increased liver size after activation of an inducible transgene (reversible effect). Hepatocytes resistant to FAS-mediated apoptosis.	
	Double transgenic ApoE/rtTA-YAP		Lethal HCCs.

Table 1.2 Description of the different phenotypes after overexpressing *YAP* or inactivation of the *Sav1* and *Mst1/2* in mouse liver (Avruch et al, 2011).

YAP deregulation also has a strong impact on other organs. For example, forced expression of a constitutively active form of Yap in the embryonic heart increases cardiomyocyte number and heart size (Xin et al, 2013). YAP expression can also expand basal epidermal progenitors in mouse skin and inhibit their terminal differentiation (Beverdam et al, 2013; Zhang et al, 2011). Inactivation of YAP in colon causes no obvious intestinal defects under normal homeostasis, but severely impairs dextran sodium sulfate (DSS)-induced intestinal regeneration, while hyperactivation of YAP in colon by colon specific knock out of *Sav1* would result in widespread early-onset polyp formation following DSS treatment (Cai et al, 2010).

These data clearly point out the important role of Hippo pathway in organ size control, tissue regeneration and cancer development. The emerging role of Hippo pathway in cancer has become more and more recognized these days. Reduced MST1 and MST2 expression was observed in human hepatocellular carcinomas, soft-tissue sarcoma and colorectal cancer (Seidel et al, 2007; Zhou et al, 2009a; Zhou et al, 2011). Down-regulation and mutations of LATS1 and LATS2 was observed in breast cancer, lung cancer and astrocytoma (Jiang et al, 2006; Sasaki et al, 2010; Takahashi et al, 2005). Abnormally high expression or increased nuclear localization of YAP is frequently observed in major human cancers like liver cancer, breast cancer and lung cancer (Su et al, 2012; Wang et al, 2012; Xu et al, 2009).

1.4.4 TEAD-YAP complex and gastric cancer

According to the result of immunohistological studies performed using gastric cancer specimen, YAP expression is significantly up-regulated in gastric carcinoma as well as precancerous lesions, and YAP overexpression has a positive correlation with the overexpression of survivin, a member of the inhibitor of apoptotic protein (IAP) family (Da et al, 2009; Zhang et al, 2012). YAP overexpression or nuclear accumulation is also correlated with poor prognosis for gastric cancer patients, due to YAP's role in promoting proliferation, anchorage-independent colony formation, cell invasion and cell motility , which is shown by ectopic expression or knockdown experiments performed in gastric cancer cell lines (Kang et al, 2011).

According to a recent report studying gene expression pattern of gastric cancer specimens utilizing both epigenomic and transcriptomic approaches, TEAD4 upregulation due to reduced promoter methylation has a significant association with poor prognosis of gastric cancer patients. Knockdown of TEAD4 results in the reduced growth of gastric cancer cells both *in vitro* and *in vivo*, since TEAD4 triggers the expression of various genes in favour of cell proliferation and migration (Lim et al, 2014).

Currently, our understanding about the role of TEAD-YAP complex in gastric cancer is still mainly based on observations other than the study on the mechanism. More thorough research should be conducted to explain the cause of TEAD-YAP hyperactivation in gastric cancer as well as the tissue specific biological functions of TEAD-YAP complex in stomach.

1.4.5 The development of TEAD-YAP antagonists with potential clinical applications

Some antagonist targeting TEAD-YAP complex has already been developed for their potential application as cancer treatment. In a screening using the Johns Hopkins Drug Library, which is a collection of >3300 drugs that have been approved by the United States Food and Drug Administration or its foreign counterparts or have entered phase II clinical trials, two small molecular compounds, named protoporphyrin IX (PPIX) and verteporfin (VP) were identified to be able to effectively disrupt the interaction between TEAD and YAP (Liu-Chittenden et al, 2012b). When VP was tested in mice bearing liver-specific knockout of *NF2/Merlin*, which exhibited bile duct overproliferation due to activation of endogenous YAP, the liver overgrowth was suppressed by VP treatment (Liu-Chittenden et al, 2012b).

A peptide called Super-TDU, which mimicked the TEAD interaction domain of VGLL4, was tested on both human primary gastric tumour cells as well as a gastric cancer mouse model treated with H. *pylori* and a cocarcinogen called N-methyl-N0-nitro-N-nitrosoguanidine (MNNG). This Super-TDU peptide showed dramatic inhibition on tumour cells' growth in both systems without obvious systematic toxicity (Jiao et al, 2014).

The potent effects of TEAD-YAP antagonists on animal cancer models revealed the importance of TEAD-YAP complex in the progression of cancer, which might have a broader application in targeted cancer therapies in the near future, since this oncogenic complex is usually inactive in normal adult tissues (Jiao et al, 2014).

1.5 The interaction between YAP and RUNX proteins

YAP-RUNX interaction is among the earliest protein-protein interactions identified for YAP. In 1999, YAP was identified as a potential binding partner for RUNX proteins in a yeast two-hybrid screening. Further analysis revealed that the tryptophan-tryptophan (WW) domain of YAP, could bind with the PY motif (PPxY) located at the carboxyl terminal of RUNX proteins (Chen & Sudol, 1995; Sudol et al, 1995). Via its strong intrinsic transcription activation domain, YAP could function as a strong transcription coactivator for RUNX proteins on the *osteocalcin* promoter (Yagi et al, 1999).

Later, the interaction between RUNX2 and YAP was described in osteoblast cells. The RUNX2-YAP interaction was crucial for regulating osteocalcin expression under the control of Src/Yes tyrosine kinase signalling during the skeleton development, since Src could phosphorylate YAP and weaken the binding between YAP and RUNX2, causing the reduction of osteocalcin expression (Zaidi et al, 2004).

It is now recognised that TEAD-YAP complex plays a crucial role in organ size control as well as cancer progression, so the interaction between RUNX3 and TEAD-YAP complex warrants re-examination for a more comprehensive understanding of the regulation and function of TEAD-YAP complex.

Chapter 2

Materials and Methods

2.1 Cell Culture

HEK293T cell line, HS746T gastric cancer cell line and AZ521 gastric cancer cell line were maintained in Dulbecco's modified Eagle's media (DMEM, Nacalai tesque) with 10% fetal bovine serum (FBS, Hyclone), 100 U/ml penicillin and 100μg/ml streptomycin antibiotics (Life Technologies). YCC1 and YCC6 gastric cancer cell lines were maintained in OPTI-MEM media (Life Technologies) with 10% FBS, 5mM Sodium Pyruvate (Life Technologies), 100 U/ml penicillin and 100μg/ml streptomycin antibiotics. The other gastric cancer cell lines used in this thesis (including MKN28, MKN74, AGS, NUG-C3, NUG-C4, NCI-N87, HS746T, MKN45, HGC27), were maintained in RPMI-1640 media (Nacalai tesque) with 10% FBS, 100 U/ml penicillin and 100μg/ml streptomycin antibiotics.

2.2 Plasmid and siRNA transfection

esiRNA human YAP1 was purchased from Sigma-Aldrich under catalogue number: EHU113021-50UG. esiTEAD1 RNA was purchased from Sigma-Aldrich under catalogue number: EHU079871-50UG. esiRNA control was purchased from Sigma-Aldrich under catalogue number: EHUEGFP-50UG. The siRUNX3 (LU-012666-00-0002, set of 4) and siScramble (D-001810-10-50, set of 4) were purchased from Thermo.

Plasmid transfection was performed using Lipofectamin 2000 (life technologies) according to manufacturer's instructions. For every 1 μg of plasmid, 2 μl Lipofectamin 2000 was used to achieve a desired transfection

efficiency. siRNA transfection (including esiRNA) was performed using Lipofectamin iMAX (Life Technologies). For every 20 pmol of siRNA, 1 μl Lipofectamin imax was used to achieve a desired knocking down efficiency.

The oligos were diluted in serum free media and mixed with Lipofectamin 2000 or Lipofectamin iMAX, incubated at room temperatrue for 20 min, then added into the cells.

2.3 Retrovirus particles preparation and infection

Retrovirus was packaged using HEK293T cells. Retrovirus vectors and pCL10A packaging vector were cotransfected to HEK293T cells at 1 : 1 ration. The supernatant containing retrovirus particles was harvested at 48 hours and 72 hours after transfection. The supernatant was filtered through 0.45 μm filter units. The filtered supernatant containing virus particles could be used directly for infections or aliquoted and stored in -80 ℃ freezer for long-term storage.

For retrovirus infection, target cells were seeded at 30% confluence. Filtered HEK293T supernatant containing retrovirus was mixed with fresh media at 1 : 1 ratio and polybrene was added to a final concentration of 5 ng/μl. This mix was added to the target cells and kept overnight, then replaced with fresh media. Target cells would undergo two rounds of overnight infections followed by drug selection (puromycin or hygromycin according to the requirement of vector) at the minimal dose which could kill all uninfected cells within 48 hours. After 2 days of selection, the remaining cells would be recovered in fresh medium without selection drugs for 1 day, and then they

would be used for downstream assays.

For TEAD1/3/4 knocking down experiment, shRNA retrovirus plasmids were obtained from Origene Technologies. Forward oligo sequences are listed in the 5'-3' direction: ShTEAD1/3/4: GATCAACTTCATCCACAAGCT; shScramble: GCACTACCAGAGCTAACTCAGATAGTACT.

2.4 Protein preparation and Western blot

For protein preparation, cell pellets were lysed in lysis buffer, containing 20mM Tris PH8, 150mM NaCl, 10% glycerol, 0.1% NP-40, 0.1mM EDTA proteinase inhibitor cocktail (Roche), 1mM DTT and Benzonase nuclease (Novagen) on ice for 45 minutes. The cell lysates were centrifuged at 12,000 rpm for 10 minutes at 4℃, and the supernatents, which contained all the soluble proteins in the cell pellets, were collected into fresh microfuge tubes.

The amount of protein was quantified using Bradford Assay. Briefly, 2ul of cell lysate was mixed with 1ml Bradford dye and incubated for 5 minutes at room temperature prior to measurement using GeneQuant 1300 spectrophotometer (GE Healthcare). The absorbance at 595nm would be recorded and the protein concentration would be determined using a standard curve by standard proteins with known concentrations.

After quantification, the amount of proteins was normalised against the most diluted sample before the addition of SDS-containing loading buffer. Cell lysates were heated at 100℃ for 10 minutes to denature the proteins.

Whole cell lysates were resolved in 10% SDS-polyacrylamide gels and

electrophoresed at 100V for approximately 2 hours. Proteins were transferred onto PVDF membranes (Biorad) at constant 100V for 2 hours prior to blocking using 5% skim milk in PBST (PBS containing 0.1% tween, Sigma-Aldrich) for 1hour.

Primary antibodies were diluted using 5% skim milk in PBST and incubated with the PVDF membrane overnight at 4℃, and were washed away using PBST 3 times for 30 minutes. After washing, the secondary antibodies which were diluted using 5% skim milk in PBST were applied to the membrane. After 1 hour of incubation at room temperature, the secondary antibodies were washed away using PBST 3 times for 30 minutes. Then chemiluminescent signals were captured using Immobilon Western Chemiluminescent HRP substrate reagents (Millipore).

The antibodies used in this thesis were listed in the chart below (**Tab** 2.1).

Antibody	Company	Catalog Number
HA (Y-11)	Santa Cruz	sc-805
GFP (FL)	Santa Cruz	sc-8334
YAP (H-125)	Santa Cruz	sc-15407
TEF1(H-4)X	Santa Cruz	sc-37611X
Myc (9E10)	Santa Cruz	sc-40
TEAD3	Abcam	ab75192
TEAD4	Abcam	ab58310
α-Tubulin (DM1A)	Sigma	T9026
β-Actin (AC-74)	Sigma	A5316
Flag M2	Sigma	F3165
Flag M2 Rabbit	Sigma	F7425
RUNX3 (R3-5G4)	MBL	D235-3
CBFBeta	MBL	D127-3
GAPDH (14C10)	Cell Signaling Technology	2118S
TEAD1	BD BioScience	610923

Table 2.1 The antibodies used for western blot detection in this thesis.

2.5 Immunoprecipitation (IP)

2.5.1 Endogenous IP

Endogenous immunoprecipitation using RUNX3 antibody (R3-6E9, MBL) was performed using HGC27 gastric cancer cells. The IP buffer contains 20mM Tris PH8, 150mM NaCl, 10% glycerol, 0.1% NP-40, 0.1mM EDTA proteinase inhibitor cocktail (Roche), 1mM DTT and Benzonase nuclease (Novagen).

Megnatic Protein A+G beads (Millipore) was pre-coated with antibodies overnight in 300 µl IP buffer containing 5mg/ml BSA as blocking reagent at 4℃. 4 µg of RUNX3 monoclonal antibody (6E9) was used to incubate with 20ul of Megnatic Protein A+G beads slurry (50% slurry), while 4 µg of normal mouse IgG was used as control at the same setting. The antibody-bond beads (named as anti-RUNX3-beads or mouse-IgG-beads) were washed three times with chilled IP buffer before usage.

HGC-27 cell line was cultured in 150mm dish to a confluency of 70%. One dish of cells were harvested by trypsin digestion, and washed with chilled PBS once. The cell pellet was lysed in 2.5 ml of IP buffer. After 3 times of brief sonication (3 seconds/time, output set at 3, microsonTM Ultrasonic Cell Disruptor), the lysate was incubated on ice for 45 min, and centrifuged at 12’000 rpm in a chilled centrifuge (TOMYTM, MX-305) to remove all the debris. 30 µl cell lysate was mixed with 10 µl 4X Laemmli Sample buffer, boiled at 100 ℃ for 10 min and kept as input control.

1 ml of cell lysate (protein concentration at about 1mg/ml) was used for one IP sample to incubate either with anti-RUNX3-beads or mouse-IgG-beads at

4 ℃ for 1 hour. After the incubation, the beads were washed with 1 ml chilled IP buffer for 6 times, and boiled in 40 ul of 1X Laemmli Sample buffer at 100 ℃ for 10 min. The supernatants were collected and used for Western blot analysis.

2.5.2 Exogenous IP

HEK293T cell was cultured to 40% confluent in 100mm dish, and transfected with plasmids according to the needs of experiments. After 24 hours, cells were harvested by trypsin digestion and washed with chilled PBS once. Cell pellet was lysed using 450 µl IP buffer/sample (same IP buffer used for endogensou IP) and kept on ice for 45 min. The debris was removed by centrifuging at 12'000 rpm in a chilled centrifuge (TOMYTM, MX-305). 30 µl of cell lysate was mixed with 10 µl 4X Laemmli Sample buffer, boiled at 100 ℃ for 10 min and kept as input control. The remaining 400 ul cell lysate was incubated with 20 ul agarose beads (50% slurry) conjucated with different tag antibodies. The tubes were kept with constant rotation at 4 ℃ for 2 hours. After the incubation, beads were washed with 1 ml chilled IP buffer for 6 times, and boiled in 40 ul of 1X Laemmli Sample buffer at 100 ℃ for 10 min. The supernatant was collected and used for Western blot analysis.

The agarose beads conjugated with tag antibodies used in this thesis were listed in the chart below (**Tab** 2.2).

Antibody	Company	Catalog Number
Flag M2 beads	Sigma	A4596
GFP beads	Chromotek	gta-100
HA beads	Roche	11815016001

Table 2.2 The agarose beads conjugated with tag antibodies used in this thesis.

2.5.3 Sequential IP

The Flag-RUNX3, GFP-YAP and HA-TEAD4 proteins were prepared using TnT® Quick Coupled Transcription/Translation System (Promega) as describe in Chapter 2.17. The final volume of protein product mixture was 56 µl. 25 µl of each protein was used for one IP mixture, and the total volume was topped up with chilled IP buffer to 400 µl. 12 µl of the IP mixture was kept separated (4 ul of 4X Laemmli Sample buffer was added) as input control for the first round of IP, and the rest IP mixture was incubated with 20 µl Flag-M2 agarose beads at 4 ℃ for 1 hour.

The beads were washed for 6 times with IP buffer, and all the bound proteins was eluted with 100 ul IP buffer containing 3X Flag Peptide (500 µg/ml) at 4 ℃ for 1 hour. 5 µl of the eluted product was kept separated (2 ul of 4X Laemmli Sample buffer was added) as the input control of the second round of IP, and the rest 95 µl elute was incubated with GFP beads at 4 ℃ for 1 hour. Then the GFP beads were washed with IP buffer for 6 times, and boiled in 30 ul of 1X Laemmli Sample buffer at 100 ℃ for 10 min.

16 µl of input control of first IP, 7 µl of input control of second IP and 30 ul of final IP products were loaded to SDS-PAGE for antibody detection.

2.6 Immonohistochemistry (IHC)

The paraffin fixed slides were offered by Prof. Yeoh Khay Guan's group in National University Hospital.

In IHC experiments, after deparaffinization and rehydration, antigen was

retrieved in retrieval solution (Target Retrieval Solution, DAKO) at 100 ℃ for 20min. After 3 washes in PBS, endogenous peroxidase activity was blocked by incubating with 1% H_2O_2 for 15min at room temperature. The slides were washed with PBS (3 X 5min) and then incubated with blocking buffer (DAKO) for 1hour at room temperature.

Primary antibodies were diluted in the blocking buffer (DAKO) at proper concentration (YAP, cell signalling, 1A12, 1:400; TEAD1, LSBIO, LS-B6651, 1:100), added on the top of slides and incubated in a humid box at 4 ℃ overnight. The slides were washed in PBST (3 X 5min) and incubated with secondary antibody (DAKO, polymer HRP-labelled anti mouseEnvision) for 1 hour at room temperature. After 3 washes using PBST, DAB (3,3'-Diaminobenzidine, DAKO) substrate was used to detect the bound peroxidase on the slides.

2.7 Dual-luciferase reporter assay

HEK293T cells were cultured to 40% confluent in 24-well plates, and transfected with plasmids according to experiment design. Triplication was made for each sample. After 24 hours, cells were lysed in 50 μl passive lysis buffer, and the luciferase activity was measured using Dual-Luciferase® Reporter Assay System (Promega) according to manufacturer's instructions.

Briefly, 10 μl of cell lysate was mixed with 50 μl of LAR II solution and the tube was placed into GloMax® Single-Tube Multimode Reader (Promega) to measure the light generated by firefly luciferase. The same tube was taken out

and 50 µl of Stop and Glo reagent was added and mixed. It was placed back to the reader for the measurement of the light generated by renilla luciferase. The ratio between Firefly and Renilla luciferase activity was calculated and normalized according to the value of vector control.

2.8 Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed using LightShift Chemiluminescent EMSA Kit (Thermo) according to manufacturer's instructions. The forward oligo of TEAD probe (5'-3') was TTCGGGACCAGGCCTGGAATGTTTCCACC/Biotin/.

The buffer for EMSA was 1X TGE buffer. To prepare 1 liter of 5X TGE buffer, 30.28 g of Tris base, 142.7 g Glycin, 3.72 g EDTA were dissolved in water and the final volume was adjusted to 1 litre. The 6% EMSA gel (10ml) was prepared by mixing 5.7 ml H_2O, 2 ml 30% acrylamide/Bis solution, 2 ml 5X TGE buffer, 200 µL 10% APS, 20 µL TEMED.

The EMSA reaction mix was prepared by mixing 1 µL 10X binding buffer, 0.5 µL 1µg/µL Poly (dI•dC) , required amount of HA-TEAD4-1-125 protein and Flag-RUNX3 protein, HA antibody if required, and H_2O till the total volume reached 8 µL. After incubating it on ice for 15 minutes, 2 µl 0.1 pmol/µl TEAD probe was added to obtain the final reaction mix, which would be loaded on the gel after incubating on ice for 30 minutes.

All the gel running and transferring steps were carried out at 4℃. The gel was pre-run in 1X TGE buffer at 65V for 30 minutes, and the reaction mix was loaded to the gel, run at 65V for another 90 minutes. The gel was transferred to

Hybond N+ membrane (GE health care) in 1X TGE buffer at 100V, 350mA for 1 hour. The membrane and DNA probes on it were crosslinked using Stratalinker (Stratalinker® UV Crosslinker) for 10 minutes. The blocking, labelling and detection steps were all conducted according to the instructions of LightShift Chemiluminescent EMSA Kit (Thermo).

2.9 Transcription factor enrichment experiment

Transcriptional Factor Enrichment Experiment was performed as previously described (Oh et al, 2013). Briefly, 10ul streptavidin conjugated agarose beads (Thermo) were used for each sample. The beads were washed with PBS briefly twice. Then beads were preabsorbed with the blocking/coupling buffer for 30 min at room temperature with constant rotation. 100ul blocking buffer was used for every 10ul beads. The beads were dispensed into individual tubes together with the blocking buffer (about 100ul mix/ tube). 7ul biotin label probe (0.1 pmol/μl) was added into each tube, and incubated for 30 min at room temperature with constant rotation. At this stage, the beads were already coated with DNA probes. These beads were washed three times with 1X oligonucleotide binding buffer before usage.

The reaction mix (300 μL scale) was prepared by mixing 3 X oligonucleotide binding buffer with cell nuclear extraction. The reaction mix was incubated with probe-coated beads for 3 hours in cold room. Then the beads were washed three times with 1ml of 1X oligonucleotide binding buffer, and boiled in 40 ul of 1X Laemmli Sample buffer at 100 ℃ for 10 min. All

precipitated proteins were preserved in the final supernatant, which could be resolved using SDS-gels.

The compositions of buffers were listed below.

Oligonucleotide binding buffer (3 X OBB) : 36mM HEPES, pH7.9, 12mM Tris, 450mM KCL, 36%Glycerol, 3mM EDTA, 3mM DTT.

Blocking/coupling Buffer (3ml): 3X OBB 1ml, 5mg/ml BSA 1.2ml, 0.5mg/m dIdC 60ul, 10mg/ml Salmon Sperm DNA 60ul, 10%NP-40 60ul, DDW 620ul. Add proteinase and phosphotase inhibitor or DTT if necessary.

Reaction Buffer (3ml): 3XOBB 1ml, 5mg/ml BSA 1.2ml, 0.5mg/ml dIdC 60ul, 10%NP-40 60ul, DDW plus protein: 680ul. Add proteinase and phosphotase inhibitor or DTT if necessary.

The forward oligo of TEAD probe (5'-3') was TTCGGGACCAGGCCTGGAATGTTTCCACC/Biotin/. The forward oligo of TEAD mutated probe (5'-3') was TTCGGGACCCAGGCCTAGAATGTTTCCACC/Biotin/. The biotin conjugated probes were annealed with the reverse complementary non-biotin conjugated probes to generate double strand probes.

2.10 Purification of RNA using Trizol

Cells were rinsed with cold PBS once and directly lysed in Trizol reagent (Life Techologies). 1 ml of Trizol reagent was used for every 3.5 cm diameter dish. Pass the cell lysate several times through a 1ml pipette tip, and collect all the lysate in the dish into fresh eppendorf tubes.

0.2 ml of chloroform was added into every 1 ml of Trizol lysate. The mix was votexed vigorously for 15 seconds and incubated at room temperature for 3 min. The samples were spin at 12’000g for 15 min at 4 ℃. The colourless upper aqueous phase was collected, and mixed with equal volume of isopropanol. Then the samples were incubated at room temperature for 10 min and spin at 12’000g for 10 min at 4 ℃. At this stage, the RNA precipitant formed a gel-like pellet on the side and bottom of the tube, which should be washed with at least 1 ml of 75% ethanol. The RNA pellet was air-dried after washing, and dissolved in RNAse free water.

The yield of RNA was measured by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, MA, USA).

2.11 Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Complementary DNA (cDNA) was synthesized from 2.0μg of total RNA using SuperScript II Reverse Transcriptase (Life Technologies). The reaction mixtures were prepared according to the manufacturer’s instructions and incubated at 42 ℃ for 50 min. The reverse transcriptase was inactivated at 70 ℃ for 15min.

Quantitative PCR was performed in 7500 Real-time PCR system using Power SYBR Green Master Mix (Applied Biosystems, CA, USA) and 2μl of 5X diluted cDNA as template.

The list of gene-specific oligonucleotide primers used for SYBR Green-based measurements were shown below (**Tab** 2.3).

GENE	Forward primer (5'-3')	Reverse primer (3'-5')
CTGF	TCGTCTGCCCAGCCCTGACT	GGCGCTCCACTCTGTGGTCTG
GAPDH	ATCATCCCTGCCTCTACTG	TGCTTCACCACCTTCTTG

Table 2.3 gene-specific oligonucleotide primers used for SYBR Green-based real-time PCR.

2.12 Chromatin Immunoprecipitation (ChIP)

ChIP was performed using TEAD1 antibody (sc-376113 X, Santa Cruz) and Magna ChIP™ A/G Chromatin Immunoprecipitation Kit (Millipore) according to manufacturer's instructions.

Briefly, 2 X 10^6 cells were used for each sample. Proteins were crosslinked to DNA by adding formaldehyde directly to culture medium to a final concentration of 1% and incubate for 10 minutes at room temperature. Aspirate medium, wash and scrape cells with ice cold PBS containing protease inhibitors. Cells were spin for 4 minutes at 700 x g at 4℃.

Cell pellets were resuspend in 100µl SDS Lysis Buffer for 10 minutes on ice. Lysate was sonicated to reduce DNA length to 500 basepairs using Bioruptor® Plus (Diagenode) with the programme set as 25 cycles of 30 seconds high output sonication followed by 30 seconds rest at 4℃. Debris was removed by centrifugation for 10 minutes at 13,000 rpm at 4℃ in a microcentrifuge. Supernatant was diluted by 10 fold with ChIP Dilution Buffer and split equally into two tubes. Either 2 mg TEAD1 antibody or 2 mg normal mouse IgG was added and incubated with the lysates overnight at 4℃.

On the next day, antibodies were immobilized by adding 10 ul of Magna ChIP™ A/G beads (bed volume) and incubating for 1 hour at 4 ℃. The beads were washed for 5 minutes using the following buffer: Low Salt Immune Complex Wash Buffer, High Salt Immune Complex Wash Buffer, LiCl Immune Complex Wash Buffer, 1X TE. Then the ChIP products were eluted from the beads twice using 250 µl elution buffer at room temperature for 15 minutes. The elute products were combined. 20 µL 5M NaCl was added into the elute products, and incubated at 65 ℃ for 4 hours to reverse the protein-DNA crosslink. After that, 10 µl of 0.5M EDTA, 20 µl 1M Tris-HCl, pH 6.5 and 2 µl of 10mg/ml Proteinase K were added to the elute products and incubated for one hour at 45 ℃.

DNA was recover from the digested elute products by phenol/chloroform extraction followed by ethanol precipitation. DNA pellets were washed with 70% ethanol and air dried. The DNA pellets were dissolved with 50 µl DNAse free water. Real-time PCR was performed using 2 µl product as template for each reaction.

The CTGF promoter primers were: CTGF ChIP Forward 5-ATATGAATCAGGAGTGGTGCGA-3; CTGF ChIP Reverse 5-CAACTCACACCGGATTGATCC-3. (Zhao et al, 2008)

2.13 Cell viability assay

Cell viability was measured using Cell Proliferation Reagent WST-1 (Roche) according to the manufacturer's instructions.

Briefly, 1000-2000 cells/well were seeded in each well of 96-well plates. (Seeding number was dependent on cell lines, and triplication was prepared for each sample.) At 0 hour, 24 hours, 48hours, 72 hours and 96 hous, 10 μl of WST-1 reagent was added into each well and incubated in CO_2 gas incubator for 2 hours. Light absorption at 450nm (620nm as reference) was determined using spectrophotometer (Infinite 200, Tecan™).

After removing basal absorbance caused by culture medium, the absorbance of 0 hour was used to adjust all samples to the same starting point. Then the values of following days would be adjusted accordingly. The adjusted absorbance would be used for the plotting of growth curve using Prism5 graph pad software.

2.14 Soft agar assay

Soft agar assay was performed using low melting agarose (Life Technologies). The basal agar was prepared by mixing hot 0.8% low melting agarose with 2X DMEM (or 2X RPMI-1640, according to the culture condition of each cell) at 1:1 ratio. 1ml basal agar was added to each well of a 12-well plate.

After the basal gel was set, the top agar, which contained 0.4% low melting agarose, 1X DMEM (or RPMI-1640) and 10% FBS, would be prepared by mixing 2X DMEM (with 20% FBS) and 0.8% low melting agarose at 1 : 1 ratio. Every component of top agar should be balanced to 37℃ before mixing, and the mixed top agar should be kept at 37℃ till usage.

2000 cells were seeded into each well of a 12-well plate (triplication should be prepared). The cells were dissolved in 400 μl of top agar, mixed thoroughly and added onto the bottom agar. The seeded plate would be kept at 4℃ for 30 minutes to ensure that the top agar had been completely set. Then fresh culture medium would be added on the top of the agar to keep moisture, which should be changed twice every week. PBS should also be added into the empty space between wells to keep moisture.

After 2 to 4 weeks, colonies would become visible for bare eyes. They could be stained with 0.005% crystal violet for 4 hours, distained with PBS for 2 days till the agar become colourless. Then pictures were taken by dissection microscope (SZX16, Olympus).

2.15 Prokaryotic protein expression and purification

cDNA of HA-TEAD4 fragments were cloned into PGEX-4T-1 vector (GE healthcare), and transformed into Rosetta™(DE3) cells. Positive clones were picked from LB plates and inoculated into 3ml LB broth, cultured with 225 rpm shaking at 37℃ overnight. The small culture was expanded to 200 ml LB broth, and cultured with 225 rpm shaking at 37℃ till the OD_{600} reached 0.8. 200ul 0.1M IPTG was added to induce the protein production at 37℃. After 3 hours of induction, the bacteria were harvested by centrifuging at 8000rmp for 15 minutes. The pellet could be stored in -80℃ freezer if necessary.

The pellet was resuspended in 20ml cold PBS (with 100 μM PMSF and 1mM DTT), and sonicated at output 3 for 30 seconds using ultrasonic cell disrupter (Microson^{TM}) for 3 times, while the lysate was kept chilled on ice all

the time. TritonX-100 was added to the sonicated lysate to a final concentration of 1%, and the cell lysate was rotated in cold room for 1 hour. Then the cell lysate was centrifuged at 20’000 rpm for 30 minutes at 4°C to remove all the debris.

The supernatant was collected and 200ul Gluthatione Sepharose (GE Healthcare) was added into it and incubated with constant rotation for 1 hour in cold room. Then the Gluthatione Sepharose was collected by brief spinning, and washed 3 times with chilled PBS. The GST-fusion proteins were now immobilized on the Gluthatione Sepharose, which could be cleaved from the beads by incubating with thrombin (GE Healthcare, 0.1U/ml to 1U/ml, dependent on the amount of protein) at room temperature for 2 hours. The beads could also be directly used for downstream applications like GST pull down assay.

2.16 GST pull down assay

GST fusion proteins were expressed and immobilized on GSH beads as described in Chapter 2.15. A small aliquot of beads from each sample was boiled in 1X Laemmli Sample buffer at 100 ℃ for 10 min. The proteins were resolved on SDS-PAGE gel and the gel was stained with Coomassie Blue staining solution for 1 hour, and distained with distain buffer for 3 hours. The relative protein amount could be determined by comparing the band intensity of each protein, and they should be normalized to the protein with lowest yield.

For each sample, only 1-4 µl of GSH beads coated with protein were used (exact amount for each sample should be adjusted according to the protein yield to guarantee same level of protein input for all samples). The beads volume was

topped up to 10 µl with empty GSH beads for easier handling. The beads were mixed with IP buffer containing the proteins of interest (either cell lysis or diluted purified proteins), and incubated with constant rotation for 1 hour at 4 ℃. The beads were washed 3 times with IP buffer and boiled in 1X Laemmli Sample buffer at 100 ℃ for 10 min. All the proteins which precipitated together with GST fusion proteins could be analyzed with Western blot.

2.17 Eukaryotic protein expression

Myc-TEAD1, Flag-RUNX3, GFP-YAP, HA-TEAD4 proteins were synthesized using TnT® Quick Coupled Transcription/Translation System (Promega).

The reaction mixture contained 7 µl water, 40 µl TNT® Quick Master Mix, 1 µl 1mM Methionine, 2 µl 0.5mg/ml plasmid DNA template. The reaction mix was incubated at 30 ℃ for 90 minutes. Then 5.5 µl 10X DNAse reaction buffer and 1 µl DNAse I (Promega) were added and incubated at room temperature for 30 minutes to remove plasmid templates.

The final mixture contains eukaryotic proteins synthesized by rabbit reticulocyte extract, which can be used for other applications like the study of protein-protein interaction *in vitro*.

2.18 Chemical transformation of Escherichia coli

Three microlitres of ligated products or one mirolitre of purified plasmid

was added into 50µl of home-made chemically-competent Escherichia coli XL10-Gold strain originally obtained from Stratagene (CA, USA). The mixture was kept on ice for 30min prior to heat-shock at 42 ℃ for 90 seconds. Then the mixture was incubated on ice immediately for 5min and recovered in 800µl Luria-Bertani (LB) medium at 37 ℃ with constant shaking for 1h.

The transformation mixture was subsequently plated onto LB agar plates containing 100µg/ml of ampicillin (Sigma-Aldrich, MO, USA) or 50 µg/ml kanamycin (Sigma-Aldrich, MO, USA) and incubated overnight at 37 ℃ for selection of successfully transformed bacterial cells.

2.19 Purification of plasmid DNA

DNA purification was performed at small-scale from 3ml of starter cultures with QIAprep Spin Miniprep columns (QIAGEN, Hilden, Germany) or large-scale from 50ml of overnight cultures with QIAfilter Plasmid Midi kit (QIAGEN, Hilden, Germany) according to manufacturer's protocols.

The yield of plasmid was measured using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, MA, USA).

2.20 Statistical analysis

Experimental data are presented as the mean±standard deviation (SD). All statistical analyses were performed using analysis of a two-tailed Student's t test with GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Survival

curves were calculated using the Kaplan-Meier method. Differences were considered statistically significant when P values were less than 0.05. * indicates $P<0.05$; ** indicates $P<0.01$; *** indicates $P<0.001$;

Chapter 3

The direct protein-protein interaction between RUNX3 and TEAD proteins

3.1 Introduction

In this chapter, the protein-protein interaction between RUNX3 and TEAD proteins (mainly TEAD1 and TEAD4) would be examined in depth. The major experimental approach which was used in this chapter was immunoprecipitation, a technique of precipitating a protein antigen out of solution using an antibody that specifically binds to that particular protein. In a mild solution without strong detergent like SDS, the protein complexes containing this antigen can be preserved, so they can be specifically pulled down by this antibody. The antibody can be further enriched using agarose or magnetic beads coated with Protein A (originated from bacterium *Staphylococcus*) or Protein G (originated from bacterium *Streptococcal*), both of which showing strong affinity to Immunoglobulin. The total precipitant, which contains all the components having protein-protein interactions with the precipitated antigen, could be analyzed with techniques like Western Blot.

3.2 RUNX3 and TEAD1 can coexist in the same complex

In previous studies, it was already shown that RUNX3 and YAP had physical interaction with each other. Since TEAD proteins and YAP had strong protein-protein interaction with each other, it was highly possible that TEAD proteins and RUNX3 could coexist in the same protein complex. I wanted to test this hypothesis by performing endogenous immunoprecipitation (IP) in HCG-27 gastric cancer cell line, in which both RUNX3 and TEAD1 proteins were expressed at a high level.

The immunoprecipitation was performed using a monoclonal antibody targeting RUNX3 (clone 6E9) or normal mouse IgG control as described in the Chapter 2.5. For the detection of RUNX3, a special Biotin-labled primary antibody (clone 5G4) was used to avoid contamination caused by IgG's heavy chain to achieve a better signal. The rest part of the Western blot protocol was standard as described in Chapter 2.4.

After the whole IP procedure, TEAD1 could be detected in the precipitant of anti-RUNX3 antibody, indicating that TEAD1 and RUNX3 coexist in the same protein complex in HGC-27 cell (**Fig** 3.1).

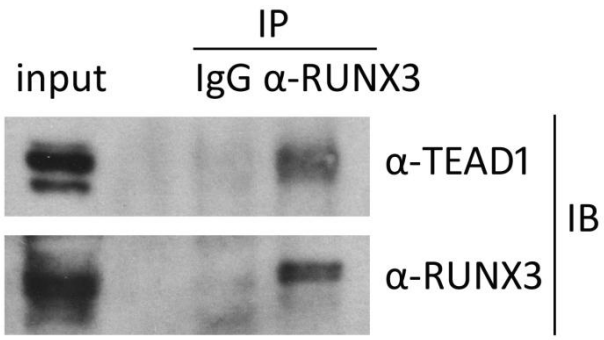


Figure 3.1 TEAD1 and RUNX3 coexist in the same protein complex in HGC27 cell line. TEAD1 was co-immunoprecipitated with RUNX3 in the precipitant of anti-RUNX3 antibody, indicating that TEAD1 and RUNX3 are in the same protein complex.

3.3 The interaction between RUNX3 and TEADs is independent on YAP

In the previous IP experiment, it was already shown that TEAD1 and RUNX3 coexist in the same protein complex. It might be YAP dependent since YAP was able to interact with both TEAD1 and RUNX3.

It had been reported that RUNX3 interacted with YAP via its PY motif. By deleting the whole PY motif in RUNX3, the interaction between RUNX3 and YAP should be totally abolished.

Here, a RUNX3 mutant in which the whole PY motif was deleted was used to perform co-IP with TEAD proteins in HEK293T cells to test whether RUNX3-TEAD interaction was mediated by YAP.

The result showed that the deletion of the whole PY motif in RUNX3 could abolish its interaction with YAP, while RUNX3's binding with TEAD1/4 largely remained intact (**Fig** 3.2). This result indicated that the interaction

between RUNX3 and TEAD proteins is not mediated by YAP.

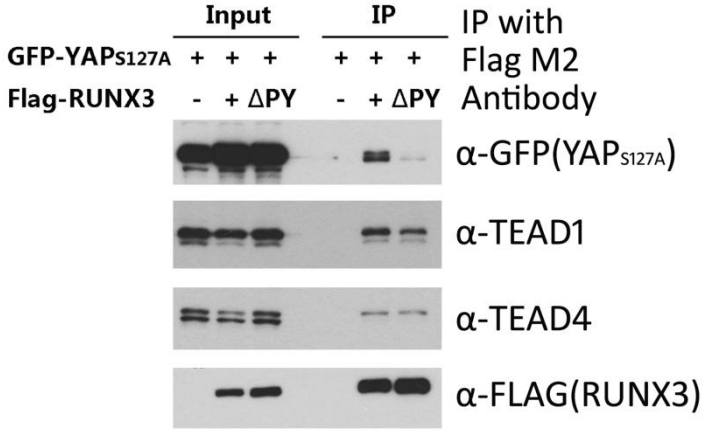


Figure 3.2 The interaction between RUNX3 and TEADs is independent on YAP. HEK293T Cell was transfected with pEGFPC2-YAP_{S127A}, pcDNA-Flag-RUNX3 or pcDNA-Flag-RUNX3ΔPY plasmids accordingly (1μg/10cm dish). Cells were harvested at 24 hours after transfection and IP was performed using M2-Flag agarose beads. ΔPY meant that the whole PY motif of RUNX3 was deleted in this construct. RUNX3ΔPY mutant failed to interact with YAP but it was still able to interact with TEAD1/4.

3.4 The N-terminal sequence of RUNX3 is critical for its interaction with TEAD proteins

The results described above showed that the interaction between RUNX3 and TEAD proteins is independent on YAP, suggesting that RUNX3-TEAD interaction was a novel protein-protein interaction which deserved more investigation.

RUNX3 is composed of many domains, including Runt domain, activation domain, inhibition domain as well as VWRPY motif. To identify which domain

was utilized for RUNX3's interaction with TEAD proteins, IP using truncated RUNX3 was performed to compare their ability to interact with TEAD proteins. The structure of truncated RUNX3 construct is shown in **Fig** 3.3A.

The IP result showed that the RUNX3-1-183 had strong interaction with TEAD1/4 while the RUNX3-182-415 had a significantly impaired binding to TEAD proteins, indicating that the sequence at the N-terminal of RUNX3 is critical for its interaction with TEAD proteins (**Fig** 3.3B).

As described in the diagram showing truncated RUNX3's structure, the RUNX3-1-187 construct mainly contained the Runt domain (**Fig** 3.3A). So we hypothesised that RUNX3 might utilize the Runt domain to interact with TEAD proteins based on the result of this experiment.

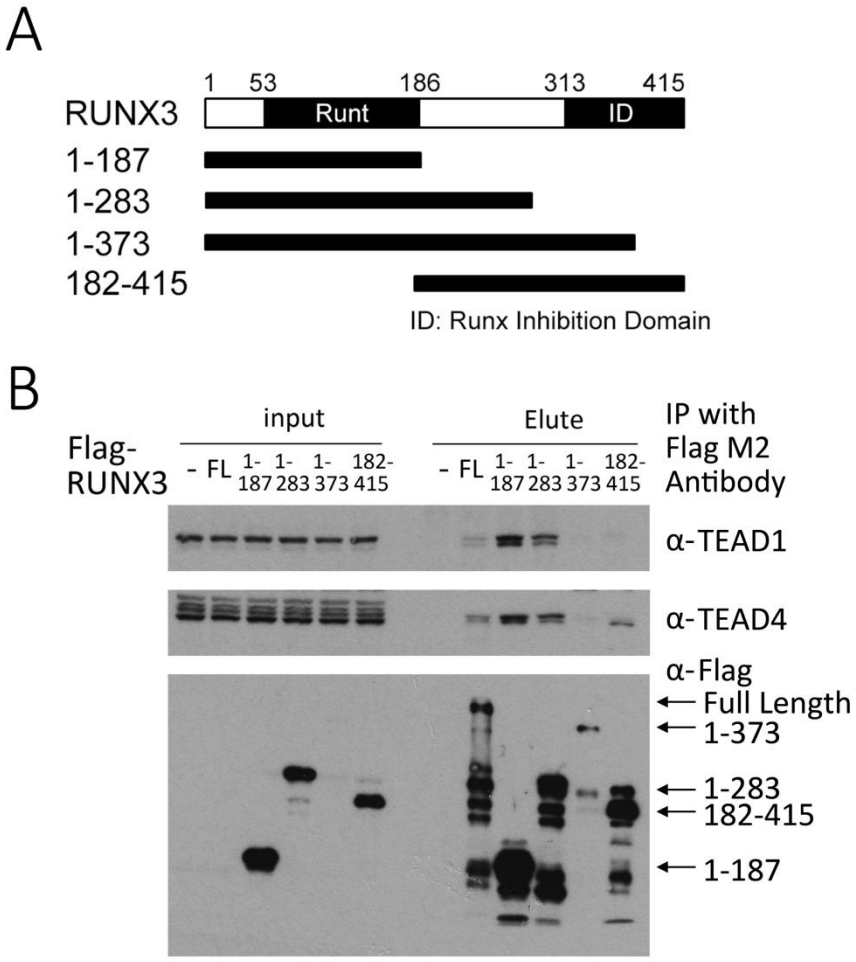


Figure 3.3 Exogenous IP using truncated forms of RUNX3 in HEK293T cell showed that the N-terminus of RUNX3 is important for the interaction between RUNX3 and TEAD proteins. (A) A diagram showing the structure of truncated RUNX3 constructs. (B) Exogenous IP result using truncated RUNX3 to pull down endogenous TEAD1/4. HEK293T cells were transfected with the truncated RUNX3 constructs (1μg/10cm dish) accordingly. Cells were harvested at 24 hours after transfection and IP was performed using M2-Flag agarose beads. The RUNX3-1-187 showed strong interaction with TEAD1/4 while RUNX3-182-415 showed significantly impaired binding to TEAD1/4.

3.5 Runt domain of RUNX3 interacts with TEAD1 directly *in vitro*

Based on the results of previous experiments, we hypothesised that RUNX3 might utilize the Runt domain (53-186 residues of RUNX3) to interact with TEAD proteins. To test this hypothesis, we designed this *in vitro* binding experiment using GST pull down method to check whether Runt domain alone was sufficient for RUNX3's interaction with TEAD1 *in vitro*.

In this experiment, Myc-TEAD1 protein was prepared using TnT® Quick Coupled Transcription/Translation System (Promega) as described in Chapter 2.17. Recombinant GST protein and GST-Runt (53-186 residues of RUNX3) fusion protein were prepared using Rosetta™(DE3) cells and immobilized on GSH agarose beads as described in Chapter 2.15. The GST pull down experiment was performed by incubating GSH beads coated with GST or GST-Runt fusion proteins with TEAD1 protein resuspended in IP buffer, as described in Chapter 2.16.

The result of this GST pull down experiment showed that recombinant GST-Runt fusion protein alone was able to bind with TEAD1 protein *in vitro*, indicating that there is a direct interaction between Runt domain and TEAD1 protein (**Fig** 3.4).

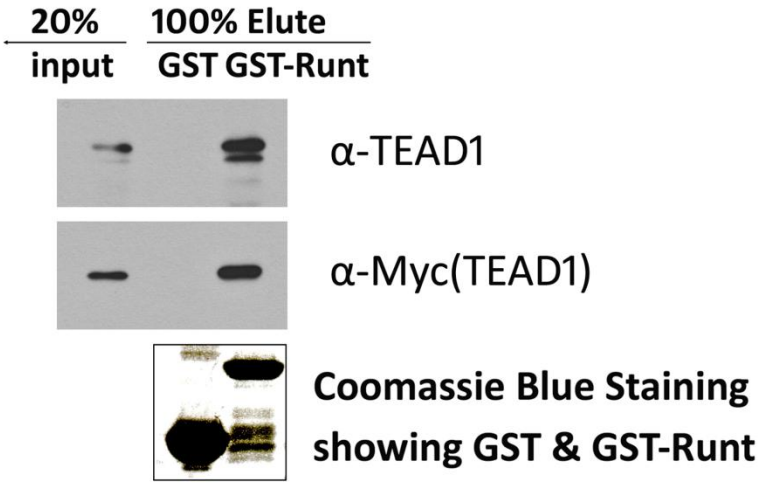


Figure 3.4 The direct protein-protein interaction between Runt domain and TEAD1 is shown by GST pull down experiment *in vitro*. GST, GST-Runt domain (53-186 residues of RUNX3) and full-length TEAD1 proteins were synthesized *in vitro*. GST and GST-Runt proteins were immobilized on GSH beads. GSH beads coated with GST-Runt protein were able to pull down TEAD1 protein *in vitro*.

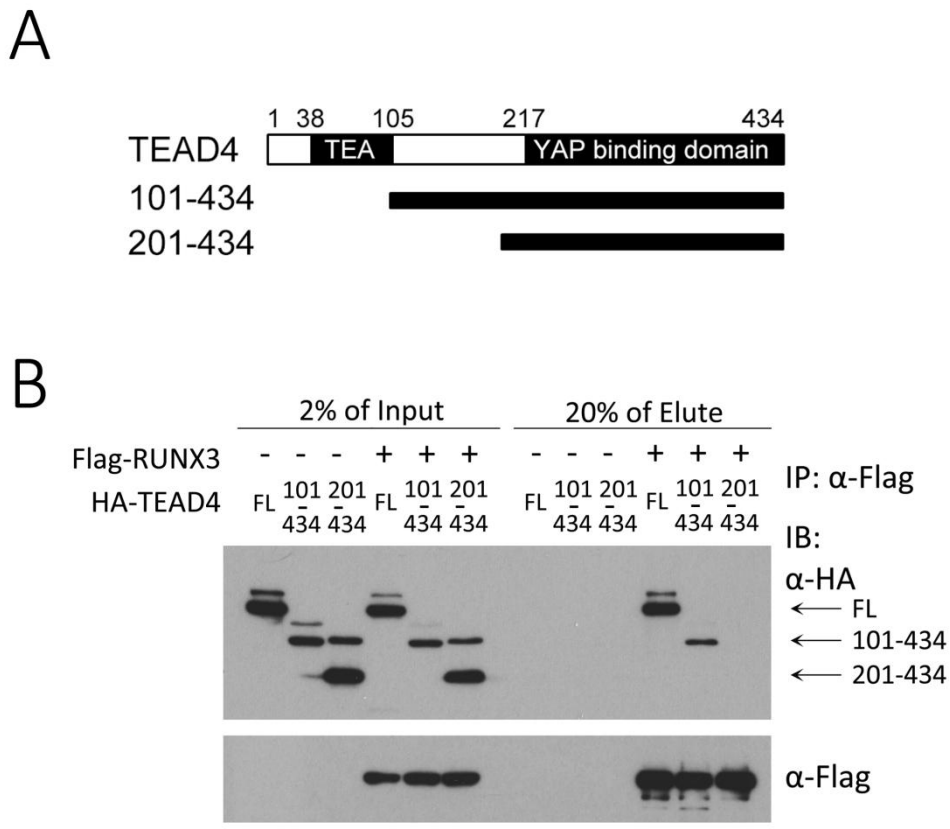
3.6 The residues 101-200 of TEAD4 are critical for its interaction with RUNX3

In order to determine the binding domain of TEAD4 for interacting with RUNX3, IP experiments using TEAD4 mutants truncated from both N-terminus and C-terminus were carried out.

In the first part of this experiment, exogenous IP was performed using TEAD4 mutants truncated from N-terminus, whose structures are shown in **Fig** 3.5A. The result showed that deleting the first 100 residues of TEAD4 did not cause any obvious reduction for TEAD4-RUNX3 interaction, while deleting the first 200 residues of TEAD4 totally abolished TEAD4's interaction with RUNX3 (**Fig** 3.5B). This result indicated that the 101-200 residues of TEAD4 are crucial for the interaction between RUNX3 and TEAD4.

In the second part of this experiment, exogenous IP was performed using TEAD4 mutants truncated from C-terminus, whose structures are shown in **Fig** 3.5C). The IP result showed that TEAD4's first 100 residues alone were not able to interact with RUNX3, while the truncated TEAD4 which contains 1-200 residues started binding with RUNX3, indicating that the 101-200 residues of TEAD4 play an important role for the interaction between RUNX3 and TEAD4 (**Fig** 3.5D).

Taken together, both experiments indicated that the 101-200 residues of TEAD4 play an important role for the interaction between RUNX3 and TEAD4.



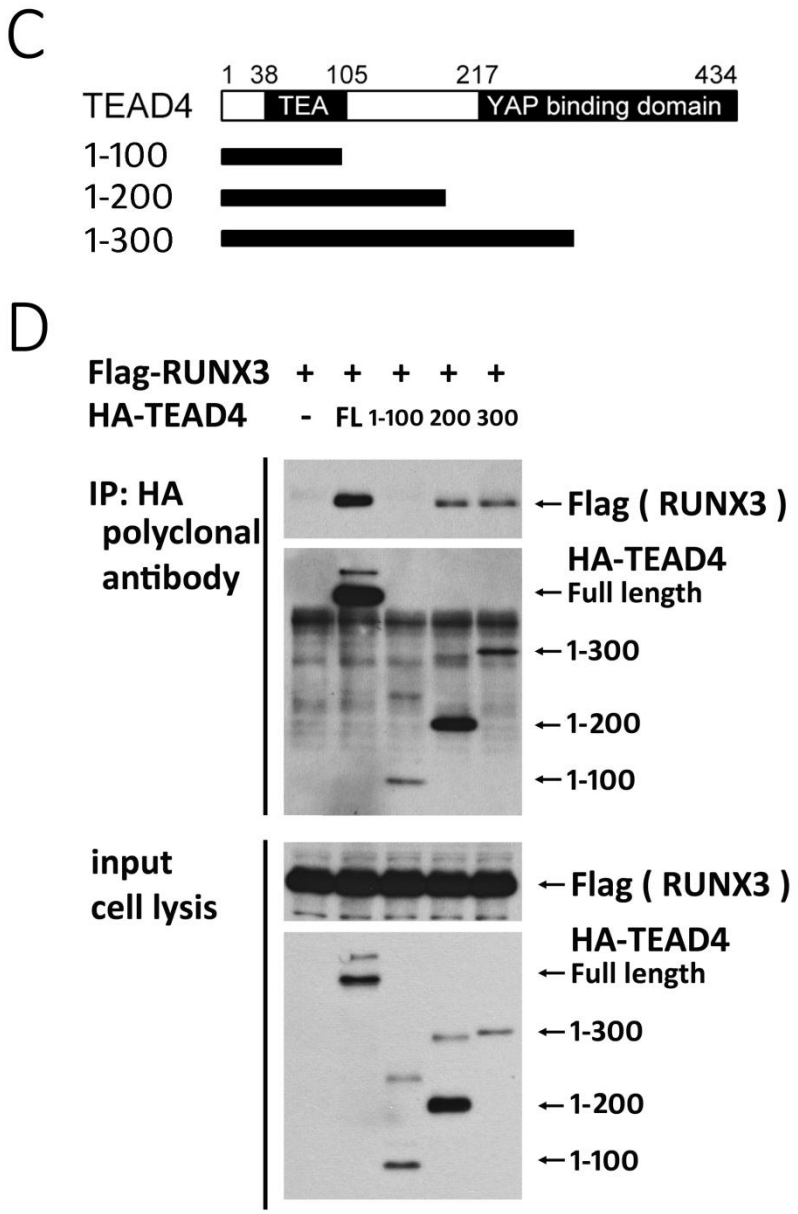


Figure 3.5 TEAD4's 101-200 residues are important for TEAD4-RUNX3 interaction. (A) A diagram showing the structure of TEAD4 mutants truncated from N-terminus. (B) Exogenous IP result using TEAD4 mutants truncated from N-terminus. HEK293T cells were transfected with the RUNX3 construct (1μg/10cm dish) and truncated TEAD4's constructs (1μg/10cm dish) accordingly. Cells were harvested at 24 hours after transfection and IP was performed using HA agarose beads. TEAD4-101-434 was still able to interact with RUNX3, while TEAD4-201-434 failed to show any interaction with RUNX3. (C) A diagram showing the structure of TEAD4 mutants truncated from C-terminus. (D) Exogenous IP result using TEAD4 mutants truncated from N-terminus. HEK293T cell were transfected with the RUNX3 construct (1μg/10cm dish) and truncated TEAD4's constructs (1μg/10cm dish) accordingly. Cells were harvested at 24 hours after transfection and IP was performed using HA agarose beads. TEAD4-1-100 did not show any interaction

with RUNX3, while TEAD4-1-200 and TEAD4-1-300 were able to pull down RUNX3.

3.7 Residues 101-125 of TEAD4 are critical for its interaction with RUNX3

Previous experiments showed that the 101-200 residues of TEAD4 were crucial for the interaction between RUNX3 and TEAD4. Aiming to identify a shorter key sequence required for this interaction, IP using TEAD4's small deletion mutants was performed.

As it was shown in the diagram, small deletions of 25 residues were introduced to TEAD4 from residue 101 to 200 sequentially (**Fig** 3.6A). According to the IP result, all the TEAD4's small deletion mutants could be equally expressed except HA-TEAD4Δ126-150. In this experiment, HA-TEAD4Δ101-200 was a negative control for RUNX3 binding according to the results of previous experiments.

The deletion of TEAD4's 101-125 residues could totally abolish its interaction with RUNX3, indicating that the 101-125 residues of TEAD4 are the shorter key sequence for RUNX3-TEAD4 interaction which we aimed to discover in this experiment (**Fig** 3.6B).

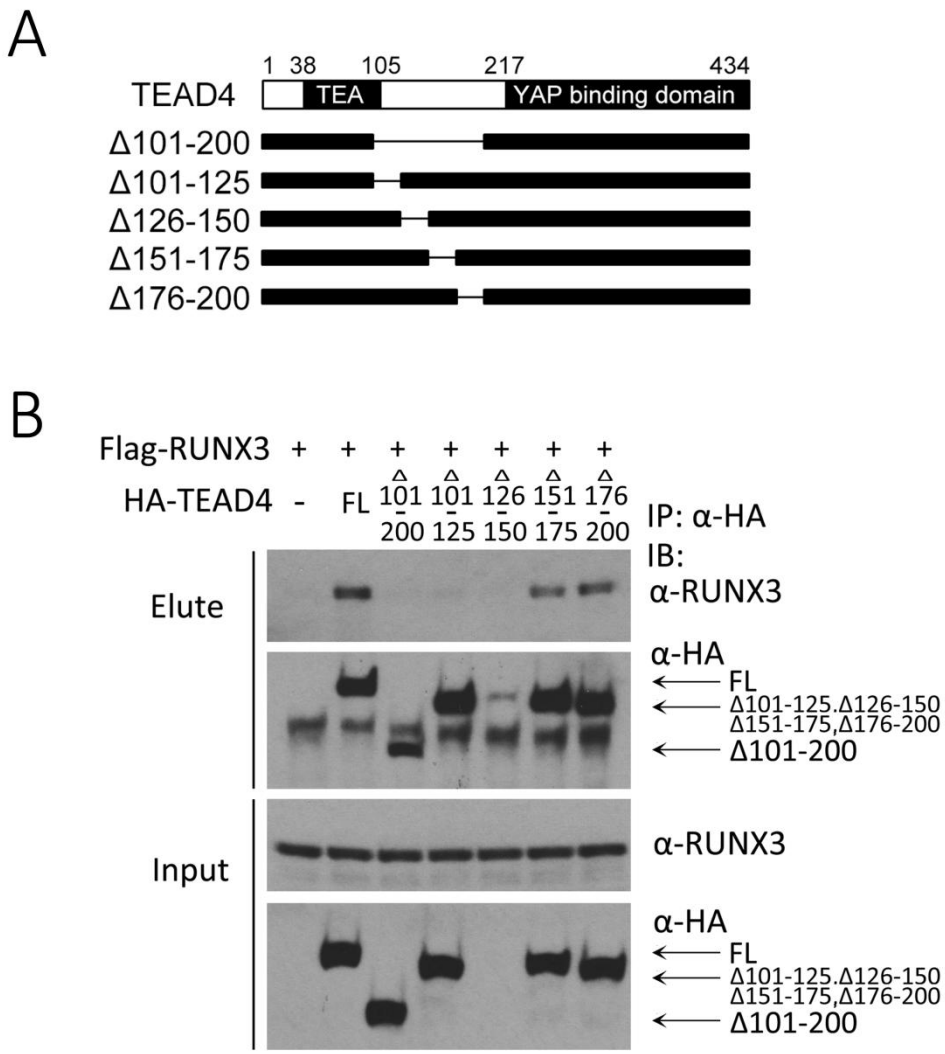


Figure 3.6 TEAD4's residues 101-125 are critical for its interaction with RUNX3. (A) A diagram showing the structure of TEAD4's small deletion mutants used in this experiment. (B) Exogenous IP using TEAD4's small deletion mutants. HEK293T cells were transfected with the RUNX3 construct (1μg/10cm dish) and TEAD4's small deletion mutants (1μg/10cm dish) accordingly. Cells were harvested at 24 hours after transfection and IP was performed using HA agarose beads. Deletion of residues 101-125 of TEAD4 could totally abolish TEAD4's interaction with RUNX3. TEAD4Δ101-200 mutant was used as a negative control here.

3.8 RUNX3, TEAD4 and YAP can form a ternary complex *in vitro*

Based on published data and the data generated in previous experiments by our own, it had been shown that RUNX3 can interact with YAP as well as TEAD4. Here, sequential IP was performed using RUNX3, YAP and TEAD4 proteins to study whether these two interactions (RUNX3-YAP and RUNX3-TEAD4) could coexist or be mutually exclusive to each other.

The Flag-RUNX3, GFP-YAP and HA-TEAD4 proteins were prepared using TnT® Quick Coupled Transcription/Translation System (Promega) as described in Chapter 2.17. The proteins were mixed in IP buffer *in vitro*. Sequential IP was performed using M2-Flag agarose beads followed by GFP agarose beads, as described in Chapter 2.5 and illustrated in the figure below (**Fig** 3.7). According to the principle of sequential IP, if three proteins could coexist in the same complex, the TEAD4 protein should still be detectable in the precipitant after two rounds of immunoprecipitations with antibodies targeting YAP and RUNX3. Otherwise, it would indicate that RUNX3-TEAD interaction and RUNX3-YAP interaction are mutually exclusive to each other.

The result of sequential IP showed that after two rounds of IP with antibodies targeting Flag-RUNX3 and GFP-YAP, the HA-TEAD4 was still detectable in the final IP products, indicating these three proteins, Flag-RUNX3, GFP-YAP and HA-TEAD4 can coexist within the same complex (**Fig** 3.8).

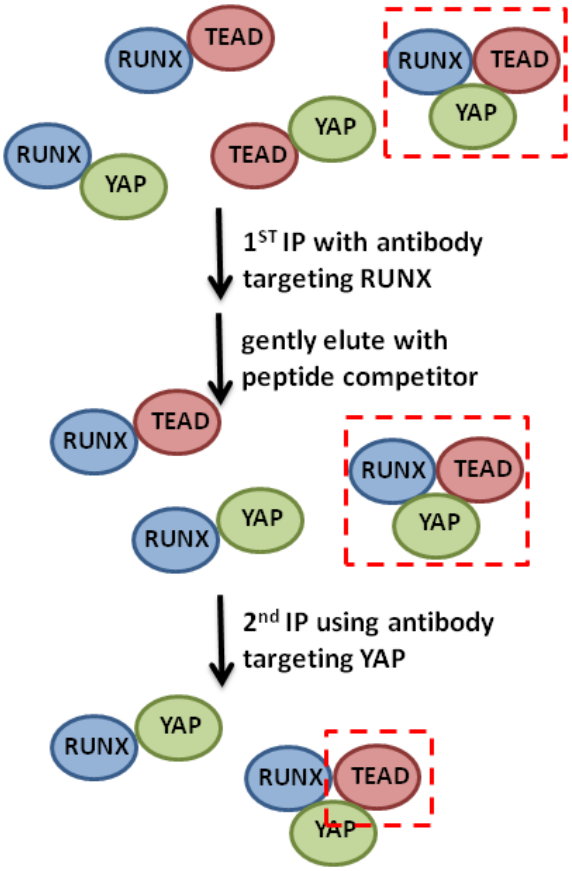


Figure 3.7 A diagram showing the work-flow of sequential IP using RUNX3, YAP and TEAD4 proteins. All protein complexes which contained RUNX3 would be precipitated first, and they would be eluted mildly from the beads by peptide competition. Then the second round of IP was performed in this eluted mixture using an antibody targeting YAP. If three proteins could coexist in the same complex, the TEAD4 protein should still be detectable after these two rounds of precipitations. Otherwise, it indicated that RUNX3-TEAD interaction and RUNX3-YAP interaction are mutually exclusive.

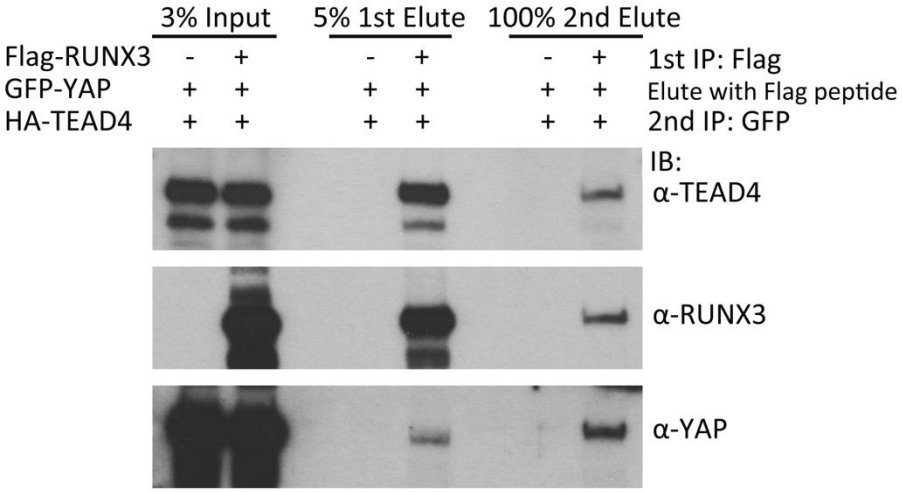


Figure 3.8 Flag-RUNX3, GFP-YAP and HA-TEAD4 proteins coexist in the same complex. In this sequential IP experiment, all three proteins were synthesized *in vitro* and mixed in IP buffer. First round of IP was performed using M2-Flag agarose beads targeting Flag-YAP, the precipitants were eluted by incubating the beads with 3X Flag peptide. The second round of IP was performed using GFP agarose beads targeting GFP-RUNX3 in the eluted products. After two rounds of IP, HA-TEAD4 was still detectable in the second IP product.

3.9 The mutation at Leucine^{121} of RUNX3 can abolish its interaction with TEAD proteins

RUNX3 is highly versatile, and its interaction with TEAD proteins might only represent a small part of its functions. For a better evaluation of the biological functions of RUNX3-TEAD interaction, a proper mutant RUNX3 control should be used to prevent potential bias caused by other functions of RUNX3. The ideal mutant RUNX3 control should fulfill the following two requirements: first, it can not interact with TEAD proteins; second, it still retains other features of wild-type RUNX3.

In this experiment, several RUNX3 mutants with point mutations in the Runt domain were tested by immunoprecipitation to see whether these mutations could abolish RUNX3's interaction with TEAD proteins. RUNX3's other functions including heterodimerization with CBFβ, the most important co-factor which RUNX3 required for a stable DNA binding and its proper functions as a transcription factor, as well as RUNX3's ability to trigger the transcription of its target genes, were tested via immunoprecipitation and dual-luciferase reporter assay.

In the first immunoprecipitation experiment measuring RUNX3 mutants' interaction with TEAD proteins, there were four Runt domain mutants showing weaker binding to TEAD proteins (**Fig** 3.9). Among them, $RUNX3_{L121H}$ mutant and $RUNX3_{R122C}$ mutant could not interact with TEAD proteins at all, while $RUNX3_{R143Q}$ and $RUNX3_{G145E}$ showed partially impaired binding with TEAD proteins.

In the second immunoprecipitation experiment measuring CBFβ binding ability, $RUNX3_{L121H}$ mutant exhibited similar binding to CBFβ, compared with wild-type RUNX3 (**Fig** 3.10).

The dual-luciferase reporter assay, which measured RUNX3's ability to trigger the expression of downstream genes, showed that the mutation of $Leucine^{121}$ to either Histidine or Proline did not reduce RUNX3's ability to drive the expression of genes by P14 promtor or IgCα promoter (**Fig** 3.11).

Taken together, $RUNX3_{L121H}$ was chosen as the best mutant control for the future study about the biological functions of RUNX3-TEAD interaction, due to the fact that this mutation can totally abolish RUNX3's binding with TEAD proteins while it still retains RUNX3's basic functions to heterodimerize with CBFβ and to trigger the expression of downstream genes.

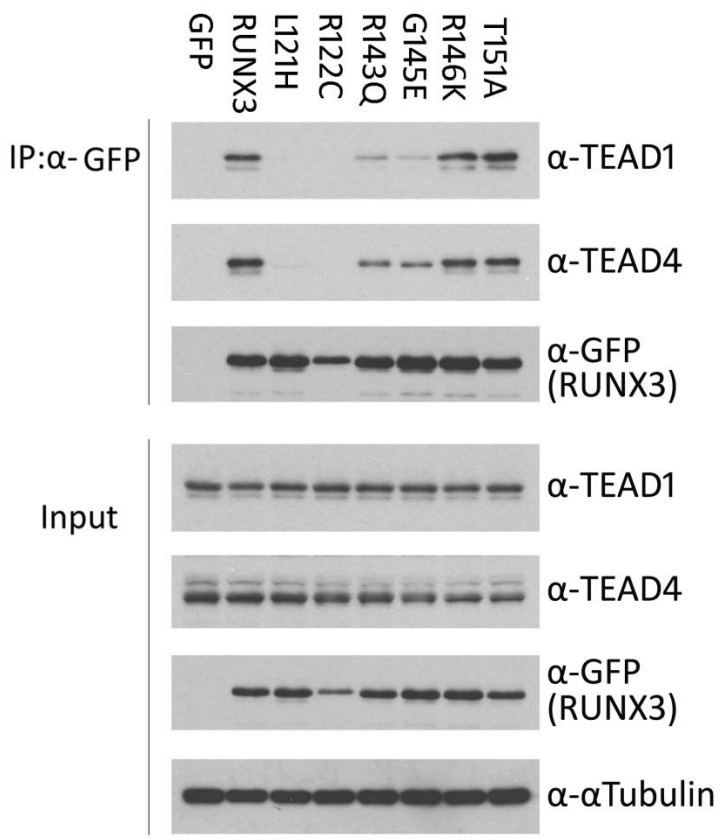


Figure 3.9 $RUNX3_{L121H}$ and $RUNX3_{R122C}$ can not interact with TEAD proteins, while $RUNX3_{R143Q}$ and $RUNX3_{G145E}$ show impaired binding with TEAD1/4. HEK293T cells were transfected with the RUNX3 mutants (1μg/10cm dish) accordingly. Cells were harvested at 24 hours after transfection and IP was performed using GFP agarose beads. The result showed that $RUNX3_{L121H}$ mutant and $RUNX3_{R122C}$ mutant could not interact with TEAD proteins at all, while $RUNX3_{R143Q}$ and $RUNX3_{G145E}$ showed partially impaired binding with TEAD proteins.

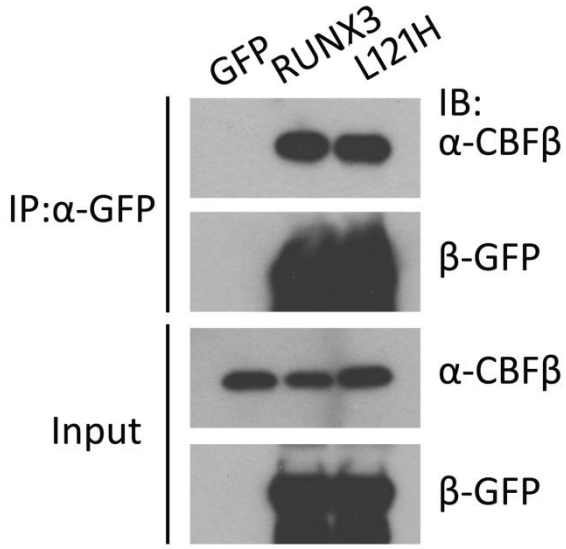


Figure 3.10 $RUNX3_{L121H}$ mutant and wild-type RUNX3 exhibit similar binding to CBFβ. HEK293T cells were transfected with the RUNX3 constructs (1μg/10cm dish) accordingly. Cells were harvested at 24 hours after transfection and IP was performed using GFP agarose beads. The result showed that $RUNX3_{L121H}$ mutant and wild-type RUNX3 showed similar binding to CBFβ.

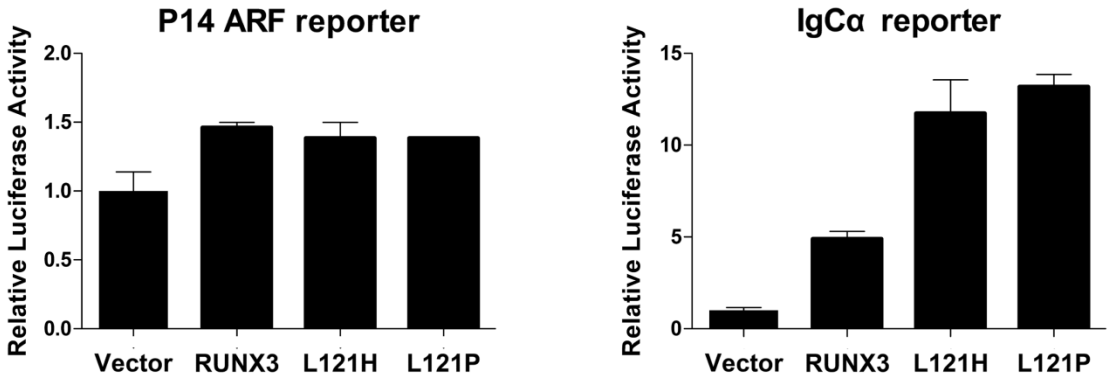


Figure 3.11 The mutation of Leucine[121] to either Histidine or Proline does not reduce RUNX3's ability to drive the expression of genes triggered by p14 promtor or IgCα promoter. HEK293T cells were transfected with reporter plasmids and RUNX3 mutant plasmids accordingly. The cells were harvested at 24 hours after transfection. The promoter activity were determined by dual-luciferase assay.

3.10 Summary and discussion

In this chapter, the endogenous IP using RUNX3 antibody showed that TEAD1 and RUNX3 coexisted in the same protein complex (**Fig** 3.1). Then RUNX3-TEAD's co-IP experiment using RUNX3 mutants lacking YAP binding ability was employed to show that TEAD-RUNX3 interaction was a novel protein-protein interaction which was independent on YAP-RUNX3 interaction (**Fig** 3.2). Mapping experiments using both truncated mutants as well as purified proteins showed that the Runt domain of RUNX3 as well as residues 101-125 of TEAD4 were crucial for TEAD4-RUNX3 interaction (**Fig** 3.3 - 3.6).

TEA domain is responsible for TEAD proteins' recognition and binding of consensus sequence, which is crucial for the transcriptional function of TEAD proteins. The structure of TEA domain has already been solved (Anbanandam et al, 2006). It is a three-helix bundle with a homeodomain fold (**Fig** 3.12A). For TEAD4, residues 47-57, residues 78-88 and residues 97-109 form three helixes, among which the third one is the DNA-recognition helix (Anbanandam et al, 2006). The sequence of the DNA-recognition helix and the key sequence required for TEAD4-RUNX3 interaction are compared in **Fig** 3.12B. Interestingly, a considerate portion of this helix (residues 97-109) locates within the key sequence (residues 101-125) which TEAD4 requires for its interaction with RUNX3, suggesting that RUNX3 might have an influence on the DNA binding of TEAD4. This hypothesis would be further explored in the next chapter.

RUNX3's Runt domain is a highly versatile domain, which is responsible for binding with DNA, heterodimerizing with CBFβ and interacting with

multiple partners like Bmi-1 (B lymphoma Mo-MLV insertion region 1 homolog), GATA-1 (GATA-binding factor 1) and LEF-1 (Lymphoid enhancer-binding factor-1) (Elagib et al, 2003; Howcroft et al, 2005; Yu et al, 2012b). The structure of Runt domain (RUNX1, residues 50-183) has already been solved (Ito, 2004; Nagata & Werner, 2001; Warren et al, 2000). It forms a 12-tranded (10 antiparallel and two parallel strands) β-barrel that adopts an s-type immunoglobulin (Ig) fold (**Fig** 3.13). According to our data, the RUNX3's Leucine^{121} and Arginine^{122} are critical for RUNX3-TEAD interaction, whose conservative sites should be Leucine^{117} and Arginine^{118} in RUNX1. Both of these residues localize in the Runt domain's loop βC-D, which has not been implicated in DNA binding. This loop makes a number of contacts with CBFβ through the side chain of Tyrosine^{113}, while the rest amino acid residues are available for making interactions with other proteins (Nagata & Werner, 2001; Warren et al, 2000).

According to the sequential IP result, RUNX3, TEAD4 and YAP could coexist in the same complex. The schematic diagram below is a summary of all possible interactions among these three proteins (**Fig** 3.14). RUNX3 and YAP interact through PY motif of RUNX3 and WW domain of YAP. RUNX3 and TEAD4 interact through Runt domain of RUNX3 and residues 101-125 of TEAD4. The C-terminal sequence of TEAD4 interacts with YAP at the TEAD binding domain which is located at the N-terminal of YAP. Even though it is shown here that three proteins can form stable ternary complex, more complicated regulation might exist to modulate the binding preference of each protein in different biological contexts.

RUNX3's ability of binding to TEAD-YAP complex instead of TEAD alone is important, due to the fact that TEAD-YAP complex is the transcriptionally active form which is able to drive the expression of target genes promoting proliferation and metastasis, while TEAD proteins alone do not have any intrinsic transcription activation domain (Jiang & Eberhardt, 1996; Xiao et al, 1991). RUNX3's role in this ternary complex would be further explored in the next chapters.

The study of TEAD proteins' partners has been going on for decades, and several proteins have been found to be interacting with TEAD. For example, myc-associated factor X (Max) interacts with TEAD1 to trigger the expression of cardiac alpha-myosin heavy-chain (α-HMC) gene (Gupta et al, 1997). The steroid receptor co-activator-1 (SRC-1) is also able to increase the transcriptional activity of TEAD2 by 2-3 folds (Belandia & Parker, 2000). On the other hand, TATA-binding Protein (TBP) binds to TEAD1 to repress chorionic somatomammotropin (hCS) promoter activity in choriocarcinoma (BeWo) cells (Jiang & Eberhardt, 1996). Till the discovery of YAP, there has always been an argument about TEAD's co-activator which could universally and robustly trigger the transcriptional activity of TEAD proteins (Vassilev et al, 2001).

TAZ is homologous to YAP and shares many functional similarities with YAP. TAZ heterodimerizes with TEAD proteins in a similar manner with YAP, and it also contains conserved WW domain (Mahoney et al, 2005). Our finding about RUNX3's interaction with TEAD-YAP complex should also be applicable

for TEAD-TAZ complex, even though no experiments have been performed to prove it.

These days, a new pursuit looking for the repressor for TEAD-YAP complex has begun. Recently, vestigial-like family member 4 (Vgll4) is discovered to antagonize YAP by competitively binding to TEAD in a similar manner with TEAD-YAP interaction (Jiao et al, 2014; Zhang et al, 2014). This discovery offers us a new understanding of how TEAD-YAP activity is regulated in cell nucleus. Like Vgll4, RUNX3 is also a nuclear protein. It would be interesting to test whether RUNX3-TEAD interaction also plays a regulatory role for TEAD-YAP activity.

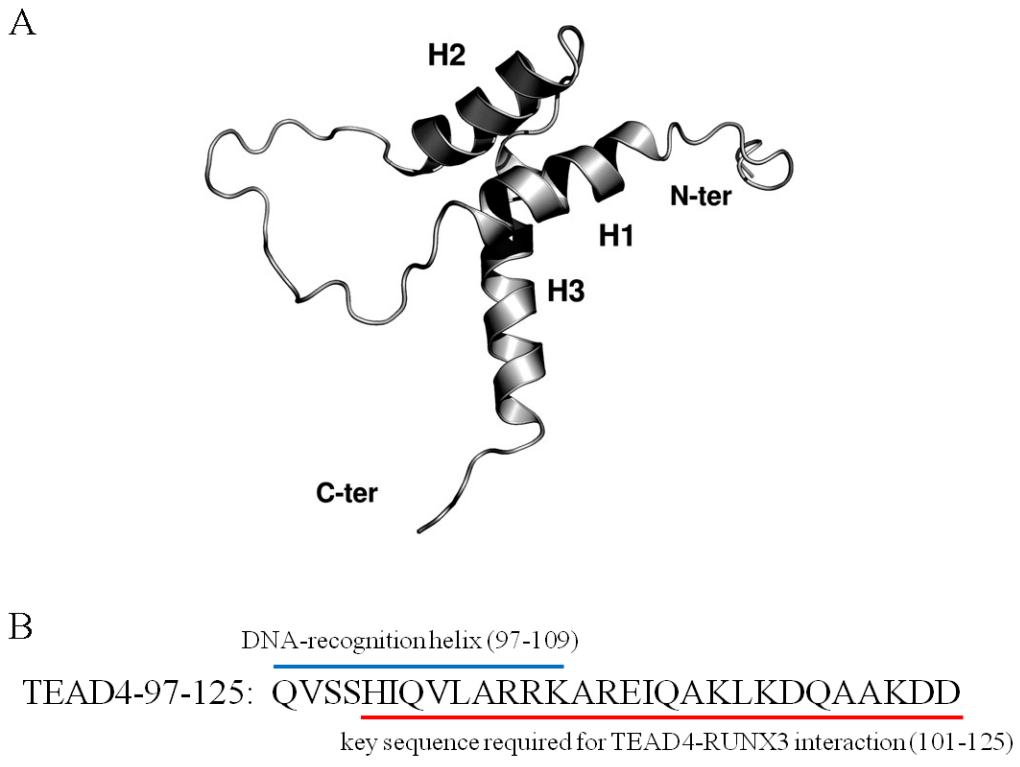


Figure 3.12 Structure of TEA domain. (A) Solution NMR structure of TEA domain. Ribbon diagram shows front view of TEA domain's fold in which helices H1 and H2 pack against H3. N and C termini are labelled (Anbanandam

et al, 2006). (B) The sequence of the TEAD4's DNA-recognition helix and the key sequence required for TEAD4-RUNX3 interaction.

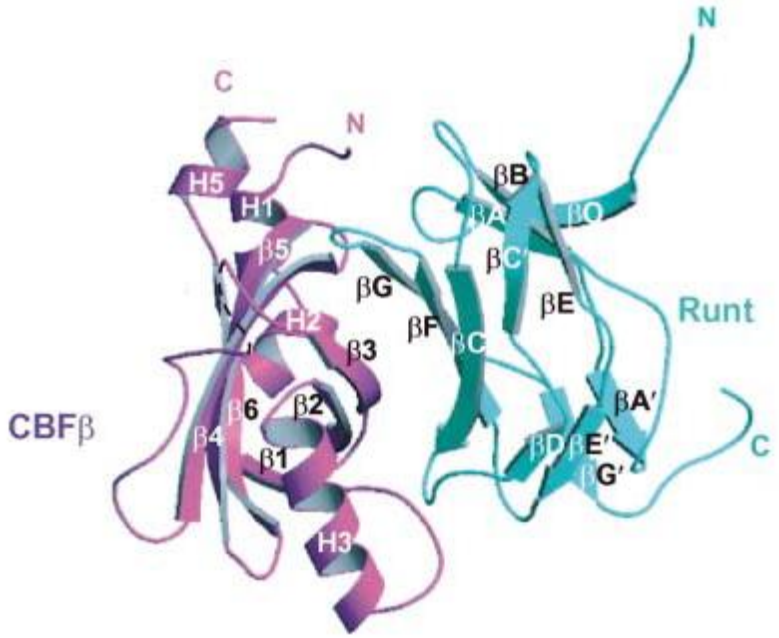


Figure 3.13 Solution NMR structure of Runt domain (cyan) and CBFβ (magenta), viewed perpendicular to the long axis of CBFβ. The concave surface of the Runt domain β-sheet, formed strands βG, βF and βC, packs against the complementary convex strand β3 of CBFβ (Warren et al, 2000).

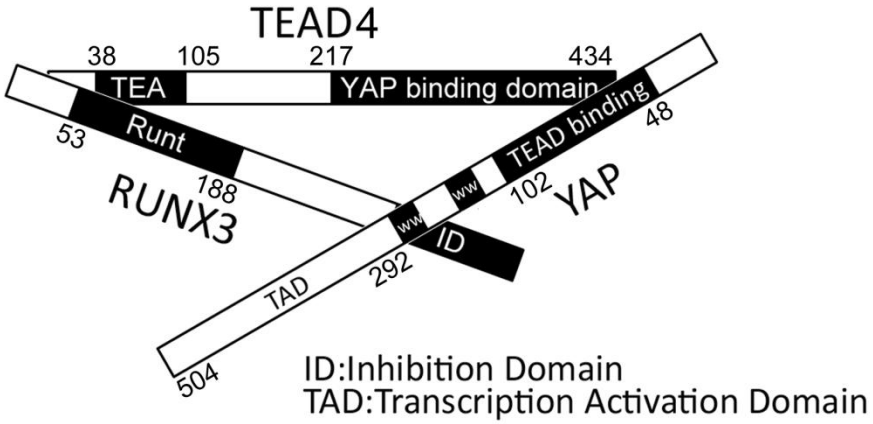


Figure 3.14 A diagram showing all possible interactions among RUNX3, TEAD4 and YAP. RUNX3 interacts with TEAD4 through Runt domain at the N-terminus, while interacting with YAP via the PY motif at the C-terminus, forming a ternary complex.

Chapter 4

RUNX3 negatively regulates TEAD proteins' transcriptional activity by direct protein-protein interaction

4.1 Introduction

In the previous chapter, the novel protein-protein interaction between RUNX3 and TEAD proteins was discovered. However, the consequence of this interaction still remained unknown.

In broad terms, RUNX3 tends to reduce cell growth by various mechanisms and TEAD proteins can trigger the expression of genes promoting cell proliferation as well as migration. It seems that RUNX3 and TEAD proteins play opposite roles for cell growth, which suggests that RUNX3 might inhibit the function of TEAD proteins.

Here, to test the hypothesis that RUNX3 could inhibit the transcriptional activity of TEAD proteins, a series of experiments were designed, including luciferase reporter assay, EMSA and transcription factor enrichment assay. In the last part of previous chapter, the $RUNX3_{L121H}$ mutant was identified as an ideal mutant lacking the ability to interact with TEAD proteins while retaining other major functions of RUNX3, which would be widely used in this chapter.

4.2 RUNX3 can reduce the transcriptional activity of TEAD-YAP complex

A TEAD luciferase reporter plasmid containing eight TEAD binding boxes can be used to measure the transcriptional activity of TEAD proteins in cells by measuring the intensity of light generated by the luciferase enzyme whose expression is driven by TEAD proteins (**Fig** 4.1A). Here, this reporter plasmid was used to test whether RUNX3 had any influence on the transcriptional activity of TEAD.

According to the result of dual-luciferase reporter assay in which RUNX3 was overexpressed in HEK293T cells at increasing doses, wild-type RUNX3 could reduce the transcriptional activity of TEAD4 at a dose dependent manner, while $RUNX3_{L121H}$ (RUNX3 mutant which could not interact with TEAD proteins) could not, indicating that RUNX3's inhibitory effect on the transcriptional activity of TEAD4 is highly dependent on RUNX3's direct interaction with TEAD4 (**Fig** 4.1B, C).

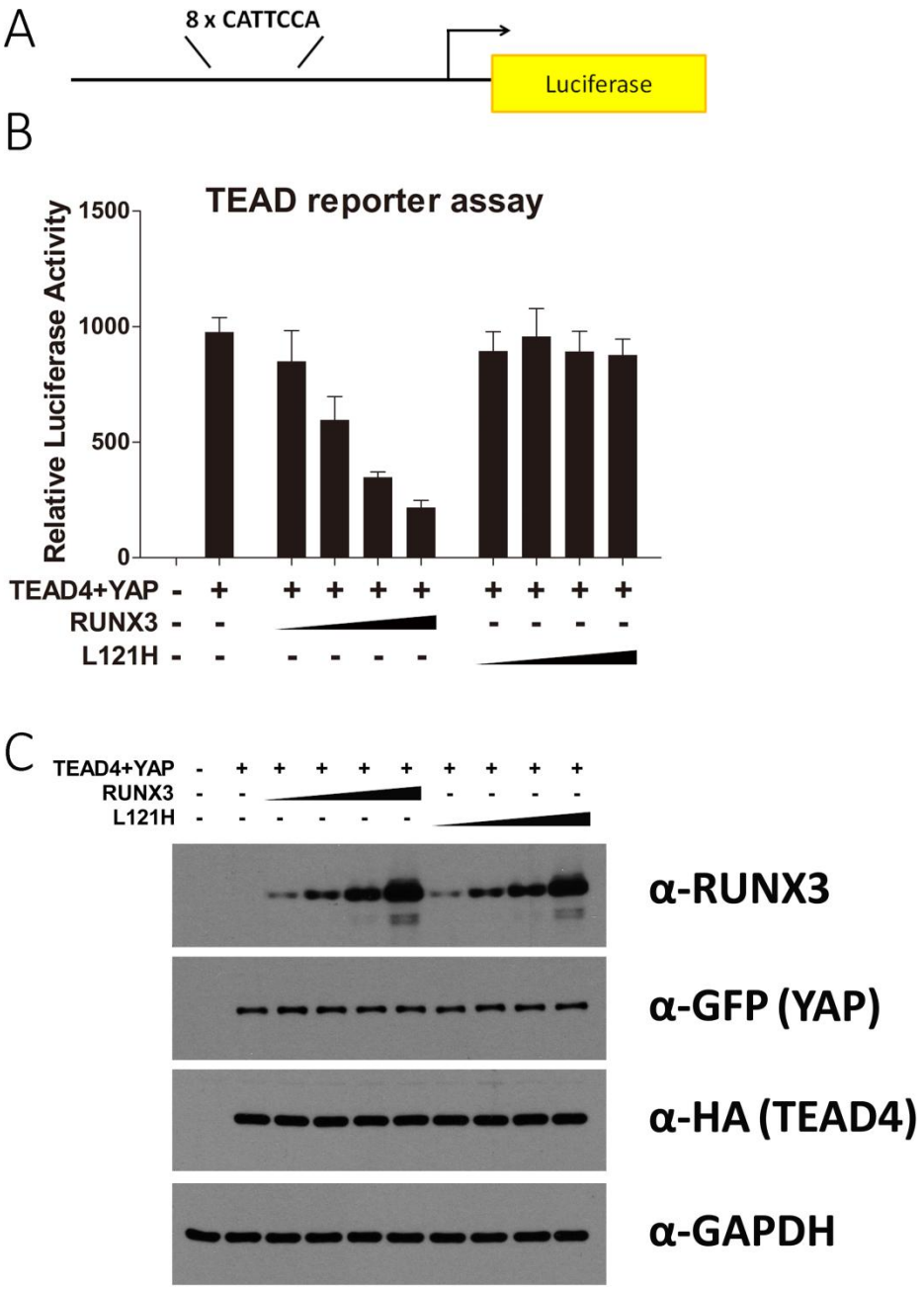


Figure 4.1 RUNX3 reduces the transcriptional activity of TEAD-YAP complex. (A) Schematic representation of TEAD luciferase reporter with 8 TEAD binding boxes in pGL3 vector. (B) Dual-luciferase reporter assay measuring TEAD's transcriptional activity after overexpressing wild-type RUNX3 or $RUNX3_{L121H}$ mutant at increasing doses in HEK293T. Wild-type RUNX3 could reduce the transcriptional activity of TEAD4 at a dose dependent manner, while $RUNX3_{L121H}$ could not. (C) Western blot result of cell lysates from dual-luciferase reporter assay measuring TEAD's transcriptional activity after overexpressing wild-type RUNX3 or $RUNX3_{L121H}$ mutant.

4.3 Overexpression of RUNX3 does not have any negative effect on the interaction between TEAD and YAP

In previous experiments, it had already been shown that RUNX3 could reduce the transcriptional activity of TEAD4 at a dose dependent manner. Since the transcriptional activity of TEAD proteins was highly dependent on its binding with its co-activator YAP, it was worthy to study whether RUNX3 could impair the binding between TEAD and YAP. The interaction between TEAD and YAP could be evaluated using immunoprecipitation method.

According to the result of TEAD-YAP_{S127A} co-immunoprecipitation assay in which RUNX3 was overexpressed at different doses, the interaction between YAP_{S127A} and TEAD4 did not show any significant reduction, indicating that RUNX3 does not interrupt YAP-TEAD interaction (**Fig** 4.2 A, B). This result suggested that RUNX3 reduces the transcriptional activity of TEAD by other means.

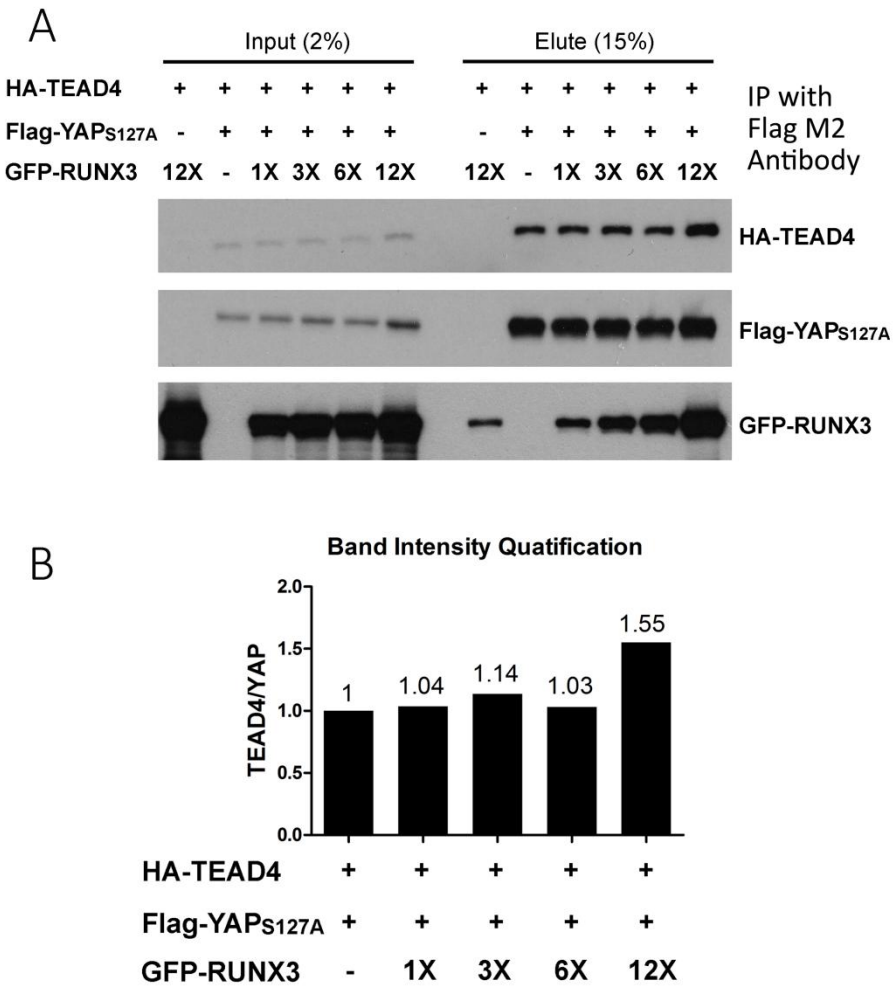


Figure 4.2 RUNX3 overexpression does not interrupt the interaction between TEAD and YAP_{S127A}. (A)Western blot result of TEAD-YAP_{S127A} co-immunoprecipitation assay in which RUNX3 was overexpressed at different doses. HEK293T cells were transfected with the pBABE-HA-TEAD4 (1 μg/10cm dish), pCDNA-Flag-YAP_{S127A} (1 μg/10cm dish) and pEGFP-C1-RUNX3 (0, 0.5, 1.5, 3, 6 μg/10cm dish) accordingly. Cells were harvested at 24 hours after transfection and IP was performed using M2-Flag agarose beads. The IP result showed that TEAD4 could be equally pulled down by YAP_{S127A} while the overexpression doses of RUNX3 increased. (B) Band intensity quatification for the Western blot result. The ratio between precipitated YAP_{S127A} and TEAD4, which reflected the binding between YAP_{S127A} and TEAD4, did not show any significant reduction as the doses of RUNX3 overexpression increased.

4.4 RUNX3 can reduce the TEAD4-1-125's interaction with DNA *in vitro*

EMSA (Electrophoretic mobility shift assay) is a traditional method to study the binding between proteins and DNA *in vitro*. Here, we utilized this method to study whether RUNX3 could affect the binding between TEAD4-1-125 (containing the DNA recognition helix of TEAD4) and the consensus sequence of DNA which TEAD proteins usually recognized.

GST-HA-TEAD4-1-125 protein was purified using E.Coli and GSH beads as described previously in Chapter 2.15. HA-TEAD4-1-125 was cleaved from GSH beads by Thrombin digestion (0.1U/ml) for 1 hour at room temperature. Flag-RUNX3 protein was expressed in HEK293T and precipitated using M2-Flag beads as described in chapter 2.5. Then RUNX3 protein was eluted from the beads by incubating with 3XFlag peptide (500 μg/ml) at 4 ℃ for 1 hour. EMSA was performed using LightShift Chemiluminescent EMSA Kit (Thermo) as described in Chapter 2.8.

According to the result of EMSA experiment, the shifted band could be super-shifted by the adding of HA antibody, ensuring that this band shift was caused by the binding of HA-TEAD4-1-125 (as shown in the first lane of **Fig** 4.3). When high doses of RUNX3 was added to the reaction mix, the shift band became weaker and finally disappeared, indicating that RUNX3 could reduce the DNA binding ability of TEAD proteins. This phenomenon was not observed at low dose of RUNX3, which might be due to the amount of RUNX3 protein was not high enough to generate any obvious competition effects (**Fig** 4.3).

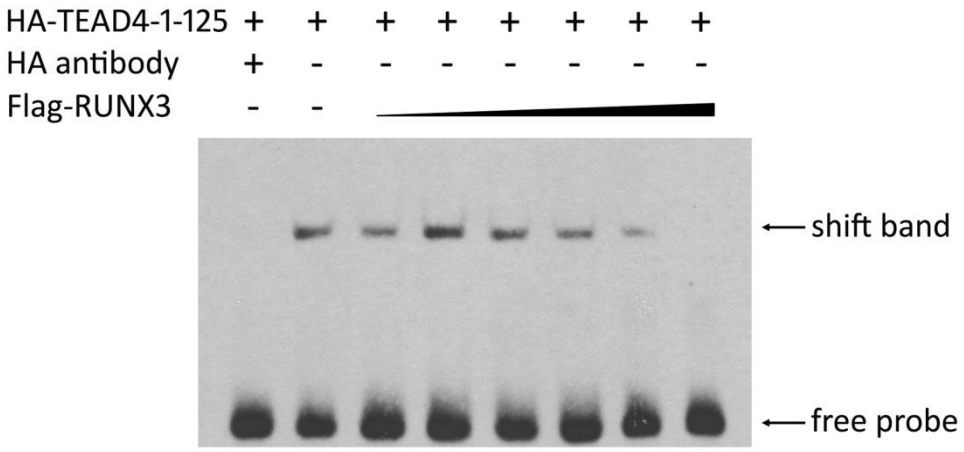


Figure 4.3 RUNX3 could reduce the binding between TEAD4-1-125 and TEAD probe. The band shift was caused by the binding of HA-TEAD4-1-125. Increasing doses of purified RUNX3 were added to the EMSA reaction mix. The shifted bands were weakened and finally disappeared at high doses of RUNX3.

4.5 RUNX3 reduces TEAD1's interaction with DNA *in vitro*

Transcription factor enrichment assay is an alternative way to evaluate the binding between DNA and transcription factors. In this assay, the synthesized DNA probe is immobilized on beads and incubated with cell lysis, so all the proteins which can bind to this probe will be precipitated together with the beads and they can be analyzed by techniques like Western blot and silver staining.

Here we performed this transcription factor enrichment assay to study whether RUNX3 could reduce the DNA binding ability of endogenous TEAD1. In this experiment, HEK293T cells were transfected with RUNX3 or RUNX1 plasmids, and nuclear fraction was extracted and incubated with beads coated with synthesized TEAD probe or mutated TEAD probe. The precipitants were analysis by Western blot to measure the amount of endogenous TEAD1 which could bind to the TEAD probes.

According to the result of this experiment, wild-type RUNX3 significantly reduced the amount of TEAD1 protein which bound to the TEAD probe, while $RUNX3_{L121H}$ mutant could not achieve this (**Fig** 4.4). This observation showed that RUNX3 reduces the endogenous TEAD1-DNA interaction, supporting the conclusion we obtained from the previous EMSA experiment, which was done using recombinant TEAD4-1-125 protein. Similar results could be obtained using RUNX1 constructs (Leucine^{117} of RUNX1 is conserved to Leucine^{121} of RUNX3), since the Runt domain is highly conserved between RUNX1 and RUNX3. This result was predicted and it suggested that RUNX1 can also disrupt the interaction between TEAD1 and DNA.

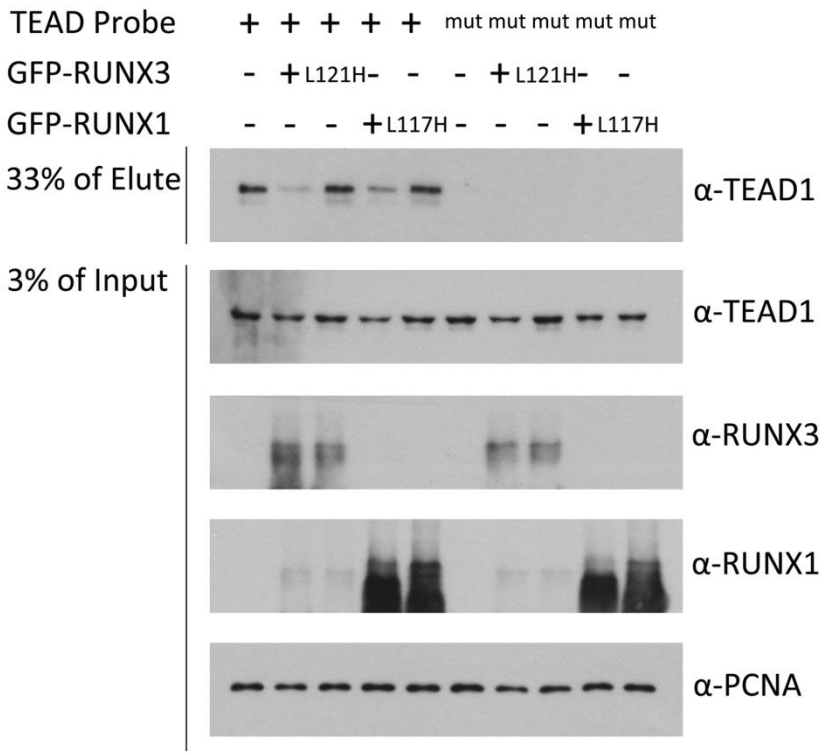


Figure 4.4 RUNX3 reduces the endogenous TEAD1-DNA interaction. HEK293T cells were transfected with RUNX3 or RUNX1 plasmids (1 μg/6cm dish), and nuclear fraction was extracted and incubated with beads coated with synthesized TEAD probe or mutated TEAD probe. The precipitants were analyzed by Western blot to measure the amount of endogenous TEAD1 proteins which bound to TEAD probe. Western blot result showed that wild-type RUNX3 and RUNX1 could reduce the DNA binding ability of TEAD1, while $RUNX3_{L121H}$ or $RUNX1_{L171H}$ mutants could not achieve this.

4.6 Summary and discussion

In this chapter, the dual-luciferase reporter assay using TEAD reporter showed that RUNX3 reduced the transcription activity of TEAD4 (**Fig** 4.1). However, the *in vitro* competition assay showed that the protein-protein interaction between TEAD4 and YAP remained intact (**Fig** 4.2). In contrast, both EMSA experiment and transcription factor enrichment assay showed that RUNX3 reduced the DNA binding ability of TEAD proteins (**Fig** 4.3, 4.4).

All these observations led to the final conclusion that RUNX3 can impair the DNA binding ability of TEAD4 by direct protein-protein interaction which masks the DNA binding surface of TEAD4, and reduce the transcriptional activity of TEAD4.

Protein-protein interaction is an important part for the regulation of transcription. Even though the majority of transcription factors are initially characterized as a stimulating factor for gene expression, more and more evidence shows that inhibitory transcription factors play an equally important role in the regulation of gene expression (Harrison, 1991). There are several ways for the inhibitory transcription factors to suppress an activating factor: (1) interfering with the DNA binding of activating factors by reorganising the chromatin structure, masking the binding site, or forming a protein-protein complex that does not bind to DNA (Hagen et al, 1994; Yang-Yen et al, 1990; Zink & Paro, 1995); (2) quenching the transcription activation by competing with transcription co-activators (Wagner & Green, 1991). Obviously, our finding about RUNX3's regulation on TEAD activity belongs to the first category, while the VGLL4, which interrupts TEAD-YAP interaction by

occupying YAP binding domain of TEAD proteins, belongs to the second category (Jiao et al, 2014; Zhang et al, 2014). The comparison between RUNX3 and VGLL4 is summarized in **Fig** 4.5.

RUNX3 is a highly versatile transcription factor, which is able to activate or suppress the expression of target genes in different biological contexts (Wheeler et al, 2002). Furthermore, RUNX3 can suppress gene expression in multiple ways. For example, RUNX3 is able to bind to SUV39H1 (Histone-lysine N-methyltransferase SUV39H1), which can recruit histone methyltransferases, contributing to the silencing of target genes including *CD4* (cluster of differentiation 4), *VEGF* (Vascular Endothelial Growth Factor) and *AKT1* (v-akt murine thymoma viral oncogene homolog 1) (Lin et al, 2012; Peng et al, 2006; Reed-Inderbitzin et al, 2006). RUNX3 is also able to associate with other transcription factors, forming complexes which have a lower DNA binding ability. For example, RUNX3 forms a ternary complex with β-catenin/TCF4 and attenuates Wnt signaling activity in intestinal epithelial cells (Ito et al, 2008). In The $CD4^+$ helper T cells, RUNX3 interacts with GATA3 and attenuates GATA3 transcriptional activity (Kohu et al, 2009). Our novel finding about the interaction between RUNX3 and TEAD proteins adds another example to this category.

Recently, RUNX3 was found to be able to physically interact with SAV1, and to form close association with MST2, leading to a higher activity of MST2/SAV1 to cause cell death (Min et al, 2012). This report mainly emphasized the role of RUNX3 in the upstream Hippo pathway, while our research mainly focused on RUNX3's influence on the effecters of Hippo

pathway. The EMSA experiment and the transcription factor enrichment experiment which we performed directly studied the DNA binding ability of TEAD proteins, without any bias from upstream signals of Hippo pathway. However, both upstream and downstream studies point to the same endpoint that RUNX3 can negatively regulate TEAD-YAP activity, indicating that RUNX3 is indeed having an important role in the regulation of TEAD-YAP activity at multiple levels.

Currently, our understanding about the regulation of TEAD-YAP activity is mainly focused on the localization of YAP. Hippo pathway, angiomotin and ZO-2 all negatively regulate the transcriptional activity of TEAD by confining YAP in cytoplasm either by physical interaction with YAP or phosphorylating YAP (Zhao et al, 2010a). One exception is VGLL4, which blocks TEAD-YAP interaction by occupying YAP binding domain in TEAD proteins (Jiao et al, 2014; Zhang et al, 2014) (**Fig** 4.5). Considering the vital role of TEAD-YAP complex in maintaining a proper homeostasis for metazoan, its regulation must be multilayered and error-proofing. The discovery of VGLL4's role filled a vacancy in our understanding of TEDA-YAP's regulation within cell nucleus. Now our discovery about RUNX3's role in blocking TEAD's binding to DNA reveals an alternative way of suppressing TEAD-YAP activity within cell nucleus, complementary to VGLL4.

According to our new understanding about the regulation of TEAD-YAP complex, there are three layers of regulations for an error-proofing control of TEAD-YAP activity: (1) restricting YAP's nuclear localization by multiple pathways; (2) altering the physical interaction between TEAD and YAP by

changing the amount of competitor for YAP; (3) limiting the DNA binding ability of TEAD proteins by RUNX3 (**Fig** 4.6). These three layers of regulations vigorously control the activity of TEAD-YAP complex at an extremely low level in the quiescent cells in adult tissues, whose malfunction is closely correlated with the development of cancer (Zhao et al, 2010a). The correlation between loss of Hippo pathway or VGLL4 expression with human cancers has already been discussed in Chapter 1.4.3, and RUNX3-TEAD interaction's role in gastric cancer would be discussed in the next chapter.

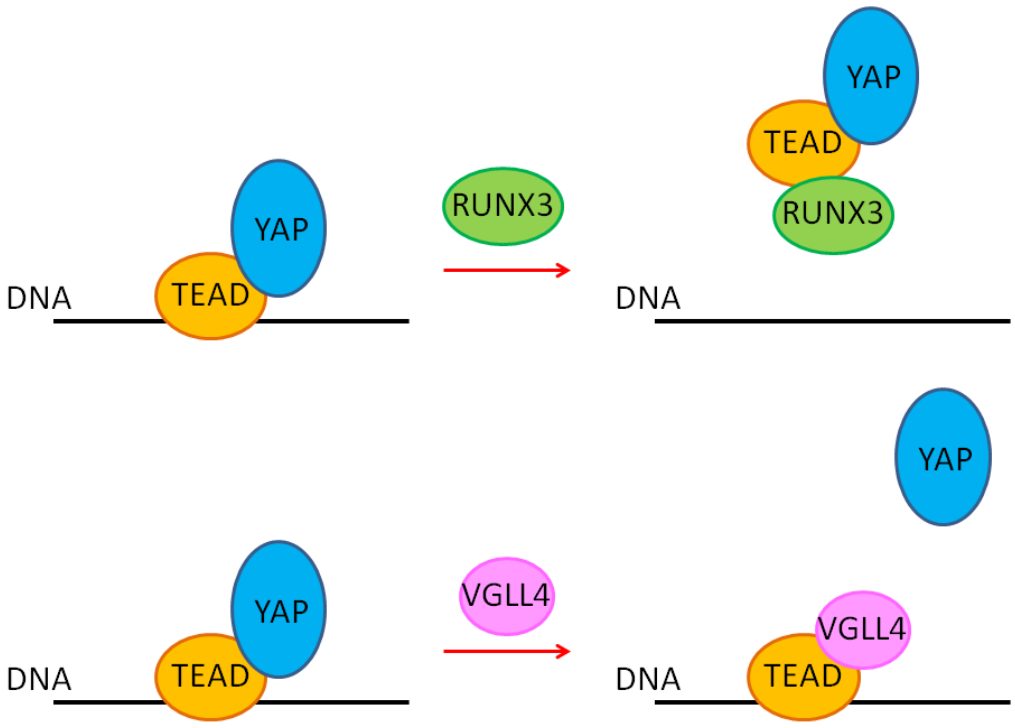


Figure 4.5 A comparison between RUNX3's and VGLL4's mechanisms of restricting TEAD-YAP activities. RUNX3 blocks TEAD proteins from binding to DNA, while VGLL4 blocks TEAD-YAP interaction by occupying YAP binding domain in TEAD proteins.

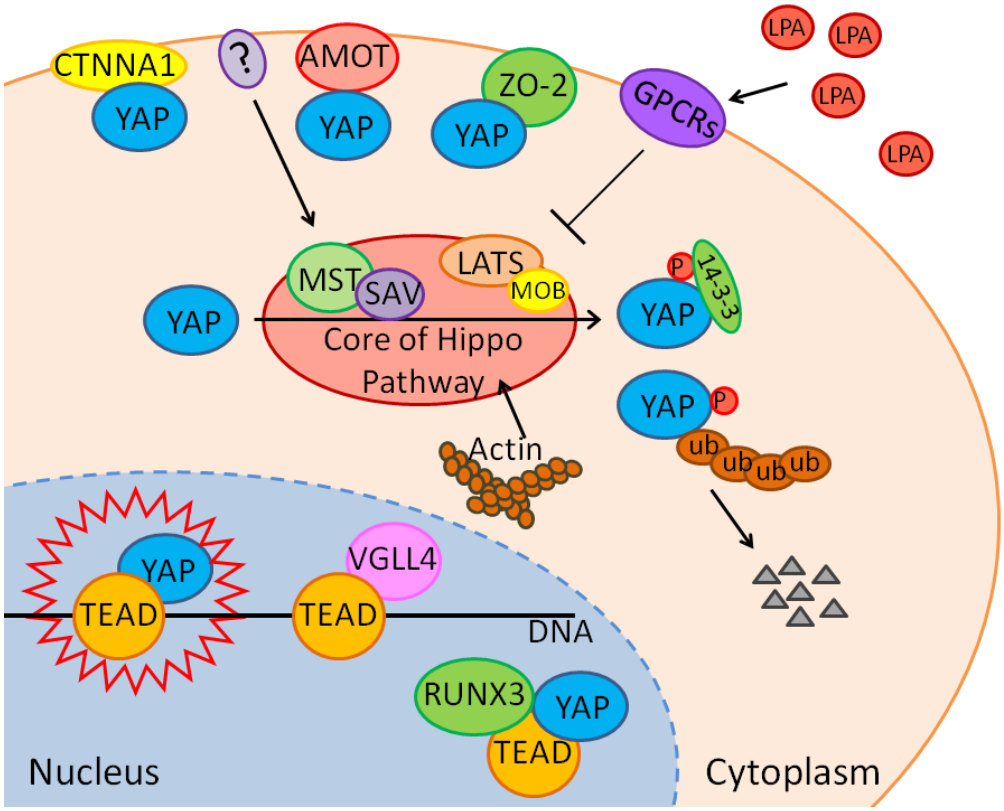


Figure 4.6 A summary of pathways and proteins regulating TEAD-YAP activity.

Chapter 5

The biological significance of RUNX3-TEAD interaction in gastric cancer

5.1 Introduction

Based on previous two chapters, RUNX3 can suppress the transcriptional activity of TEAD-YAP complex by preventing TEAD proteins from binding to DNA. This might be one of the novel mechanisms explaining how RUNX3 functions as a tumour suppressor, whose inactivation is frequently observed in gastric cancer.

In this chapter, the role of TEAD-YAP complex in gastric cancer would be examined first, since there were few reports studying this when we started this project in 2012. After we confirmed that TEAD-YAP complex was oncogenic in gastric epithelial cells, a series of experiments would be performed to test whether RUNX3 could inhibit the oncogenic activity of TEAD-YAP complex in the context of gastric cancer.

5.2 Knock down of TEAD or YAP causes growth retardation in a large portion of gastric cancer cell lines

TEAD-YAP complex has been shown to exhibit strong oncogenic activity in epithelial cells in various organs like liver, breast and colon (Zhao et al, 2010a). Recently, YAP's oncogenic role has been reported in stomach (Jiao et al, 2014; Lim et al, 2014). However, the mechanism by which TEAD-YAP complex is involved in gastric carcinogenesis is poorly understood. Since gastric cancer cells are highly heterogeneous (Ji et al, 2002), a comprehensive study on the role of TEAD and YAP in gastric carcinogenesis would be necessary.

We performed stable knock down of TEAD1/3/4 in 13 gastric cancer cell lines, by using a retrovirus carrying shRNA targeting TEAD1/3/4. Cell growth was monitored using WST-1 assay as described in Chapter 2.13. The relative cell viability was determined by calculating the ratio between the growth rate of cells carrying shTEAD1/3/4 and the growth rate of cells carrying shScramble.

At the same time, Western blot analysis was also performed using cell lysis extracted from these cell lines, and the expression levels of YAP, TEAD1, TEAD3 and TEAD4 were determined by measuring the band intensity. The bands' intensity was quantificated and the YAP-TEAD1/3/4 index was calculated as the sum of band intensity of YAP and TEAD1/3/4, normalized to the band intensity of GAPDH.

To validate our observations generated from stable knock down of TEAD proteins using shTEAD1/3/4, transient knock down of YAP and TEAD1 was also performed in 5 gastric cancer cell lines (MKN74, MKN28, AGS, HGC-27

and HS746T), and similar cell growth analysis was performed after these cells were treated with esiRNA targeting YAP and TEAD1.

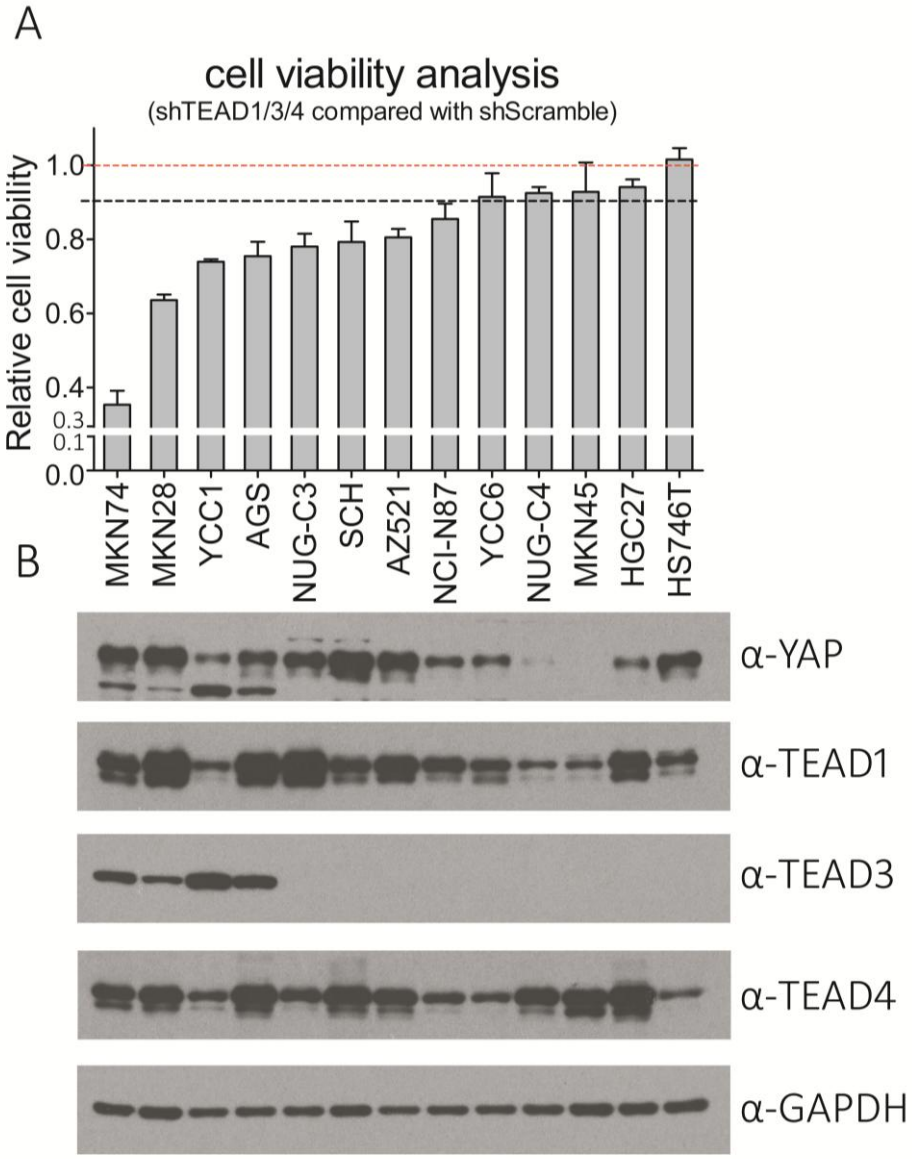
According to the results of cell growth analysis, 8 out of 13 gastric cancer cell lines (MKN74, MKN28, YCC1, AGS NUG-C3, SCH, AZ521 and NCI-N87) showed significant growth reduction after TEAD1/3/4's knock down, while the other 5 cell lines (YCC6, NUG-C4, MKN45, HGC-27 and HS746T) only exhibited minor response to the knock down (**Fig** 5.1A, D).

Similar observation could be achieved by transient knock down of TEAD1 using esiRNA. MKN74, MKN28 and AGS responded more clearly to TEAD1 knock down, compared with HGC-27 and HS746T (**Fig** 5.2A, B). Interestingly, knock down of YAP did not lead to exactly the same cell response compared with knock down of TEAD1. esiYAP caused a milder growth reduction than esiTEAD1 in MKN74, but it caused a much stronger growth reduction than esiTEAD1 in HS746T. In the rest of the three cell lines (MKN28, AGS and HGC-27), esiYAP and esiTEAD1 led to similar cell growth reduction (**Fig** 5.2A, B).

The Western blot analysis showed that most cell lines which responded well to TEAD's knock down had a relatively higher expression level of YAP and TEAD proteins, as indicated in the quantification figure of YAP-TEAD1/3/4 index (**Fig** 5.1B, C).

In summary, gastric cancer cell lines showed heterogenous responses to the stable knock down of TEAD1/3/4 as well as the transient knock down of YAP and TEAD1. Cell lines with high YAP and TEADs' expression tended to exhibit

significant growth inhibition with shTEAD1/3/4, while cell lines with low YAP and TEADs' expression did not respond appreciably to shTEAD1/3/4.



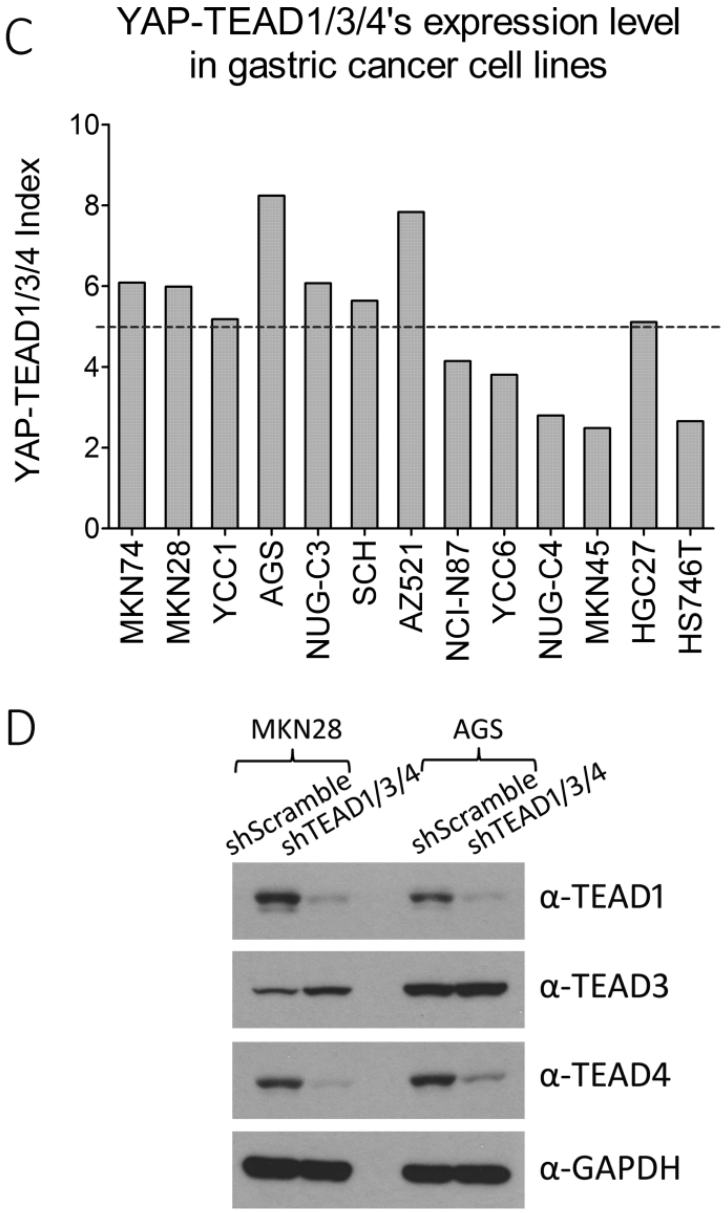


Figure 5.1 Knock down of TEAD1/3/4 causes growth retardation in a large portion of gastric cancer cell lines. (A) Cell growth analysis using WST-1 assay. The relative cell viability was determined by calculating the ratio between the growth rate of cells carrying shTEAD1/3/4 and the growth rate of cells carrying shScramble. (B) Western blot result of cell lysis extracted from gastric cancer cell lines. (C) The band intensity was quantificated and the YAP-TEAD1/3/4 index was calculated as the sum of band intensity of YAP and TEAD1/3/4, normalized to the band intensity of GAPDH. (D) Western blot result showing the knocking down efficiency of shTEAD1/3/4 in MKN28 and AGS cell lines.

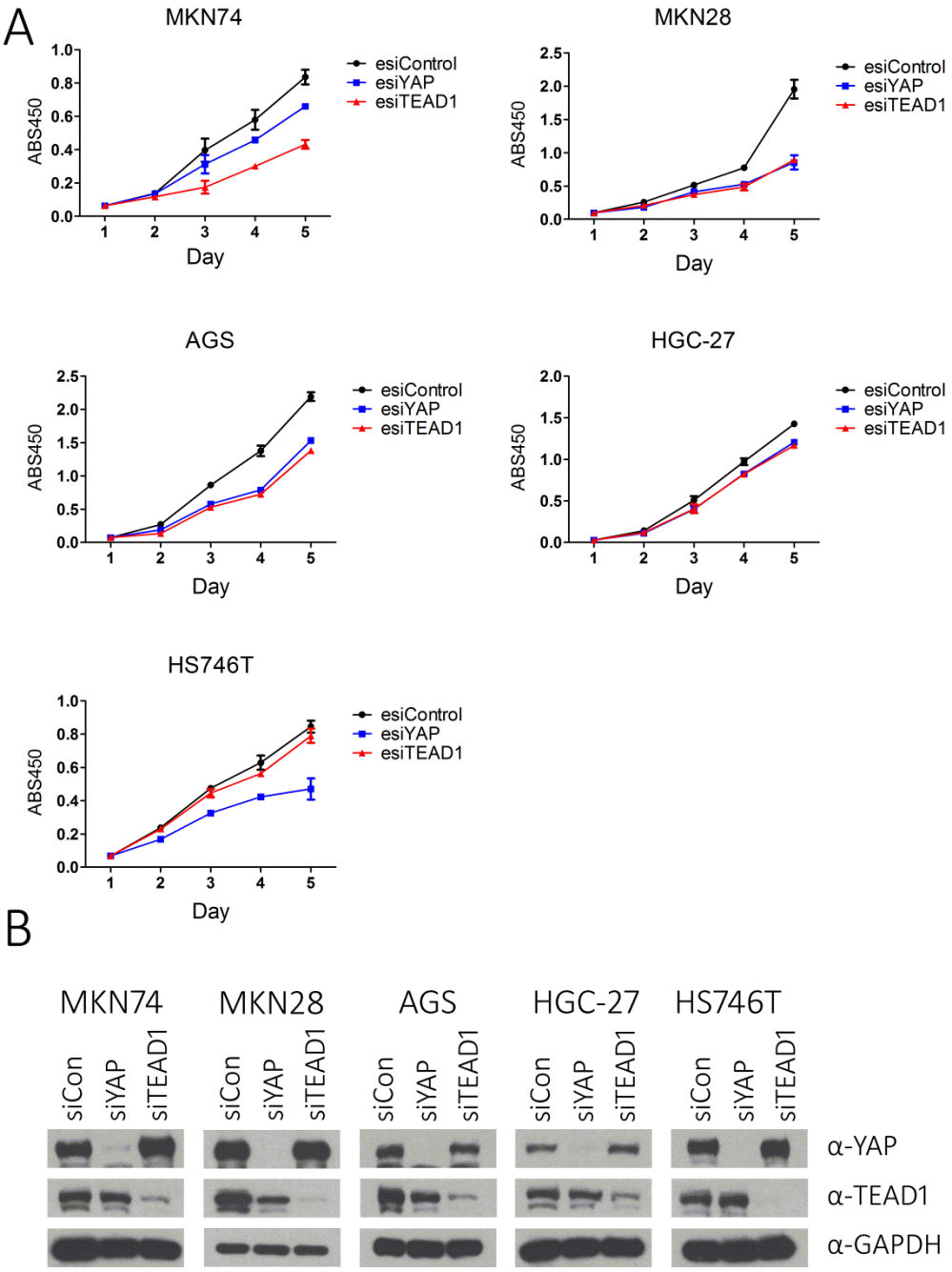


Figure 5.2 Knock down of YAP or TEAD1 by esiRNA reduces cell growth in gastric cancer cell lines. (A) Cell growth analysis using WST-1 assay. Cell lines were transfected with esiRNA accordingly, and seeded into 96-well plate for cell proliferation assay for 5 days. (B) Western blot results showing the knocking down efficiency in these cell lines.

5.3 YAP and TEAD1 are frequently overexpressed in gastric cancer specimen

IHC (Immunohistochemistry) is a standard method to study the protein expression in fixed tissues of clinical specimens. YAP has been reported to be overexpressed in many cancers, including liver, breast and colon cancers. YAP overexpression would directly lead to an enhanced transcriptional activity of TEAD proteins. Here, we performed IHC staining using YAP and TEAD1 antibodies to obtain expression levels as well as expression patterns of YAP and TEAD1 in gastric cancer samples.

50 pairs of gastric cancer specimens and matched normal gastric tissues were stained using YAP and TEAD1 antibodies as described in Chapter 2.6. The overall clinical information was summarized in the chart below (**Tab** 5.1).

The result of IHC experiment revealed that YAP could only be weakly stained in a small portion of normal gastric tissues, while YAP staining was extraordinarily strong in gastric cancer tissues. Overexpressed YAP was localized in both cytoplasms as well as cell nucleus (**Fig** 5.3A). The IHC scoring was performed for YAP staining, and the result of Student T-test showed that the expression of YAP was significantly up-regulated in gastric cancer tissues compared with adjacent normal stomach tissues (**Fig** 5.3B).

TEAD1 was ubiquitously expressed in the cell nucleus of most epithelial cells in both normal and cancer tissues. The signal was slightly stronger in gastric cancer tissues than normal tissues, but the difference was not significant (**Fig** 5.3A).

	variables	number of cases
age (years)	<=65	19
	>65	31
sex	male	32
	female	18
tumour size (mm)	<=50	28
	>50	21
staging	1 a&b, 2	22
	3,4	28
current H.pylori infection	positive	7
	negative	29
lauren classify	diffuse	16
	intestinal	26

Table 5.1 Summary of clinical information for patients offering gastric cancer specimen.

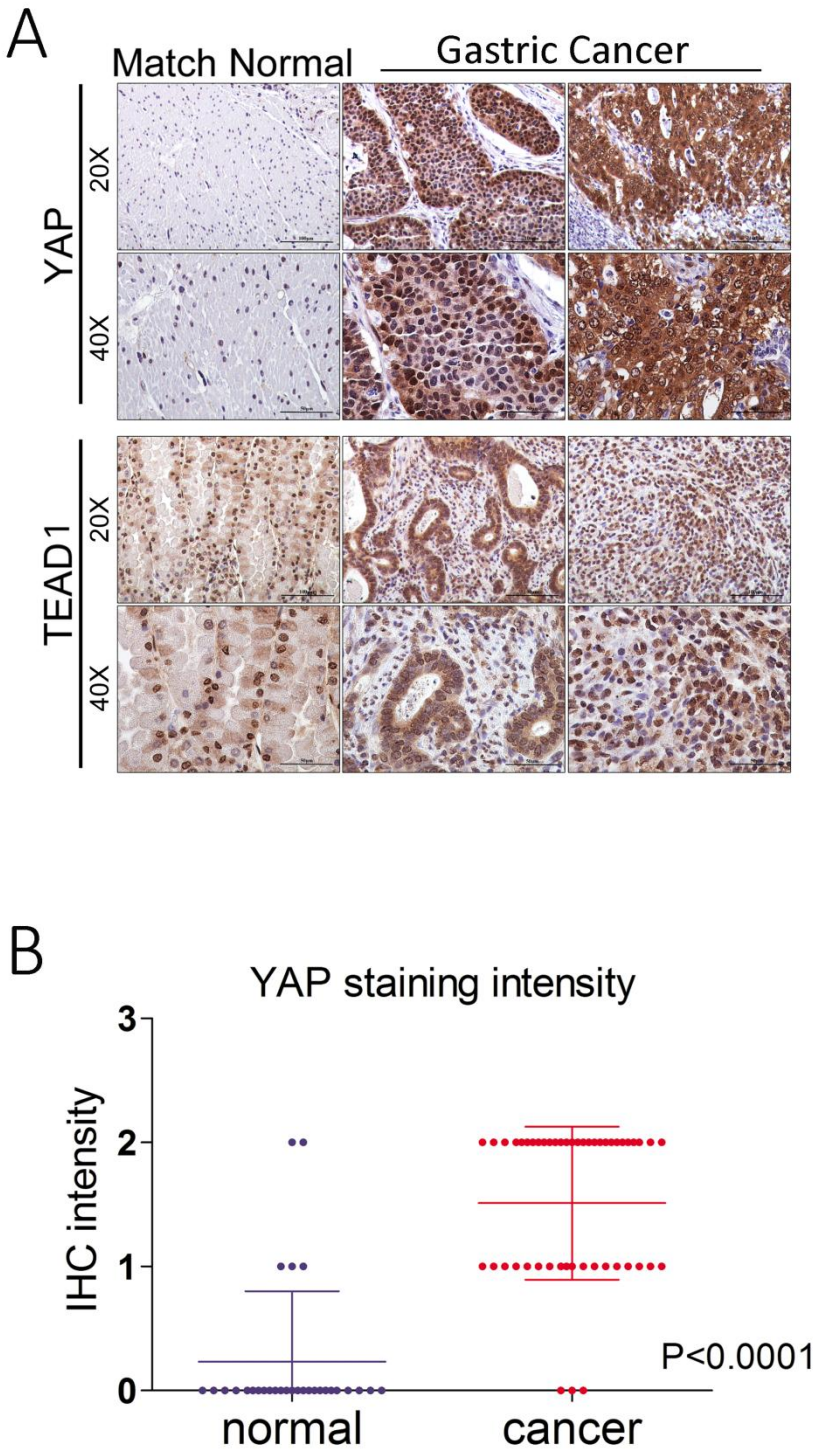


Figure 5.3 YAP and TEAD1 expressions are frequently up-regulated in gastric cancer, compared with adjacent normal stomach tissues. (A) Representative IHC staining results using YAP and TEAD1 antibodies in gastric cancer specimen or adjacent normal stomach tissues. (B) The student T-test result comparing the scoring results of YAP IHC staining intensity in both gastric cancer tissues and adjacent normal stomach tissues.

5.4 Higher YAP-TEAD mRNA expression is significantly correlated with poorer prognosis of gastric cancer patients

Higher mRNA expression of YAP and TEAD proteins would lead to an enhanced transcriptional activity of TEAD, which might have a negative influence on the prognosis of patients. Here we wanted to test this hypothesis by study whether the overall survival time was shorter for gastric cancer patients with higher TEAD-YAP expression.

Primary gastric cancer (GC) samples (n = 200) profiled on Affymetrix Human Genome U133 Plus 2.0 Genechip arrays (GSE15459) were preprocessed in a standard manner. After preprocessing, 185 GC samples were available for downstream analyses. GC samples were clustered using a K-medoids approach and Kaplan-Meier survival analysis, with overall survival as the outcome metric, was employed to compare GCs with "high" TEAD (TEAD1, TEAD2, TEAD3, TEAD4) and YAP gene expression to GCs with "low" expression of these genes. The log-rank test was used to assess the significance of the Kaplan-Meier analysis. Univariate and multivariate analyses were performed using Cox regression.

According to the result of clustering, 185 gastric cancer patients were clearly grouped into two groups according to the mRNA level of YAP, TEAD1, TEAD2, TEAD3, and TEAD4 (**Fig** 5.4A). Patients with higher TEAD-YAP expression (134 patients) showed significantly shorter overall survival time compared with patients with lower TEAD-YAP expression (51 patients), after adjusting for age, stage and tumour site (**Fig** 5.4B).

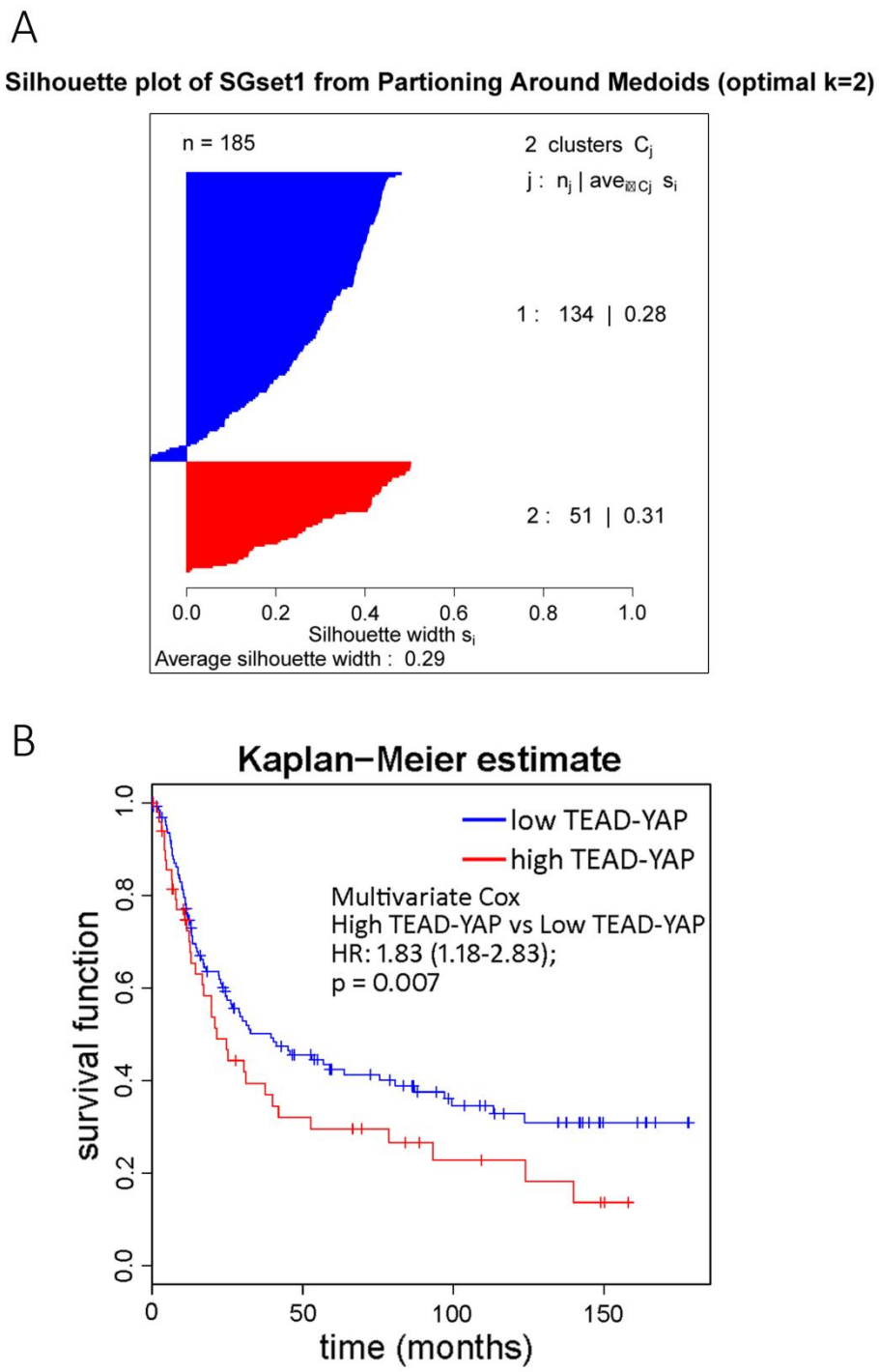


Figure 5.4 Higher YAP-TEAD mRNA expression is significantly correlated with poorer prognosis of gastric cancer patients. (A) Silhouette plot of micro array data set showed that all patients were divided into two groups according to the expression of YAP, TEAD1, TEAD2, TEAD3 and TEAD4. (**B)** Kaplan-Meier survival analysis of gastric cancer patients with different TEAD-YAP expression levels. The log-rank test was used to assess the significance of the Kaplan-Meier analysis. Cox regression was used to adjust for age, stage and tumour site.

5.5 The expression levels of both RUNX1 and RUNX3 show significant reduction in gastric cancer patients with higher YAP-TEAD expression

Here we examined whether the expression level of RUNX1/3 and TEAD-YAP expression levels were correlated, which could offer some clues indicating whether RUNX1/3 and TEAD-YAP were functionally related.

The same micro array cohort of Chapter 5.4 was used to investigate the expression levels of RUNX1 and RUNX3. Weights were first computed for each sample using expression of PTPRC (Protein Tyrosine phosphatise receptor type C, CD45), which was a measure of lymphocyte contamination within the tissue. Weight (W) per sample was computed using the formula: $W = 1/(PTPRC_i/PTPRC_{median})$, where $PTPRC_i$ is the expression level of PTPRC in i^{th} sample and $PTPRC_{median}$ is the median expression of PTPRC across all samples. The weighted RUNX gene expression was then computed multiplying the weight and RUNX expression for the each sample. A Welch two-sample t-test was employed to calculate the significance of a difference between RUNX expressions in “high” versus “low” TEAD-YAP expressors. All two-sided p-values were reported. $P < 0.05$ was taken to be significant.

According to the result of statistical analysis, the expression levels of both RUNX1 and RUNX3 were significantly lower in gastric cancer patients with higher TEAD-YAP expression, indicating that RUNX3 inactivation might be functionally correlated with enhanced TEAD-YAP activity (**Fig** 5.5).

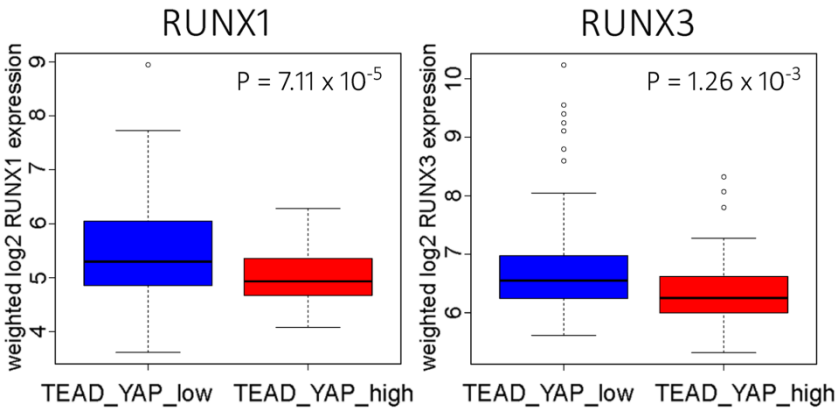


Figure 5.5 The expressions of both RUNX1 and RUNX3 show significant reduction in gastric cancer patients with higher YAP-TEAD expression. RUNX1 and RUNX3 expression was adjusted to PTPRC (CD45) expression level. A Welch two-sample t-test was employed to calculate significance of a difference between RUNX expressions in "high" versus "low" TEAD-YAP expressors.

5.6 A large portion of potential TEAD-YAP target genes in gastric cancer specimen can be suppressed by RUNX3 in SNU16 gastric cancer cell line

Results described in chapter 4 indicated that RUNX3 can reduce the transcriptional activity of TEAD proteins. For a better understanding of which TEAD target genes were under such regulation of RUNX3, we used a series of bio-informatics analysis to compare microarray data from multiple resources, including the micro array performed in MCF10A cell line after YAP overexpression (Zhao et al, 2008), microarray performed using gastric cancer specimen, and microarray performed in SNU16 gastric cancer cell line after RUNX3 knocking down, aiming to identify a group of TEAD-YAP target genes which could be inhibited by RUNX3 in gastric cancer cell. This bioinformatic analysis consisted of two major steps, as described in details below.

Step 1: Identify candidate TEAD-YAP target genes which are significantly up-regulated in gastric cancer specimens with high TEAD-YAP expression.

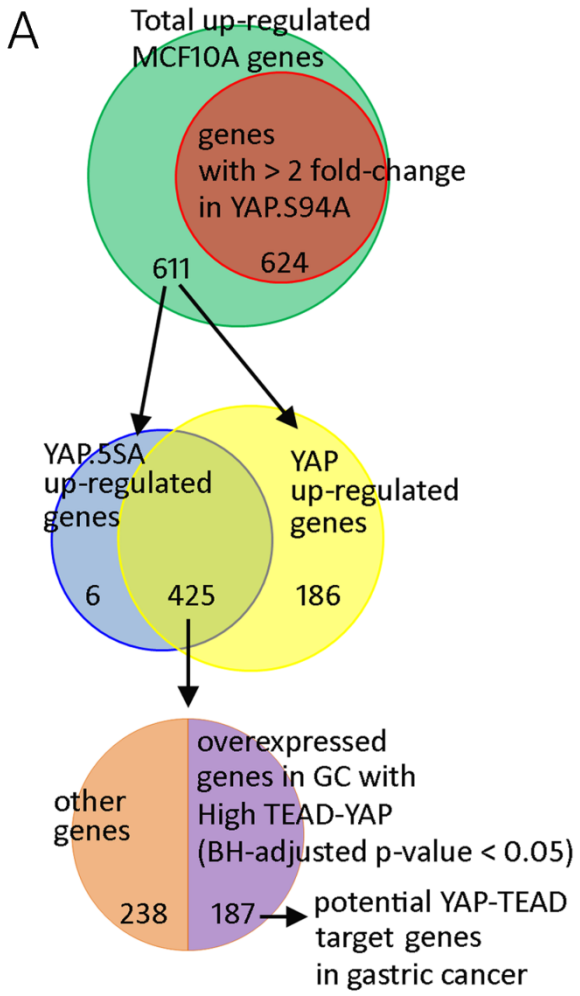
The gene expression signature promoted by YAP was previously established In MCF10A mammary epithelial cells. Using this published results, we reanalyzed gene expression data from cDNA microarray analyses which were performed on 185 gastric cancer tissues (GSE15459). This identified 187 candidate TEAD-YAP target genes in gastric cancer specimens that met the following two criteria: 1) significantly up-regulated by YAP and YAP_{5SA}, but not by TEAD-interaction mutant YAP_{S94A} in MCF10A; 2) Significantly overexpressed in gastric cancer patients with high TEAD-YAP expression (**Fig** 5.6A).

Step 2: Identify candidate TEAD-YAP target genes which can be inhibited by RUNX3 in SNU 16 gastric cancer cell line.

To perform microarray using SNU16 cell line, Controls (n = 3) and RUNX3-Knockdown (n = 3) samples were profiled on Illumina Human Ref-8 arrays. Genes which were up-regulated in RUNX3-Knockdown samples were used to intersect with genes identified previously to be up-regulated in "TEAD-YAP" high vs low expressors. The expression data from intersected genes were next subjected to hierarchical cluster analysis with average correlation as the distance measure.

According to the result of these bioinfomatics analysis, among the 187 candidate gastric TEAD-YAP targets, 69 genes (36.7%) were up-regulated in SNU16 cell lines when RUNX3 was knocked down, indicating that a large portion of TEAD-YAP target genes were under the suppression of RUNX3

when RUNX3 was expressed at a physiological level. CTGF (connective tissue growth factor) was one of the most significantly induced TEAD-YAP target genes following RUNX3 knockdown (**Fig** 5.6B).



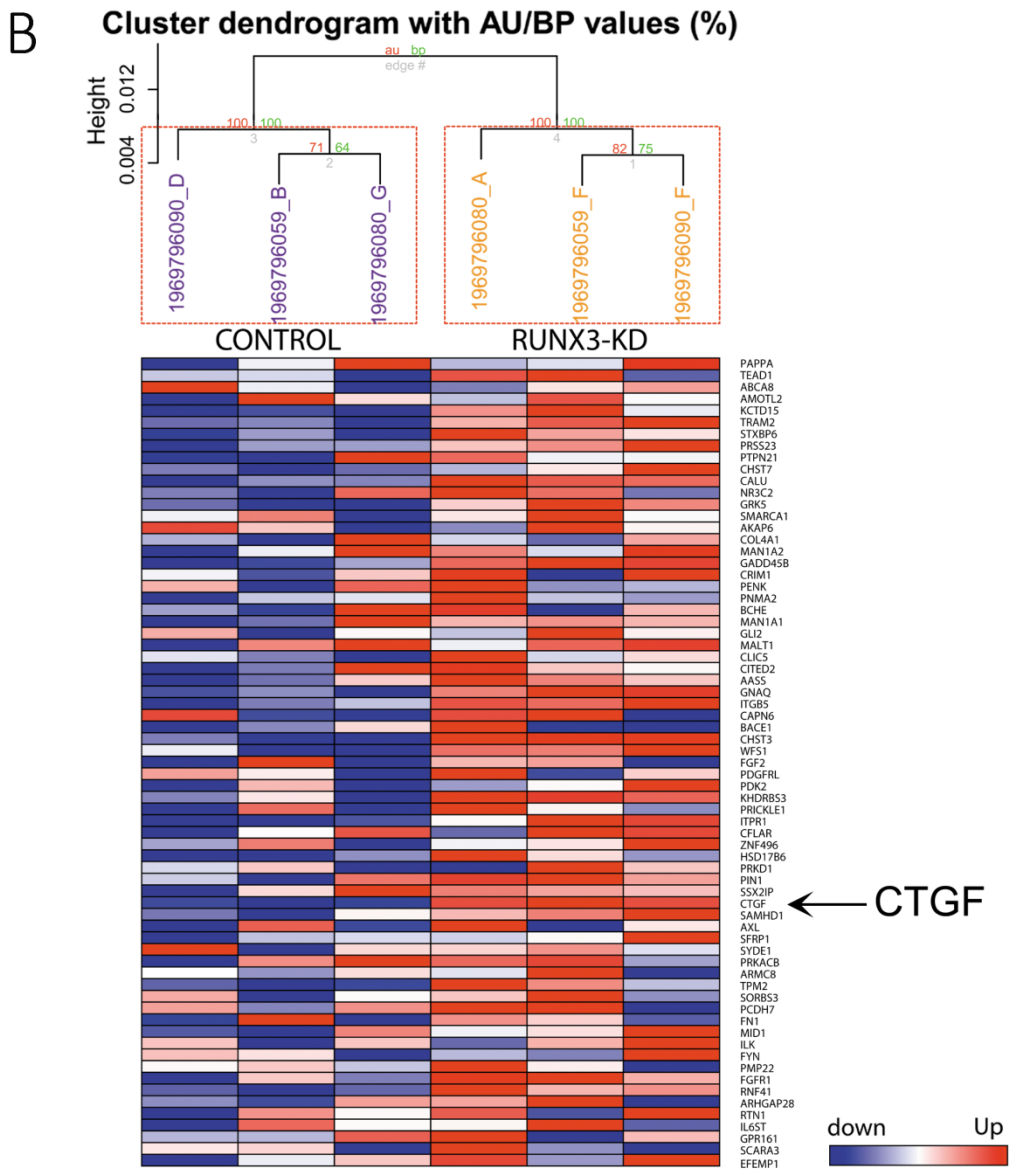


Figure 5.6 A large portion of potential TEAD-YAP target genes in gastric cancer specimens are suppressed by RUNX3 in SNU16 gastric cancer cell line. (A) The strategy for identifying candidate TEAD-YAP target genes in gastric cancer specimens. (B) The candidate TEAD-YAP target genes set whose expression got up-regulated after RUNX3 knocking down in SNU16 cell line.

5.7 CTGF is negatively regulated by RUNX3 in gastric cancer cell lines

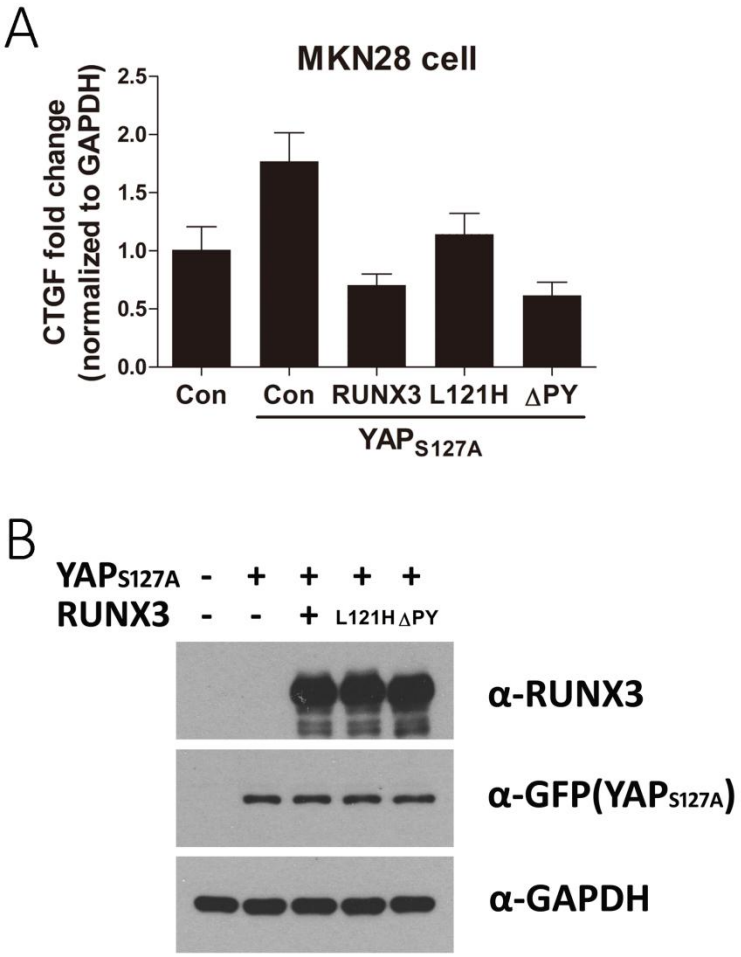
CTGF (connective tissue growth factor) is highlighted in the previous bioinformatic analysis as one of candidate gastric YAP-TEAD target genes which could be suppressed by RUNX3 in SUN16 gastric cancer cell line. At the same time, CTGF is a well-established TEAD-YAP target gene whose detailed mechanism by which it is regulated by TEAD-YAP complex has been clearly illustrated. TEAD-YAP complex triggers the expression of CTGF by binding to the TEAD motifs located in the promoter region of CTGF gene (Zhao et al, 2008). Due to these two reasons, CTGF was selected as a model target gene for further analysis about how RUNX3 regulated TEAD-YAP complex's function.

Two gastric cancer cell lines were used in this experiment, MKN28, which does not express any endogenous RUNX3, and HGC-27, in which endogenous RUNX3 is expressed at a high level. Exogenous RUNX3 would be introduced into MKN28 while endogenous RUNX3 would be knocked down in HGC-27, and the expression level of CTGF would be determined by real-time PCR.

According to the result obtained in MKN28 cell line, CTGF expression was induced by YAP_{S127A}, which could be reversed by reintroducing wild-type RUNX3. This inhibitory effect became weaker if the $Luecine^{121}$ of RUNX3 was mutated to Histidine, which abolished RUNX3's interaction with TEAD proteins, indicating that RUNX3-TEAD interaction plays an important role in this process. However, deleting the PY motif, which would impair the RUNX3-YAP interaction, did not make any difference to the inhibitory effect caused by RUNX3, indicating that RUNX3-YAP interaction does not contribute to RUNX3's inhibition on CTGF expression (**Fig** 5.7A, B).

According to the result produced in HGC-27 cell line, knocking down of RUNX3 increased the expression of CTGF, indicating that the endogenous RUNX3 has an inhibitory effect on CTGF expression. When YAP_{S127A} was overexpressed, the expression of CTGF was further up-regulated. This additive effect indicated that RUNX3's negative regulation on CTGF expression by RUNX3 is not dependent on YAP (**Fig** 5.7C, D).

Taken together, these results obtained from both gastric cancer cell lines pointed to one common conclusion that RUNX3 suppresses the expression of CTGF, and RUNX3-TEAD interaction plays an important role in this process.



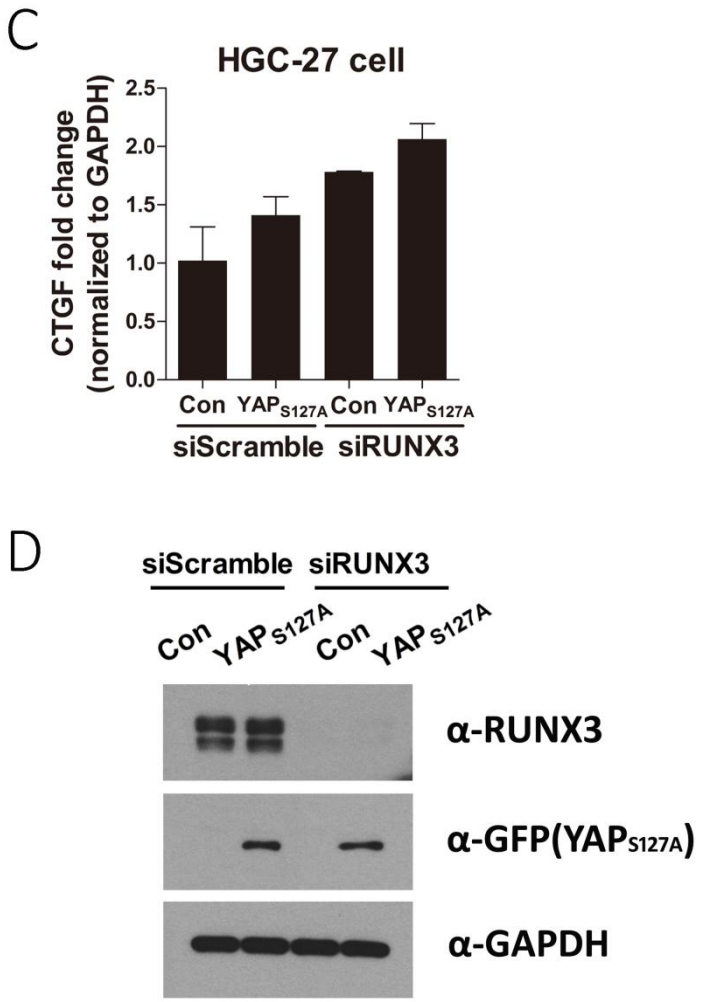


Figure 5.7 CTGF is negatively regulated by RUNX3 in gastric cancer cell lines. (A) Real-time PCR analysis of CTGF expression in MKN28 after RUNX3 overexpression in MKN28 cell line. MKN28 cell was transfected with pcDNA-Flag-YAP_{S127A} (1 μg/well in a 6-well plate), pcDNA-Flag-RUNX3 (1 μg/well), pcDNA-Flag-$RUNX3_{L121H}$ (1 μg/well), pcDNA-Flag-RUNX3ΔPY (1 μg/well) plasmids accordingly. RNA was extract using Trizol at 48 hours after transfection. cDNA was reverse-transcripted and real-time PCR was performed and the result was normalized to GAPDH. Triplication was performed for each sample. Mean and standard deviation was shown in the figure. (B) Western blot result showing protein expression in the MKN28 cells. (C) Real-time PCR analysis of CTGF expression after RUNX3 knocking down in HGC-27 cell line. HGC-27 cell was transfected with siRUNX3 (100 pmol/well in a 6-well plate), pcDNA-Flag-YAP_{S127A} (1 μg/well) accordingly. RNA was extract using Trizol at 48 hours after transfection. cDNA was reverse-transcripted and real-time PCR was performed and the result was normalized to GAPDH. Triplication was performed for each sample. Mean and standard deviation was shown in the

figure. (D) Western blot result showing protein expression in HGC-27 cells used for real-time PCR analysis of CTGF expression after RUNX3 knocking down.

5.8 RUNX3 suppresses the expression of CTGF by preventing TEAD1 from binding to the promoter of CTGF

From previous experiments, we had already known that RUNX3 could suppress the expression of CTGF. At the same time, the data from Chapter 4 showed that RUNX3 impairs the DNA binding ability of TEAD proteins. Based on these observations, we hypothesized that RUNX3 might inhibit the expression of CTGF by preventing TEAD proteins from binding to the promoter of CTGF. To test this hypothesis, Chromatin IP was performed using antibody targeting TEAD1 in MKN28 cell, as described in Chapter 2.12

The result of chromatin IP showed that CTGF promoter was enriched by TEAD1 antibody in MKN28 cell line, and this enrichment was reduced when RUNX3 was overexpressed, indicating that RUNX3 down-regulates the expression of CTGF by directly inhibiting TEAD proteins from binding to the promoter of CTGF (**Fig** 5.8).

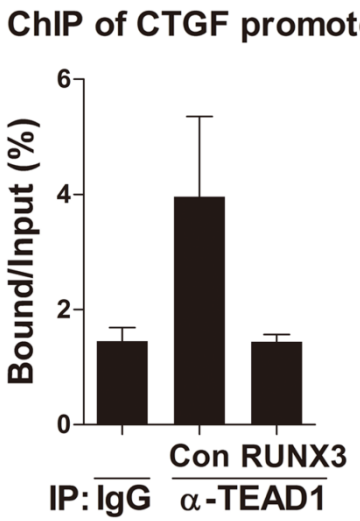


Figure 5.8 RUNX3 suppresses the expression of CTGF by preventing TEAD1 from binding to the promoter of CTGF. MKN28 cells were transfected with pEGFP-C1-RUNX3 (5 μg/10cm dish). Cells were harvested at 48 hours after transfection and chromatin IP was performed using TEAD1 antibody or normal mouse IgG as described in Chapter 2.12. The precipitated DNA was analyzed using real-time PCR. The result showed that overexpression of RUNX3 could impair the enrichment of CTGF promoter by TEAD1 protein.

5.9 RUNX3 overexpression significantly reduces growth of gastric cancer cell lines in soft agar

Gain of anchorage-independent growth is one of the key steps of malignant transformation. The soft agar colony formation assay is a common method to measures cell proliferation in an anchorage-independent manner. By analyzing the soft agar colony formation after RUNX3 overexpression in gastric cancer cell lines, the role which RUNX3 played in the malignant transformation of cancer cells could be evaluated.

MKN28 and AGS cell lines were used in this experiment since neither of them expresses endogenous RUNX3. Both cell lines were able to form hundreds

of colonies if they were infected with vehicle virus control, but their ability to grow in an anchorage-independent manner was remarkably impaired when RUNX3 was overexpressed. The mutation of Leucine^{121} of RUNX3 could partially neutralize the negative effect of RUNX3 on cell growth, indicating that RUNX3's interaction with TEAD proteins played an important role for RUNX3's ability to inhibit the anchorage-independent growth of gastric cancer cells (**Fig** 5.9A, B).

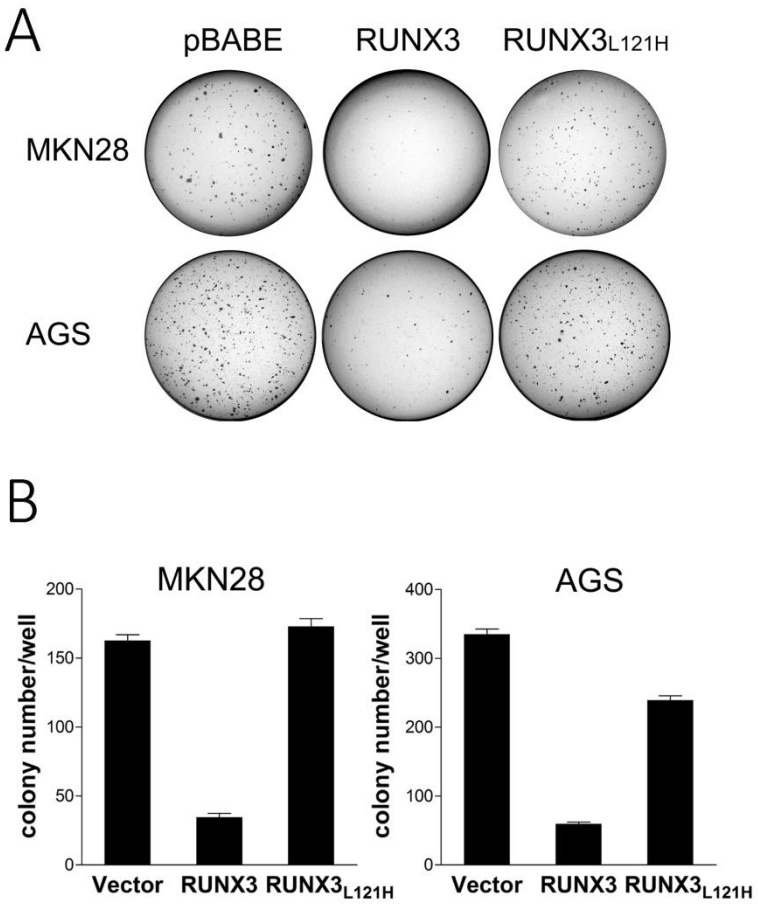


Figure 5.9 RUNX3 overexpression significantly reduces the growth of gastric cancer cell lines in soft agar. (A) Soft agar colony formation assay after RUNX3 overexpression in MKN28 and AGS cell lines. Cells were infected with retrovirus overexpressing GFP-RUNX3 and stable cell lines were selected using puromycin. Cells were seeded in soft agar and colonies were stained after 3 weeks. (B) Quantification for the results of the soft agar colony formation assay

after RUNX3 overexpression in MKN28 and AGS cell lines. Triplication was made for each sample. Mean and standard deviation were shown.

5.10 RUNX3 inhibits the overgrowth of gastric cancer cells caused by TEAD-YAP complex

The results reported earlier by others showed that overexpression of TEAD4-YAP fusion protein should result in better cell survival in soft agar, since TEAD4-YAP fusion protein would trigger the expression of downstream genes promoting cell survival and proliferation (Zhao et al, 2008). Here, we wanted to test whether RUNX3 was able to prevent the enhancement of anchorage-independent cell growth caused by TEAD4-YAP fusion protein. This experiment was carried out in MKN28 cell line, in which endogenous RUNX3 was not expressed.

The result showed that overexpression of TEAD4-YAP fusion protein led to the formation of more colonies growing in soft agar, compared with vehicle control. The growth of colonies was significantly reduced when RUNX3 was overexpressed, regardless of whether TEAD4-YAP fusion protein was expressed or not. However, when the Leucine^{121} of RUNX3 was mutated, the inhibitory effect of RUNX3 was lost (**Fig** 5.10A, B).

Taken together, these results indicated that RUNX3 inhibits the activity of TEAD4-YAP fusion protein and reduces the growth advantage caused by TEAD-YAP fusion protein.

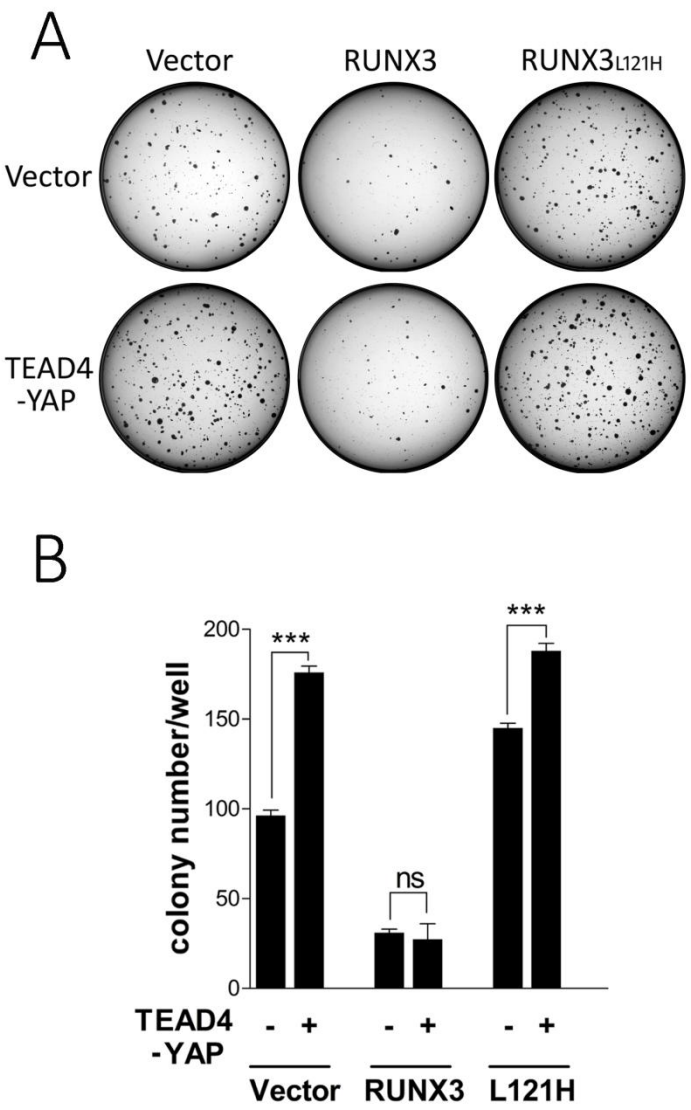
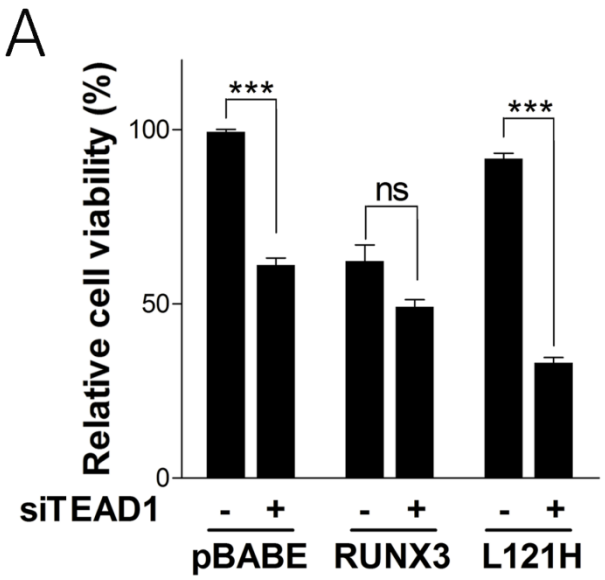


Figure 5.10 RUNX3 inhibits the overgrowth of gastric cancer cells caused by TEAD-YAP complex. (A) Soft agar colony formation assay after the overexpression of TEAD4-YAP fusion protein and RUNX3 in MKN28 cell line. Cells were infected with retrovirus overexpressing TEAD4-YAP and GFP-RUNX3 accordingly. Stable cell lines were selected using hygromycin and puromycin. Cells were seeded in soft agar and colonies were stained after 3 weeks. (B) Quantification for the soft agar colony formation assay after the overexpression of TEAD4-YAP fusion protein and RUNX3 in MKN28 cell line. Triplication was made for each sample. Mean and standard deviation were shown.

5.11 RUNX3 and TEAD1 work in the same pathway to regulate the growth of gastric cancer cells

Both knocking down TEAD1 and ovrexpressing RUNX3 would cause the reduction of cell growth. Here we wanted to test the combined effects of both treatments to see whether TEAD1 and RUNX3 worked in the same pathway. If the effects of knocking down TEAD1 and overexpressing RUNX3 were additive, it meant that they worked in separate pathways. Otherwise, it meant that they worked in the same pathway.

According to the result of cell proliferation assay, both knocking down of TEAD1 and overexpression of RUNX3 would cause 40% reduction of cell growth in MKN28 gastric cancer cell line. However, the combined treatment did not cause a significant reduction any further, compared with single treatment. This result indicated that TEAD1 and RUNX3 work in the same pathway (**Fig** 5.11A, B).



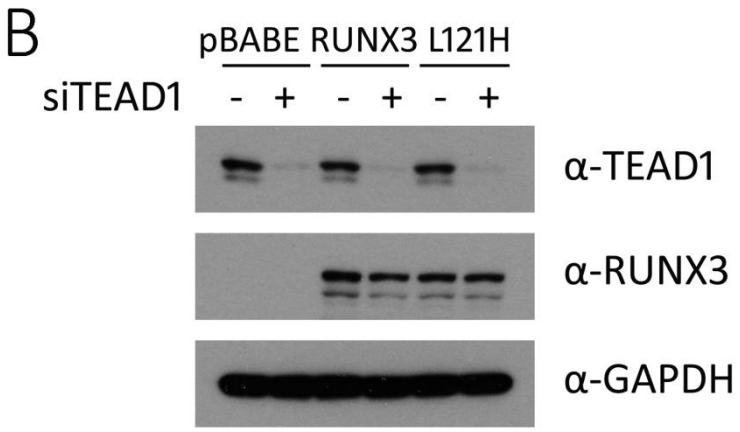


Figure 5.11 RUNX3 and TEAD1 work in the same pathway to regulate the growth of MKN28 cell. (A) Relative cell viability after knocking down TEAD1 and overexpressing RUNX3 in MKN28. Stable cell lines expressing GFP-RUNX3 or GFP-RUNX3$_{L121H}$ were transfected with siTEAD1 (100 pmol/well in a 6-well plate). The cells were seeded into 96-well plate and cell proliferation was monitored by WST-1 assay at 96 hours. Triplication was made for each sample. Mean and standard deviation were shown. (B) Western blot result showing protein expression after knocking down TEAD1 and overexpressing RUNX3 in MKN28.

5.12 Overexpressing TEAD1 compensates for RUNX3's negative effect on cell growth

From previous experiments, it was shown that TEAD1 and RUNX3 function in the same pathway to regulate cell growth. Here we performed another cell growth analysis by overexpressing TEAD1 and RUNX3 together to test whether extra TEAD1 could compensate for the negative effect on cell growth caused by RUNX3's suppression on TEAD-YAP activity.

The result of cell proliferation assay revealed that overexpressing TEAD1 alone did not exhibit any significant influence on cell growth, but it could increase cell growth in the context of RUNX3 overexpression, compensating for

RUNX3's negative effect on cell growth (**Fig** 5.12A, B). This result indicated that extra TEAD1 could partially rescue the negative effect on cell growth caused by RUNX3 overexpression, supporting our previous observations that RUNX3 reduces cell growth by suppressing the activity of TEAD proteins.

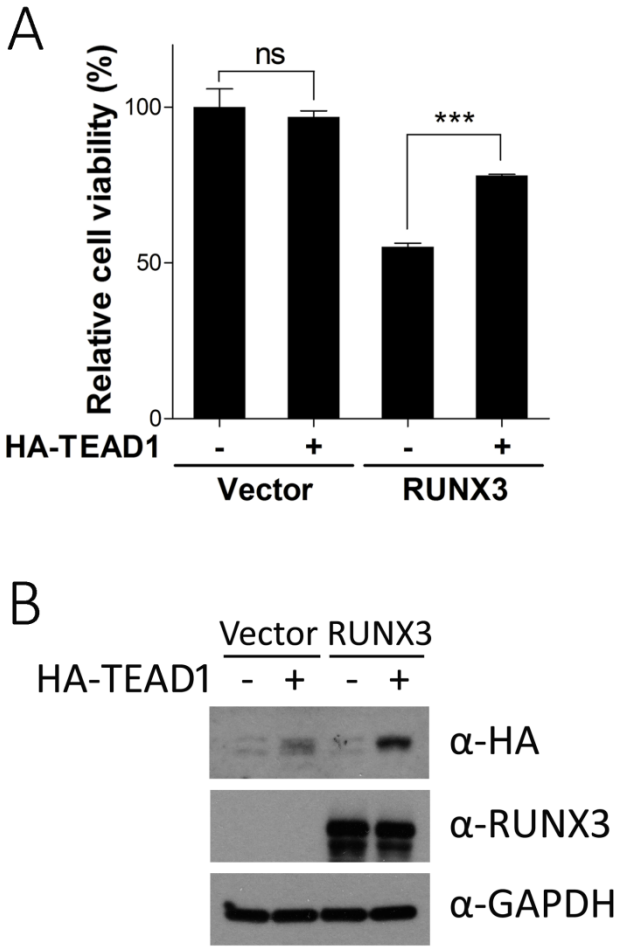


Figure 5.12 Overexpressing TEAD1 compensates for RUNX3's negative effect on cell growth. (A) Relative cell viability after overexpressing TEAD1 and RUNX3 in MKN28 cell. Cells were infected with retrovirous overexpressing HA-TEAD1 and GFP-RUNX3. Stable lines were selected using hygromycin and puromycin, and seeded into 96-well plates for proliferation assay using WST-1 method. Triplication was made for each sample. Mean and standard deviation were shown. (B) Western blot result showing protein expression after overexpressing TEAD1 and RUNX3 in MKN28 cell.

5.13 Summary and discussion

According to our analysis, TEAD-YAP complex is highly oncogenic for gastric epithelial cells, whose overexpression is important for the proliferation of gastric cancer cell lines (**Fig** 5.1, 5.2). Overexpression of YAP and TEAD proteins is frequently observed in gastric cancer specimens, ant it is significantly correlated with a shorter overall survival time for gastric cancer patients (**Fig** 5.3, 5.4). The expression of RUNX3 is relatively lower in patients with high YAP-TEAD expression, and RUNX3 suppresses the expression of 36.7% (69/187) candidate TEAD-YAP target genes in gastric cancer (**Fig** 5.5, 5.6). Further analysis on RUNX3's regulation on CTGF, one TEAD-YAP target gene, revealed that RUNX3 suppresses the expression of CTGF by directly preventing TEAD proteins from binding to the promoter of CTGF (**Fig** 5.7, 5.8).

According to the soft agar colony formation assays performed using gastric cancer cell lines, RUNX3 reduces the anchorage-independent cell growth by inhibiting the activity of TEAD-YAP complex (**Fig** 5.9, 5.10). The result of cell proliferation assays also supported the conclusion that RUXN3 and TEAD proteins work in the same pathway to control the growth of gastric cancer cell lines, and overexpressing TEAD1 partially compensates for RUNX3's negative effect on cell growth (**Fig** 5.11, 5.12). All these results suggested us a novel mechanism for understanding the role of RUNX3 as a tumour suppressor in gastric cancer.

It is interesting to find that knocking down TEAD1 and knocking down YAP did not lead to exactly the same response in gastric cancer cell lines (**Fig** 5.2), which might be due to several reasons. First, different cells might have

different amount of four TEAD proteins. In some cells, TEAD1 might be the major form, while other TEAD proteins dominate in other cell lines. In the latter situation, knocking down TEAD1 alone might lead to a smaller effect due to the compensation by other TEAD proteins. Secondly, TEAD proteins might utilize co-activators other than YAP, for example, TAZ or SRC-1, in gastric cancer epithelial cells, which could explain why sometimes knocking down of YAP causes a milder effect for cell growth, compared with knocking down TEAD1. Thirdly, YAP is multi-functional. It is involved in proproliferative as well as pro-apoptotic pathways. Knocking down YAP might reduce the signal outputs for both sides, and the mixed effects could be neutralized and weaker. However, since both stable knocking down TEAD1/3/4 by shRNA and transient knocking down TEAD1 or YAP alone by esiRNA led to reduced growth in most gastric cancer cell lines, it seems that the major function of YAP is still promoting the survival of gastric cancer cells.

Even though TEAD1 is universally expressed at a high level in gastric cancer cell lines, YAP overexpression is only observed in a portion of gastric cancer cell lines (**Fig** 5.1B), Ironically, overexpression of YAP is much more severe and frequently observed in clinical samples than overexpression of TEAD1. This can probably be explained by the fact that YAP is always under strict posttranslational regulations by multiple pathways, causing its rapid degradation, and YAP is also able to increase the expression of its own, rapidly forming a positive feedback loop. So once YAP's negative regulations are lost, YAP gets accumulated quickly. However, there is little evidence showing that TEAD proteins undergo such a stringent regulation. There is only one report about TEAD4's overexpression due to loss of promoter methylation in gastric

cancer. According to their IHC data, TEAD4's up-regulation is not as dramatic as YAP's accumulation, just like what we observed here (Lim et al, 2014).

According to our analysis, the expression of both RUNX1 and RUNX3 is significantly reduced in gastric cancer patients with higher TEAD-YAP expression (**Fig** 5.5). This finding is very interesting, and difficult to explain, because micro array analysis can not offer any information about causal relationship. It is possible that reduction of RUNX1/3 expression causes hyper activation of TEAD-YAP complex and vice versa. We did not explore this question any further here since we already knew that RUNX3 was able to inhibit the activity of TEAD-YAP complex based on Chapter 4, but actually more work could be done to study whether there is a direct relationship between TEAD-YAP and RUNX proteins at the transcriptional level.

To identify potential TEAD-YAP target genes which could be up-regulated by TEAD-YAP complex in gastric cancer, we compared micro array data generated in gastric cancer tissues with data generated in MCF10A cell line after YAP overexpression, looking for genes which could be up-regulated by YAP in MCF10 and showed significant correlation with TEAD-YAP's overexpression in gastric cancer specimen (**Fig** 5.6A). This strategy is not perfect due to two reasons: first, MCF10A is a mammary epithelial cell lines, which might have different YAP-TEAD target genes with gastric epithelial cells due to the difference of cell context; Secondly, significant correlation does not guarantee the existence of a direct regulation of one gene on another, which might be biased by the third factor which is correlated with the expression of these two genes at the same time. However, we still used this strategy due to the lack of

alternative choices. Other experiments like ChIP-Seq need to be performed if we want to confirm whether these genes are direct targets of TEAD-YAP complex. However, our major purpose here is to identify a gene expression pattern correlated with enhanced TEAD-YAP activity, so the current strategy is already enough to achieve this goal.

After that, these candidate TEAD-YAP target genes was compared with micro array data generated in SNU16 gastric cancer cell lines in which RUNX3 was knocked down by shRNA, and 36.7% of these genes were up-regulated after RUNX3 knocking down, indicating that a considerate portion of potential target genes are suppressed by RUNX3 in SNU16 cell (**Fig** 5.6B). However, for most of these genes, the down-regulation was not very dramatic, which might be due to three reasons: first, these target genes might not be fully activated in SNU16 due to cell context or impropriate cell density; secondly, the regulation of RUNX3 on TEAD-YAP target genes might not be the major way of regulation; thirdly, the regulation of RUNX3 on TEAD-YAP target genes might be selective, which means that the strong regulatory effect would be limited to a small number of genes due to promoter context. Based on the available information, it is difficult to judge which hypothesis is right, but at least we are sure that RUNX3 has a negative effect on a considerate portion of candidate TEAD-YAP target genes in gastric cancer.

Real time PCR and Chromatin IP experiments were performed to show that RUNX3 could suppress CTGF expression by preventing TEAD1 from binding to CTGF promoter (**Fig** 5.7, 5.8). CTGF was chosen here as an model gene due to three reasons: first, CTGF is the best characterized TEAD-YAP target genes,

whose TEAD binding site in promoter is already known (Zhao et al, 2008); Secondly, CTGF is one of the most significant up-regulated candidate TEAD-YAP target genes after RUNX3 knocking down; Thirdly, clinical studies have shown that overexpression of CTGF is significantly correlated with lymph node metastasis and poor prognosis in patients with gastric cancer (Liu et al, 2007; Liu et al, 2008). Knocking down of endogenous CTGF expression inhibits the growth and invasion of gastric cancer cells *in vitro* and attenuates peritoneal dissemination *in vivo* (Jiang et al, 2011).

More TEAD-YAP target genes should be tested to confirm whether our hypothesis is applicable to a broader field, which would require more optimization due to the lack of detailed background knowledge about how these genes are regulated by TEAD-YAP complex. In the clustering figure summarizing all the candidate TEAD-YAP target genes which could be suppressed by RUNX3 (**Fig** 5.6B), many genes attracted our attention due to their strong correlation with poor prognosis of gastric cancer or other kinds of cancer, for example, FGF2 (Fibroblast growth factor 2), AXL (AXL Receptor Tyrosine Kinase) and FGFR1 (Fibroblast growth factor receptor 1) (Andre et al, 2013; Katoh & Katoh, 2006; Wu et al, 2002).

In this chapter, both soft agar colony formation assays as well as regular cell proliferation assays were performed to evaluate cell growth. These two assays reflect two different kinds of cell growth. Regular cell proliferation assay reflects cell growth on traditional culture dish, which is a two dimensional process, while soft agar colony formation reflects cell growth in soft agar, which is a three dimensional process. Actually soft agar colony formation assay

includes two stages of cell growth. First, the cells need to overcome apoptosis caused by loss of anchorage to initiate the first few rounds of division. Then the cells would proliferate faster after they have set up a minimal population offering cell-cell contact signals in favour of survival and proliferation. The former stage is actually the opposite process of Anoikis, a form of programmed cell death which is induced by anchorage-dependent cells detaching from the surrounding extracellular matrix (ECM).

Overexpressing TEAD-YAP fusion protein in MKN28 cell increased the number of visible colonies formed in soft agar, indicating that TEAD-YAP complex could prevent the cell from Anoikis (**Fig** 5.10). However, in MKN28 and AGS cells, restoring wild-type RUNX3 expression by retrovirus infection could significantly reduce the number of visible colonies while restoring $RUNX3_{L121H}$ mutant lacking TEAD binding ability generated a much weaker inhibitory effect on the growth of colonies, indicating that TEAD-YAP complex is protecting the cell from Anoikis while RUNX3 promotes Anoikis by inhibiting TEAD-YAP complex. The inhibitory effect caused by RUNX3 in regular two dimensional culture dishes is weaker than RUNX3's effect in soft agar assay, indicating that RUNX3 plays important roles in both processes: Anoikis and inhibition of cell proliferation, and at least a part of these effects are achieved by interfering with the functions of TEAD-YAP complex.

For a better understanding of RUNX3's role in the regulation of TEAD-YAP complex, *in vivo* work should be performed to examine whether TEAD-YAP activity shows any difference when RUNX3 is knocked out in mice. However, this work can not be done due to the time limit of PhD candidate. The

internal rescue effects among all three RUNX proteins also add difficulties for the design of experiments. Currently, it has already been reported that knocking out *Runx3* would lead to neoplasia in lung epithelial cells, gastric epithelial cells and small intestinal epithelial cells in mice (Chen, 2012; Lee et al, 2010; Li et al, 2002). At the time of these publications, RUNX3's connection with TEAD-YAP complex was not discovered yet, so there was no information about Hippo pathway's status in these *Runx3* knockout mice. One quick option is to measure the Hippo pathway signature by micro array in the neoplasia tissues obtained from Runx3 knockout mice, and to compare it with the signature in normal epithelial cells in these tissues.

Taken together, based on our current findings from the analysis of gastric cancer specimens as well as experiments performed in gastric cancer cell lines, we can conclude that RUNX3 functions as a tumour suppressor in gastric cancer partially by suppressing the activity of oncogenic TEAD-YAP complex.

Chapter 6

General discussion

6.1 Summary of findings

This research project was designed to examine the protein-protein interaction between RUNX3 and TEAD proteins. The project could be divided into three parts. The first part studied how these proteins interacted with each other. The second part focused on how RUNX3-TEAD interaction influenced the transcriptional function of TEAD proteins. The third part studied the biological significance of this interaction in gastric cancer.

In the initial part of this project (Chapter 3), we showed that RUNX3 and TEAD1 could coexist in the same complex in HGC-27 cell line by endogenous IP experiment (**Fig** 3.1). Then I excluded the possibility that TEAD and RUNX3 interacted through YAP by performing exogenous IP using a RUNX3 mutant which lacked YAP binding ability (**Fig** 3.2). After confirming the direct protein-protein interaction between RUNX3 and TEAD proteins, I mapped the domains which both proteins required to interact with each other using multiple techniques, including exogenous IP using truncated constructs in HEK293T and GST pull down using purified proteins (**Fig** 3.3-3.6). According to the results, Runt domain of RUNX3 was utilized for its interaction with TEAD4, while the residues 101-125 of TEAD4 were crucial for its interaction with RUNX3. These 25 amino acid residues include a large part of Helix three (Residues 97-109) of TEAD4, which is the DNA recognition helix. The result of sequential IP experiment supported our hypothesis that TEAD4, YAP and RUNX3 could form a ternary complex, which indicated that RUNX3 is able to interact with both transcriptionally active or inactive forms of TEAD proteins (**Fig** 3.7-3.8). In the last part of this chapter, we screened several RUNX3 mutants with point

mutations within Runt domain, and discovered that a point mutation at Leucine^{121} of RUNX3 could totally abolish its binding with TEAD proteins, while retaining most of RUNX3's other functions, so the $RUNX3_{L121H}$ mutant would be widely used in the next two chapters to study the biological functions of RUNX3-TEAD interaction (**Fig** 3.9-3.10).

In the second part of this project (Chapter 4), we aimed to figure out how RUNX3-TEAD interaction affected the function of TEAD proteins. First, the luciferase reporter assay showed that RUNX3 reduced the transcriptional activity of TEAD proteins (**Fig** 4.1). However, the RUNX3-TEAD interaction did not have any obvious influence on the binding between TEAD and YAP (**Fig** 4.2). So we examined whether RUNX3 could reduce the DNA binding ability of TEAD proteins by both EMSA assay and transcription factor enrichment assay. RUNX3 was found to significantly reduce the DNA binding ability of TEAD proteins, causing the reduction of TEADs' transcriptional activity (**Fig** 4.3 - 4.4).

In the last part of this project (Chapter 5), we examined the biological significance of RUNX3-TEAD interaction in gastric cancer. First we proved that TEAD-YAP complex was oncogenic in gastric cancer cell lines (**Fig** 5.1, 5.2), and its overexpression was significantly correlated with poor prognosis in patients with gastric cancer (**Fig** 5.3, 5.4). We used bioinformatic approach to identify a group of candidate targets genes which got up-regulated by TEAD-YAP complex in gastric cancer specimen. Then we compared this group of genes with the micro array result generated in SNU16 gastric cancer cell lines in which RUNX3 was knocked down, and found that a considerable portion

(36.7%) of candidate TEAD-YAP target genes were suppressed by RUNX3 in SNU16 cell, indicating that RUNX3 played an important role to suppress the activity of TEAD-YAP complex in gastric cancer (**Fig** 5.6). We also validated RUNX3's inhibitory effect on CTGF by real-time PCR and Chromatin IP to show that RUNX3 could inhibit CTGF's expression by preventing TEAD proteins from binding to CTGF's promoter (**Fig** 5.7, 5.8). Then we performed both cell proliferation assays as well as soft agar colony formation assays to show that RUNX3 could significantly reduce the cell growth of gastric cancer cell lines partially through suppressing the activity of TEAD-YAP complex (**Fig** 5.9-5.12).

Taken together, we discovered that RUNX3 forms direct protein-protein interaction with TEAD proteins, and down-regulates the transcriptional activity of TEAD-YAP complex by preventing TEAD proteins from binding to DNA. RUNX3 suppresses the expression of a considerate portion of TEAD-YAP target genes in gastric epithelial cells and this could partially explain how RUNX3 suppresses the growth of gastric cancer cells and functions as a tumour suppressor.

6.2 Significance of findings

The present work is an attempt to thoroughly examine the protein-protein interaction between RUNX3 and TEAD proteins. This study suggested that RUNX3 could significantly inhibit the transcriptional activity of TEAD proteins by direct protein-protein interaction and the loss of RUNX3 in gastric cancer would lead to a more aggressive progression of disease due the enhanced TEAD activity.

Our finding pointed out RUNX3's novel role as a negative regulator of TEAD-YAP complex within cell nucleus, adding a new facet for a better understanding about the mechanisms how RUNX3 functions as a tumour suppressor.

Recently, great progression has been achieved for the mechanism study about how RUNX3 functions as a tumour suppressor. Our understanding about the functions of this critical protein has reached a new level.

Based on published literature as well as the research conducted in our group, loss of RUNX3, which is commonly observed in various kinds of solid tumours, would lead to severe changes for the biology of cells, paving the way for oncogenic transformation (Li et al, 2002). These changes include reduced expression of key proteins for cell homeostasis (Chi et al, 2005; Yano et al, 2006), enhanced Wnt signalling (Ito et al, 2008), stronger EMT tendency (Voon et al, 2012), failure to initiate cell defence against oncogenic K-Ras (Lee et al, 2013), trembling genome stability due to incomplete repair of DNA damage, and elevated TEAD-YAP activity (**Fig** 6.1). These changes cover major steps for oncogenic transformation, which can explain why RUNX3 is such a hotspot

for promoter hypermethylation as well as LOH (Loss of heterozygosity) in solid tumours (Carvalho et al, 2005; Fan et al, 2011).

The grave consequences caused by loss of RUNX3 suggest us the potential applications of RUNX3 for the treatment of cancer. There are two possible ways to reach this goal: first, restoring the RUNX3 expression in cancer cells, which can be achieved by gene therapy or small molecular compounds which can reverse the hyper-methylation on RUNX3's promoter; secondly, developing short peptides which mimic the critical part of RUNX3 proteins, for example, the sequence which interact with TEAD proteins or β-catenin/TCF4. Currently, several drugs which can reverse promoter methylation, including azacytidine, decitabine, and fazarabine, are already used as drugs against cancer. More research should be conducted to examine these drugs' effect on RUNX3's restoration.

Recently, peptides as well as small molecular compounds disrupting the TEAD-YAP complex have already been available, and they both shown dramatic antagonizing effect on TEAD-YAP activity in animal models (Jiao et al, 2014; Liu-Chittenden et al, 2012a). According to our findings, loss of RUNX3 would increase the risks of hyper-activated TEAD-YAP complex, which can be used as one of the criterions to decide whether anti-TEAD-YAP therapy should be used for the benefits of patients at the age of personalized medicine.

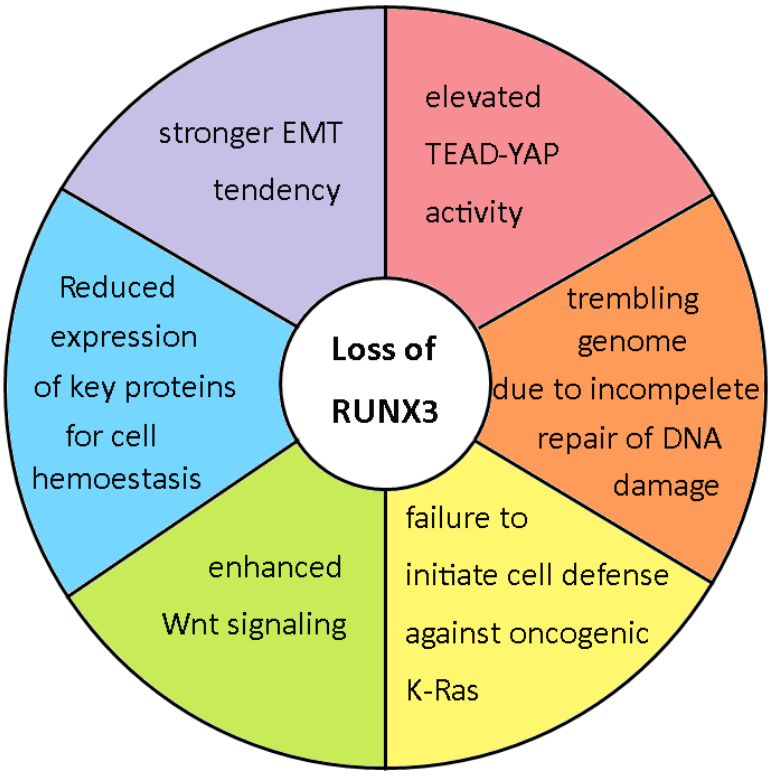


Figure 6.1 A summary of major changes caused by loss of RUNX3 in epithelial cells.

6.3 Future works

Our current understanding about the interaction between RUNX3 and TEAD proteins is still incomplete. There are several questions to answer.

First, the detailed structure of RUNX3-TEAD complex remains unknown. Several methods can be used to determine the structure of a protein, including X-ray crystallography, NMR spectroscopy, and electron microscopy (Schneidman-Duhovny et al, 2012). Collaborative work is required to fulfill such tasks. Once the structure is solved, new windows would be opened for designing small molecular compounds or short peptides mimicking RUNX3's

binding with TEAD proteins to prevent TEAD proteins from binding to DNA (Takeda-Shitaka et al, 2004).

Secondly, more TEAD-YAP target genes under the negative control of RUNX3 should be identified. In Chapter 5.6, we already generated a list of candidate TEAD-YAP target genes which were negatively regulated by RUNX3 in SNU16 gastric cancer cell line. However, our results were merely based on the analysis of micro array data. A more comprehensive study should be conducted using ChIP-Seq technique, which measures protein-genome interaction at a global level (Park, 2009). By comparing the DNA binding pattern of TEAD proteins before and after RUNX3 overexpression, all the genes which follow the regulation model which we proposed in this thesis can be identified.

Thirdly, our model should be tested in more models other than gastric cancer. It is already known that TEAD-YAP plays an oncogenic role in various kinds of solid tumours, and RUNX3 is also expressed in other tissues like skin and brain (Inoue et al, 2002; Lee et al, 2011b). It is highly possible that our model can be applied in a broader field.

Last but not least, more *in vivo* work should be done to study the significance of RUNX3 for the regulation of TEAD-YAP complex. There are many ways to achieve TEAD-YAP hyper-activation in mouse model, including knocking out Hippo pathway component Sav1 or Merlin, or knocking in constitutive activated YAP_{S127A} mutant (Cai et al, 2010; Jiao et al, 2014). It would be interesting to examine whether knocking out of RUNX proteins would cause a more severe phenotype for hyper-activated TEAD-YAP complex.

In general, the data presented in this thesis demonstrate that RUNX3 is a novel negative regulator for TEAD proteins, adding to our knowledge about how RUNX3 functions as a tumour suppressor in gastric cancer. However, more thorough investigations are still required for a comprehensive understanding of RUNX3-TEAD interaction.

Publications

Publication in International Journal

Liu AM, Wong KF, Jiang X, **Qiao Y**, Luk JM (2012) Regulators of mammalian Hippo pathway in cancer. *Biochim Biophys Acta*.**1826(2)**:357-64.

Oral Presentations in International Conferences

Qiao Y, Ito Y. The Interplay between RUNX3 and TEADs in Gastric Cancer. Keystone Symposia E2: The Hippo Tumor Suppressor Network: From Organ Size Control to Stem Cells and Cancer. 19th-23rd May 23, 2013. Monterey, California, USA.

Poster Presentations in Local/Regional Conferences

Qiao Y, Ito Y. The Interplay between RUNX3 and TEADs in Gastric Cancer. 6th Annual Scientific Meeting of Singapore Gastric Cancer Consortium. 25th -26th July, 2013.

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