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IKKalpha mediated NOTCH1 Activation Promotes Tumorigenesis via FOXA2 Suppression

Mo Liu

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**IKK α Mediated NOTCH1 Activation Promotes Tumorigenesis via
FOXA2 Suppression**

By

Mo Liu

APPROVED:

Mien-Chie Hung, Ph.D., Supervisor

Dihua Yu, M.D., Ph.D.

Elsa R. Flores, Ph.D.

Hui-Kuan Lin, Ph.D.

Andrew Bean, Ph.D.

APPROVED:

DEAN, THE UNIVERSITY OF TEXAS

HEALTH SCIENCE CENTER AT HOUSTON

GRADUATE SCHOOL OF BIOMEDICAL SCIENCES

**IKK α Mediated NOTCH1 Activation Promotes Tumorigenesis via
FOXA2 Suppression**

**A
DISSERTATION**

**Presented to the Faculty of
The University of Texas
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and
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M. D. Anderson Cancer Center
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In Partial Fulfillment
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For the Degree of**

DOCTOR OF PHILOSOPHY

**by
Mo Liu, M.S.**

**Houston, Texas
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Publication No. _____

Mo Liu

Supervisory Professor: Mien-Chie Hung, Ph.D.

Abstract

Proinflammatory cytokine TNF α plays critical roles in promoting malignant cell proliferation, angiogenesis, and tumor metastasis in many cancers. However, the mechanism of TNF α -mediated tumor development remains unclear. Here, we show that IKK α , an important downstream kinase of TNF α , interacts with and phosphorylates FOXA2 at S107/S111, thereby suppressing FOXA2 transactivation activity and leading to decreased NUMB expression, and further activates the downstream NOTCH pathway and promotes cell proliferation and tumorigenesis. Moreover, we found that levels of IKK α , pFOXA2 (S107/ 111), and activated NOTCH1 were significantly higher in hepatocellular carcinoma tumors than in normal

liver tissues and that pFOXA2 (S107/111) expression was positively correlated with IKK α and activated NOTCH1 expression in tumor tissues. Therefore, dysregulation of NUMB-mediated suppression of NOTCH1 by TNF α /IKK α -associated FOXA2 inhibition likely contributes to inflammation-mediated cancer pathogenesis. Here, we report a TNF α /IKK α /FOXA2/NUMB/NOTCH1 pathway that is critical for inflammation-mediated tumorigenesis and may provide a target for clinical intervention in human cancer.

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CHAPTER 1 INTRODUCTION

1.1 Liver cancer and inflammation

Liver cancer is the third most common cancer in the world¹. 90% liver cancer patients die within a year after diagnosis, and 5-year survival rate of liver cancer is only 6.9%^{2,3}. In 2008, it was estimated that there were about 700,000 new cases of liver cancer worldwide, and a similar number of patients died as a result of this disease⁴⁻⁶. Nativity, residential enclave status, and neighborhood SES characterize Hispanics and Asians with significantly unequal incidence rates of liver cancer, implicating behavioral or environmental risk factors, including primarily associated with geography incidence rate risk factor chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, dietary aflatoxin exposure, alcohol-related cirrhosis, fatty liver disease, obesity, smoking, diabetes, and iron overload^{7,8}.

It is not a surprise to see the tight correlation between liver inflammation and liver cancer. Back to early 1863, the founder of modern pathology, Dr. Rudolf Virchow suggested that “lymphoreticular infiltrate” reflected the origin of cancer at sites of chronic irritation^{9,10}. It is the first time that people noticed the association between inflammation and cancer. Since then, the relationship between inflammation and cancer has been observed and studied for a long time. Now, the causal relationship between inflammation and cancer is more widely accepted with general molecular and cellular mechanisms underneath. One of typical examples is

extrinsic factors induced inflammatory or infectious conditions augment the risk of developing cancer at certain anatomical sites (for example, the liver)¹¹⁻¹³. These extrinsic pathways converge resulting in the activation of transcription factors, like nuclear factor- κ B (NF- κ B), signal transducer and activator of transcription 3 (STAT3) and hypoxia-inducible factor 1 α (HIF1 α), in tumour cells. These transcription factors coordinate the production of inflammatory mediators, including cytokines and chemokines. The proinflammatory cytokines and chemokines, such as tumor necrosis factor α (TNF α), multiple interleukin factors recruit and activate various leukocytes, most notably cells of the myelomonocytic lineage. The cytokines activate the same key transcription factors in inflammatory cells, stromal cells and tumour cells, resulting in more inflammatory mediators being produced and a cancer-related inflammatory microenvironment to enhance cell proliferation, cell survival, cell migration and tumor angiogenesis, thereby promoting tumor development¹⁴⁻¹⁶. However, the mechanism of how specific type of inflammation targets unique cancer markers to promote tumorigenesis in different tumor development stages is still obscure. Therefore, uncovering the mechanism involving in inflammation-cancer process for further therapeutic and prevention study is urgently needed, and the identification of the molecules associated with inflammation-mediated tumorigenesis will provide a novel strategy for development anticancer or prevention agents.

1.2 TNF α pathway and cancer

The microenvironment surrounding tumors comprise with couple distinct types of cells, including fibroblasts, endothelial cells, and inflammatory cells. And a neoplastic clonal cell population exists in stromal and infiltrating inflammatory cells to provide sustenance and facilitate the potential metastatic process of the malignant cell^{17,18}. One of the crucial aspects of the tumor micro-environment is the cytokine-mediated communication between the tumour and stromal cells¹⁹. Tumor necrosis factor- α (TNF- α), as one of most important inflammatory factor, have large amount of activities to permit cell-cell communication and involve in inflammation-associated tumorigenesis²⁰.

The classical signaling pathway responded to TNF α is NF- κ B pathway via TNFR1. TNFR1 engagement leads to recruitment of the adaptor molecule TRADD, which subsequently recruits the signaling proteins TRAF2 and RIP1. TRAF2 mediates recruitment of the IKK complex. TAB2 and TAB3 interact with TRAF2 and TAK1 resulting in the activation of TAK1. TAK1-phosphorylated IKK β is on its activation loop. Active IKK phosphorylates I κ Bs on conserved serines, triggering their degradation through β -TRCP ubiquitin-proteasome proteolytic system and release of bound NF- κ B dimers, which translocate to the nucleus to drive gene transcription which associates with tumorigenesis²¹.

Accumulated evidences show that the low-dose, chronic TNF α production is a feature of many tumor cells²². For example, the tumor-promoting role of TNF α was

similarly investigated in models of hepatic carcinogenesis. TNFRI^{-/-} mice displayed reduced oval cell (hepatic stem cell) proliferation during the pre-neoplastic phase of liver carcinogenesis, correlating with fewer tumours than wild-type mice²³. In addition, dysregulation of TNF α -IKK-NF- κ B pathway is usually found in many human cancers. In Barrett's esophageal adenocarcinoma and hepatoma, TNF α -IKK-NF- κ B pathway is upregulated^{24,25}; Mutation of CYLD induced constitutive activation of NF- κ B occurs in cylindromatosis patients²⁶⁻²⁸; couple of mutations or loss-function I κ B are detected in couple of cancer types, including colon cancer and breast cancer²⁹⁻³³. These pathological findings indicate the clinical relevance of TNF α signaling pathway in human cancer development.

1.3 Independent Role of IKK subunits to NF- κ B pathway

IKK α and IKK β are major downstream serine/threonine kinases which have multiple functions dependent and independent on the NF- κ B pathway. Previous study show that NF- κ B-independent activities induced by TNF α ³⁴⁻⁴⁰, For example, independent IKK β positively regulate inflammation linked tumorigenesis and angiogenesis in several cancer types⁴¹⁻⁴³.

It has also been reported that IKK α can be activated by TNF α treatment independently of IKK β . For example, two Nature papers, published side-by-side, showed that IKK α , in response to TNF α , translocates from the cytoplasm to the nucleus and phosphorylates histone H3 to regulate transcription of target genes^{44,45}.

In addition to histone H3, we also reported that IKK α is able to phosphorylate CBP and switches CBP binding preference from p53 to NF- κ B upon TNF α stimulation⁴⁶. These TNF α -induced IKK α activities are independent of IKK β . There are several reports that strongly support the idea that IKK α is able to contribute to tumor development independently of NF- κ B. For instance, the nuclear function of IKK α has also been implicated in prostate cancer metastasis by inhibiting the expression of the tumor suppressor, Maspin, in mouse model⁴⁷. Moreover, IKK α is able to phosphorylate and regulate ER, AIB1, CYCLIN D1, and β -CATENIN in cell proliferation⁴⁸⁻⁵². Briefly, IKK α can increase cyclin D1 expression by phosphorylating and activating the estrogen receptor- α and SRC-3 on the cyclinD1 promoter as well as phosphorylate cyclinD1 directly as a feedback loop. These activities contribute to oncogenic effects. These findings suggest that IKK α has the unique ability in regulating versatile physiological and pathological functions, which are independent of NF- κ B.

1.4 Forkhead Box Protein (Fox) and Cancer

Fox proteins are super transcriptional regulator family that is evolutionarily conserved. This family defined by a unique DNA-binding domain (DBD) called the forkhead box or winged helix domain^{53,54}. Members of this family bind DNA as monomers and regulate downstream target gene expression. The N terminus and C terminus contain transactivation domains whose function is regulated at the level

of phosphorylation⁵⁵. So far, there are 17 Fox gene subfamilies, with at least 41 genes identified in humans^{56,57}. Fox proteins play important roles in a wide spectrum of biological processes, including metabolism, development, migration and cell cycle events⁵⁸⁻⁶². Since Fox proteins control these essential homeostatic processes, the fact that losing or gaining of their functions alter cell fate and lead to tumorigenesis is in expectation. For example, in FoxA family, which shows a unique tissue-specific expression pattern, FoxA1 is essential for differentiation of epithelial cells in normal prostate, while prostate of FOXA1 knockout mice show hyperplastic lesions^{63,64}; Overexpressed FoxA2 act as a key regulator in colon liver metastasis⁶⁵. Except FoxA, several Fox subfamilies, such as FoxO, FoxP and FoxM are verified to link to tumorigenesis and the progression of certain cancers⁶⁶⁻⁷⁰.

Post translational modification, such as phosphorylation, acetylation and ubiquitination regulate Fox proteins activities. One of most clear example is FoxO3A. Several upstream kinases including Akt, JNK, CDK2 and I κ B kinase (IKK) phosphorylate FoxO3A to induce its nuclear export and degradation. Deregulation of these kinases normally happen in tumors and contribute to tumorigenesis⁷¹⁻⁷⁴. For example, disruption of IKK affects chromatin remodeling and NF κ B signaling. Moreover, IKK has been shown to physically bind to and phosphorylate FoxO3A in tumor, and cytoplasmic FoxO3A correlates with the expression of IKK β in many tumors and associates with poor prognosis in breast cancer⁷². Recent studies also show that insulin-induced FoxA2 phosphorylation by Akt promotes FoxA2 nuclear

exclusion, inactivates downstream target genes and unrestrains hepatic gluconeogenesis in type 2 diabetes, a disease strongly associated with cancers. Not only phosphorylation, acetylation and mono-ubiquitylation of Fox proteins play critical part in Fox regulated cancers. For example, FoxP3 suppresses of target gene expression involving a histone acetyltransferase–deacetylase complex^{75,76}.

Deregulation of Fox factors can promote tumorigenesis by favoring proliferation and survival through the repression or induction of gene expression. For example, FoxO3a regulate FasL and p27 transcription^{77,78}, FoxA2 is a well known regulator of GATA3 gene.^{79,80} The advent of chromatin immunoprecipitation coupled with microarray technology (ChIP-chip) brings larger scale of views of Fox transcriptional regulatory functions than which on traditional gene-to-gene basis⁸¹⁻⁸⁵. Recently, genome-wide ChIP-sequencing experiments, which can detect transcription factor binding in any genomic region accurately, are used in FoxA2 to screen its downstream target genes in adult mouse liver. A large number of novel Foxa2 binding targets are reported for the first time, including both tumor suppress genes and well known oncogenes, such as fgf1, numb, and dlk1^{86,87}.

Based on our so far knowledge of Fox family, therapies that indirectly target Fox factors through cofactors or repressors of Fox, or direct target Fox by modifying the post-translational regulation of Fox factors, may enable a shift in Fox-induced gene expression, and change aberrantly regulated Fox proteins functions. Based on cooperativity with other regulatory factors, redundancy and tissue-specific roles of Fox proteins, Fox family proteins targeted drugs are

promising to have lower toxicity and higher tissue specificity. In clinical, some Fox family proteins are used, or have potential to be used, as both indirect and direct targets⁸⁸. Breast carcinoma chemotherapeutic drugs paclitaxel activates JNK, which modulate the stability of FoxO3A by phosphorylation DBD of FoxO3A so that blocks tumorigenic cascades regulated by FoxO3A^{89,90}. FoxA1 as a marker for luminal subtype A breast cancer is also a good therapeutic candidate⁹¹. (Fig1)

Fox proteins as therapeutic targets

Cancer Type	Drug or therapy	Fox protein targeted	Pathway affected
Hepatocellular carcinoma	ARF peptide inhibitor	FOX M1	FOX M1 downregulation and induction of apoptosis
Osteosarcoma	Siomycin A	FOX M1	Downregulation of FOX M1 expression
Breast Cancer	Paclitaxel	FOX O3A	Decrease in Akt and Increase in JNK activity, leading to FOX O3A activation and induction of BIM
Melanoma	Adenovirus mediated transfer of constitutively FOX O3A	FOX O3A	Activation of FOX O3A and induction of apoptosis
Melanoma	Vaccination with dendritic cells transfected with	FOXP3	FOXP3-specific cytotoxic T lymphocyte response and

Fig 1 FOX proteins work as therapeutic targets in clinic. FOX M1 are used in HCC and Osteosarcoma; FOX O3A are used in both breast cancer and melanoma; FOXP3 are also used in melanoma via vaccination.

1.5 NOTCH pathway and its role in cancer

The discovery of a notch on wings of *Drosophila melanogaster* in 1914 is the first time NOTCH gene get noticed⁹². With later one century studies, researchers

are opening the door to an ever-widening understanding of Notch family and related signalings which controlled multiple cellular processes, influence cell fate decision.

The Mammalian Notch family has four members, NOTCH1, NOTCH2, NOTCH3 and NOTCH4⁹³. The map of NOTCH signaling is not fully clear, but the general scheme is accepted. NOTCH signaling is initiated by the binding of NOTCH ligands with NOTCH receptors. After S1 cleavage of NOTCH precursor, mature NOTCH which comprises with two subunits, ECD (extracellular domain) and ICD (intracellular domain), changes its conformation, leading to S2 cleavage site exposure. S3 cleavage mediating by γ -secretase complex follows with S2 cleavage consequently. The S3 cleavage results in the release of NICD from cell plasma membrane and translocation of NICD to nucleus. Major function of nuclear NICD is to mediate the conversion of the CBF1–Su(H)–LAG1 (CSL) repressor complex into a transcriptional activation complex, therefore initiates a transcriptional cascade to activate or repress target genes, including p21, Myc and HES and HEY family⁹⁴⁻⁹⁶.

The first role of NOTCH found in human cancer is to be an oncogene in T-ALL. Most of T-ALL cases have activating mutation of Notch1, which leads to constitutive activating of Notch pathway to promote tumorigenesis⁹⁷. Comparing to causative role of Notch in T-ALL, the role of Notch signaling in solid tumors is vague. The status of The Notch signaling is highly dependent on the spatial and

temporal context of Notch activation and the status of other signaling pathways in the cells as well⁹⁸.

Several processes, including proteolysis, glycosylation, ubiquitylation and phosphorylation⁹⁹, control Notch activation. Ligands overexpression, abnormally activated receptors or dysregulation of negative regulators may lead to aberrant activation of the Notch pathway¹⁰⁰. Some of these circumstances are deregulated in cancers, resulting in aberrant Notch signaling¹⁰¹⁻¹⁰³.

NUMB and NUMB-like proteins function as signalling inhibitors for Notch by targeting the membrane-bound Notch for degradation following activation¹⁰⁴. Loss of NUMB has been detected in over 50% percent of breast carcinoma¹⁰⁵, and possibly results in the stabilization and hyperactivation of Notch. In addition, NUMB binds to p53 and MDM2 to prevent ubiquitylation of p53¹⁰⁶. Thus, loss of NUMB in a large proportion of breast cancers can result in increased Notch activity and loss of p53 tumor suppressor function, resulting in an aggressive tumor phenotype with poor prognosis.

In contrast to its oncogenic activities in multiple cancer types, Notch has been proved as a tumor suppressor in some cancer types as well. This tumor suppressor activity is worked as a result of crosstalk with other signaling pathways, which decrease cell proliferation, increase apoptosis or cellular differentiation¹⁰⁷. For example, NOTCH could block WNT signaling to suppress tumorigenesis thereby

driving cells toward a more differentiated phenotype¹⁰⁸.

The knowledge of the extensive crosstalk of the Notch pathway with other pathways such as the epidermal growth factor receptor (EGFR) pathway, WNT pathways, could prove useful in developing combinatorial cancer therapies¹⁰⁹.

1.6 Statement of problem, hypotheses and project goals

HCC is a typical tumor on a background of inflammation mainly triggered by exposure to infectious agents (hepatotropic viruses, inflammatory cytokines). In the past twenty years, many studies unfold significant alterations of different cytokines and their cascades in liver cirrhosis and HCC. However, the molecular links between inflammation and cancer progression are still remaining contradiction and not completely known.

In previous studies, TNF α tumor promoting effects were found in several genetic mouse model of inflammation-related carcinogenesis through activating the key inflammatory transcriptional regulator NF- κ B. Contrarily, hepatocyte-specific deletion of IKK β gene or ablation of IKK γ /NEMO leads to the spontaneous development of HCC¹¹⁰⁻¹¹³. These phenomena indicate that other cascades, other than traditional NF- κ B, are involved in TNF α induced liver cancer. Based on our previous research that IKK α /IKK β can independently regulate downstream cascades to lead tumorigenesis and angiogenesis by phosphorylating Fox family, an interesting question is rising, whether IKK α /IKK β dependent Fox cascades play

critical role in inflammation-induced liver cancer? If yes, which Fox is the player?

In addition, in search of mechanisms that may be involved in TNF α -mediated tumorigenesis, the previous study showed that controversial roles of NF- κ B in TNF α -mediated liver cancer in several genetic mouse models, thus it is still not clear whether IKK α/β has a role independent to NF- κ B in the TNF α -mediated tumorigenesis. Preliminary data showing that in HCC patients, p-IKK expression has positive correlation to activated NOTCH1 expression. Therefore, it is possible that there are other NF- κ B-independent pathways involved in TNF α -mediated NOTCH1 production and tumorigenesis. We hypothesize that TNF α induces NOTCH1 activation through IKK-mediated phosphorylation of FOX protein, therefore increase of NOTCH1 pathway activation, finally leading to tumorigenesis.

CHAPTER 2 MATERIALS AND METHODS

2.1 Cell culture

Human hepatocellular carcinoma cancer cell lines (Hep3B, Huh-7, and HepG2), human embryonic kidney cell lines (HEK-293 and HEK-293T) and mouse embryonic fibroblasts (*Ikk α ^{-/-}*, *Ikk β ^{-/-}*) were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 10% fetal bovine serum. For transient transfection, cells were transfected with DNA by either Lipofectamine with Plus reagent or electroporation.

2.2 Plasmids, antibodies, and chemicals

We constructed Myc-FOXA2- and GFP-FOXA2-expressing plasmids by

inserting hFOXA2 complementary DNA (cDNA) into pcDNA6 and pCMV-EGFP vectors containing the Myc and GFP tags, respectively. We generated the GST-FOXA2-expressing plasmid by subcloning the FOXA2 fragment into the pGEX-6P-1 GST vector. Different mutant forms of the FOXA2 construct were generated by subcloning the FOXA2 fragment into pcDNA6 and pCMV-EGFP vectors. Mutants were generated using the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene). The FOXA2 and mutant expression plasmids were constructed into retroviral pBABE-Puro and pBABE-GFP vectors for retroviral infection and expression.

Antibodies against FLAG (F3165, Sigma), Myc (11667203001, Roche), IKK α (sc-7606, Santa Cruz), IKK β (sc-8014, Santa Cruz), I κ B α (9242S, Cell Signaling Technology), TNF α (Santa Cruz Biotechnology, SC-8301), I κ B α (Santa Cruz Biotechnology, SC-371), IKK γ (Santa Cruz Biotechnology, SC-8330), RHEB (Santa Cruz Biotechnology, SC-6341), GAPDH (Santa Cruz Biotechnology, SC-20357), NOTCH1 (NICD) (sc-32745, Santa Cruz Biotechnology), plkB α (S32/S36) (Cell Signaling Technology, 9246), NUMB (ab14140, Abcam), FOXA2 (ab60721, Abcam), p-IKK α (S176/180) (2697, Cell Signaling), α -tubulin (T-5168, Sigma), and actin (A2066, Sigma) were purchased from the indicated suppliers. Antibodies to the S107/111 phosphorylation sites of FOXA2 were generated at China Medical University. Synthetic phosphorylated peptides representing portions of FOXA2 around both S107 and S111 were used as antigens for producing antibodies.

TNF α was purchased from Sigma, and Bay (43-9006) and SCI34 were purchased from CalBiochem. Full-length recombinant GST-IKK α and His-IKK β proteins were purchased from EMD Millipore. The liver tumor tissue array was purchased from US Biomax.

2.3 siRNA

Hep3B cells or Huh-7 cells were transfected with IKK α ON-TARGETplus siRNAs (LQ-003473-00-0002), NUMB ON-TARGETplus siRNAs (LQ-015902-00-0002), NOTCH ON-TARGETplus siRNAs (LU-007771-00-0002), or control SMARTpool siRNA (Upstate Biotechnology, D-001206-13-05) by lipofectamine with plus reagent and lysed 72 h after transfection.

2.4 Transfection

For transient transfection, cells were transfected with DNA by either SN liposome (59), lipofectamine with plus reagent, or electroporation using a Nucleofector 1 device (Amaxa) with electroporation buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM glucose, and 20 mM HEPES, pH 7.0). To investigate the effects of IKK on p-FOXA2 (Ser107/111), or NOTCH1 activation, the transfected cells were serum-starved overnight and extracted directly or after stimulation with TNF α (20ngl/ml), for 30 min or 24h.

2.5 In vitro pull-down assay

Recombinant IKK α proteins and IKK β proteins (EMD Millipore 21233; Upstate Biotechnology, 14-485) were incubated with *in vitro* transcription and translation lysates of myc-tagged TSC1, which was produced using a TNT coupled reticulocyte lysate system (Promega), in binding buffer (25 mM Tris-HCl, pH 7.5, 125 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 μ g of leupeptin/ml, 1 μ g of aprotinin/ml, and 1 μ g of pepstatin/ml) for 1 h at 4°C. Rabbit IgG antibody (rlgG) or anti-IKK β antibody was then added and incubation was continued at 4°C overnight. The resulting immunocomplexes were precipitated with protein A-Sepharose beads (Roche) at 4°C for 4 h and washed extensively with Tris-buffered saline, and the bound proteins were eluted with sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer and then analyzed by Western blotting.

2.6 Immunoprecipitation and immunoblotting

Cells were lysed in RIPA-B buffer (20 mM Na₂HPO₄ [pH 7.4], 150 mM NaCl, 1% Triton X-100) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 mM NaF, 2 mM sodium orthovanadate, 3 μ g/ml aprotinin, and 750 μ g/ml benzamidine). For immunoprecipitation, the samples were precleaned with either protein A or protein G agarose for 1 hr at 4 °C. Precleaned cell lysates were Proteins were immunoprecipitated by indicated antibodies for overnight at 4 °C, incubated with either protein A or protein G agarose for 3 hr at 4 °C, washed

ice-cold RIPA-buffer with protease inhibitors four times, resolved by SDS-PAGE, and transfer to PVDF membranes. For immunoblotting, membranes were blocked with TBST buffer (10 mM Tris-HCl (pH 7.9), 150 mM NaCl, and 0.05% Tween 20) with either 5% BSA or 5% skim milk, incubated with indicated primary antibodies, subsequently incubated with HRP-conjugated secondary antibodies and detected by ECL (Amersham Biosciences).

2.7 In vitro kinase assays

Briefly, 80% confluent Hep3B cells were serum starved for 16 h and then treated with/ or without 20ng/ml TNF α for 30 min. The cells were lysed and immunoprecipitated with either anti-IKK α antibodies and analyzed by *in vitro* kinase assay using the purified GST-FOXA2 as substrates, respectively, and incubate at 30°C for 30 min in the presence of 50 mM ATP in a kinase buffer with 5 μ Ci [γ -³²P]ATP. Reaction products were subjected to SDS-PAGE and analyzed by autoradiography.

2.8 Identification of in vivo phosphorylation sites by mass spectrometry

After protein gel electrophoresis, bands corresponding to FOXA2 phosphorylated by IKK α *in vivo* were identified, excised from gels, and subjected to tryptic digestion. After being isolated by immobilized metal affinity chromatography, the enriched phosphopeptides were analyzed by micro-liquid chromatography/tandem mass spectrometry.

2.9 Real-Time PCR

Primer sequences used to quantify real-time PCR were as follows: Hey2: 5'-AAACAAGGATCTGCAA-3' and 5'-CGGAATCCTATGCTCATGAA-3'; Hey1: 5'-TATCTGAGCATCATTGAA-3' and 5'-TGTGCGGGTGATGTCCGAA-3'; p21: 5'-GTGAAGGCTGTGTCTTC-3' and 5'-TACAGATTTTGTGTGCTCT-3'; p27: 5'-AATTTTCGATTTTCA-3' and 5'-CTTGCAGGCACCTTTGGGGG-3'; Hes6: 5'-ATCAACGAGAGCCTG-3' and 5'-CACTGGATGTAGCCGGCAGCGAA-3'.

2.10 ChIP and Re-ChIP Assay

Primer sequences used for ChIP-PCR were as follows: NUMB: 5'-AATGAAATTGGATGCATTGAGACCA-3' (forward) and 5'-AAGCTTATCTCTGCTAGCTTTATTCA-3' (reverse). GAPDH promoter was used as a negative control.

2.11 Anchorage-Independent Growth Assay

Briefly, the cell-growth matrix consisted of base agar and top agarose in six-well culture plates. The base layer (1.5 ml) contained DMEM/F12 medium, 10% FBS, and 0.5% agar. The top layer (1.5 ml) contained DMEM/F12 medium, 10% FBS, 0.35% agarose, and the suspension of cells (5×10^3). The foci were counted after 3 weeks. The modifications used for retrovirus-infected Hep3B cells are as follows: retrovirus-infected cells (5×10^4) were mixed with 0.5% agarose and poured onto a bed of 1% agar. Both the top and the bottom layers were prepared in DMEM/F12

medium with 10% FBS.

2.12 Immunohistochemical Staining

Briefly, human histoarrays of multiple cancerous tissues and human liver cancer tissues primary tumor specimens of HCC, colon, lung, and prostate cancer (IMH-365, US Biomax) were incubated with antibodies directed against p-IKK α or NICD (activated NOTCH1), detected with biotin-conjugated secondary antibody and avidin–peroxidase, and visualized by aminoethylcarbazole chromogen. Images were analyzed by ACIS (Dako). The samples were divided into two groups according to staining intensity: low (+/-) and high (+/+). The human liver primary tumor samples for Western blotting were received from Gaoxiong Medical School. Fisher's exact test and Spearman rank correlation were used for statistical analysis; $P < 0.05$ was considered statistically significant. According to histologic scoring, the intensity of staining was ranked into four groups: high (score 3), medium (score 2), low (score 1), and negative (score 0). The immunoreactivities for pIKK, NOTCH1 were semiquantitatively scored using a well-established immunoreactivity score system in which immunoreactivity score was generated by incorporating both the percentage of positive tumor cells and the intensity of staining.

2.13 Mouse Model for Tumorigenesis

We performed the tumorigenesis assay for retrovirus-infected cells with both a liver cancer subcutaneous mouse model and an orthotopic mouse model, which

has been described elsewhere. Cells were injected subcutaneously. After tumors reached 2–3 mm in diameter, tumors were harvested, dissected into small pieces ($1 \times 1 \times 1 \text{ mm}^3$), and transplanted into the livers of new mice ($n = 5$ per group). TV was calculated according to the formula described by Yaguchi et al.: $TV = 0.5 \times \text{length} \times \text{width}^2$. Animal experiment protocol number is approved by the UT MD Anderson Cancer Institutional Animal Care and Use Committee under protocol number ACUF 06-87-06139. Thirty patient samples are from National Cheng-Kung University Hospital. Thirty patients admitted to National Cheng-Kung University Hospital with HCC who received curative surgery between January 1, 2003, and December 31, 2006, were enrolled, and National Cheng-Kung University Hospital Institutional Review Board approved the protocol.

2.14 Statistical Analyses

Statistical analyses were performed with Student's t test, Spearman's rank correlation test, or Pearson's chi-square test as indicated. $p < 0.05$ was considered statistically significant. $R^2 > 0.5$ was considered statistically correlated.

CHAPTER 3

Phosphorylated IKK expression positively correlates with activated NOTCH1 expression and high HCC tumor grade

It is well known that both $\text{TNF}\alpha$ triggered signal pathways and NOTCH1 signaling are critical to tumor cell proliferation and tumorigenesis in different cancer types. However, the critical underneath molecular mechanism linking these two pathways in hepatocellular carcinoma (HCC), especially inflammation associated hepatocellular carcinoma is not clear. To address a potential crosstalk between these two pathways in inflammation-associated HCC tumorigenesis, we examined the expression level of p-IKK, which is an important downstream kinase of $\text{TNF}\alpha$, and the level of intracellular domain of NOTCH1, which is considered as activated NOTCH1 in 100 human primary HCC tumor specimens by immunohistochemical (IHC) staining. In 100 specimens, 47 of them were detected with high p-IKK expression, and 27 of which showed high NICD expression, indicating a significant positive correlation between p-IKK and NICD ($p = 0.003$, [Figures 2A and 2B](#)). In addition, both p-IKK and NICD had higher expression levels in higher-grade tumors ([Figures 3A and 3B](#)). To determine whether this relationship holds in other cancers, we examined human primary tumor specimens of colon, lung, and prostate cancer by IHC staining. Similar to our finding in liver tumors, we also found a positive correlation between p-IKK and NICD (see [Figure 4](#))

Fig 2. Clinic Correlation between p-IKK and NICD

(A) Correlation between levels of p-IKK α/β and activated NOTCH1 (NICD) in 100 human primary HCC tumor specimens stained with antibodies specific to NICD and p-IKK ($P = 0.003$, Pearson's chi-square test).

(B) Two representative specimens from (A); arrows point to p-IKK and NICD.

Liver tumor tissues samples		NICD (Activated NOTCH1)		Total
		Low(+/-)	High(+++)	
p-IKK	Low(+/-)	39	14	53
	High(+++)	20	27	47
Total		59	41	100

$P=0.003$

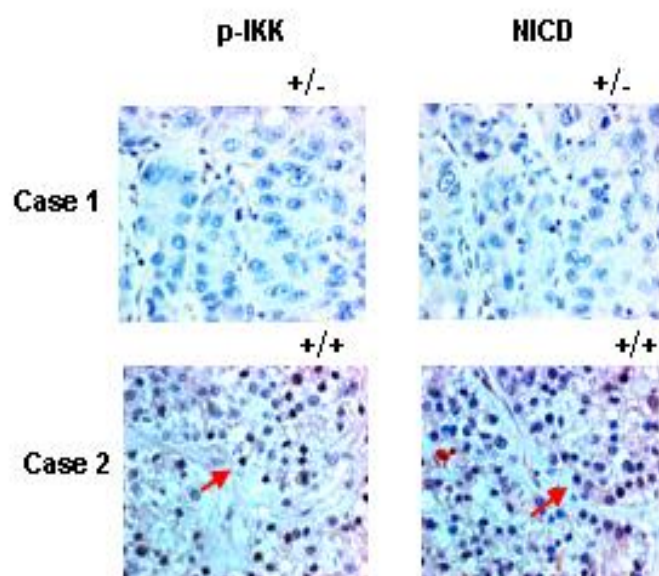


Fig 3 Clinic correlation between p-IKK/NICD and tumor grade

(A) Correlation between tumor grade and p-IKK expression. High p-IKK expression correlated significantly with higher tumor grade ($P = 0.035$, Pearson's chi-square test).

(B) Correlation between expression of p-IKK, NICD and tumor grade. High NICD expression correlated significantly with higher tumor grade ($P = 0.01$, Pearson's chi-square test).

Liver tumor tissues samples		Grade			Total
		1	2	3	
p-IKK	Low(+/-)	7	33	13	53
	High(+++)	3	21	23	47
Total		10	54	36	100

$P=0.035$

Liver cancer tissues		Grade			Total
		1	2	3	
NICD	Low(+/-)	9	35	15	59
	High(+++)	1	19	21	41
Total		10	54	36	100

$P=0.01$

Fig 4 Clinic correlation between p-IKK and NICD in multiple cancer types

Association between levels of p-IKK α/β (p-IKK) and activated NOTCH1 (NICD) in human primary tumor specimens of colon, lung, and prostate cancer stained with antibodies specific to NICD and p-IKK ($P = 0.024$, Pearson's chi-square test).

Tumor tissues of multiple cancer types		NOTCH		Total
		Low (+/-)	High (+/+)	
p-IKK	Low	11	3	14
	High	6	10	16
Total		17	13	30

$P=0.024$

CHAPTER 4 IKK α is required for TNF α -induced NOTCH1 activation

The clinical positive correlations between p-IKK and NICD raise a question in our mind: whether TNF α /IKK might upregulate NICD expression. To validate the relationship between IKK and NICD expression, we detected NOTCH1 intracellular domain, which is an activated form of NICD in mouse embryonic fibroblasts (MEFs) treating with TNF α (Figure 5). We found that after TNF α treatment, NICD expression in MEFs increase (Figure 5, top), which indicates that NOTCH1 activation was increased upon TNF stimulation. To further investigate which components of IKK complex regulates NICD expression, wild-type (WT), IKK $\alpha^{-/-}$, and IKK $\beta^{-/-}$ MEFs were treated with TNF α and compared their NICD expression level by westernblotting assay. The NICD level in IKK $\alpha^{-/-}$ MEFs was substantially lower than in WT MEFs (Figure 5, bottom; compare lanes 1 and 2). Re-expression of IKK α in the IKK $\alpha^{-/-}$ MEFs rescued NICD expression (Figure 8, bottom; compare lanes 2 and 3). However, re-expression of IKK β could not affect the NICD level in IKK $\beta^{-/-}$ MEFs (Figure 5, bottom; compare lane 1 with lanes 4 and 5). Together, these results indicate that IKK α , but not IKK β , is necessary components in TNF α -induced NICD expression, suggesting that TNF α may stimulate NOTCH1 activation through an IKK α -dependent pathway.

To further confirm that IKK α is required for TNF α -mediated increase of NICD expression, a RBP-Jk reporter luciferase assay, which could monitor the activity of NOTCH1 signal transduction pathways in cultured cells by showing activities of two NOTCH1 downstream enzymes, was used as a tool to detect NOTCH1 activity.

IKK α expression was detected by western blot analysis to make sure IKK α was knocked down by siRNA (Figure 6, top). RBP-Jk reporter luciferase assay showed the similar results that NOTCH1 is activated by TNF α stimulation and such activation was that blocked by knocking down IKK α (Figure 6).

Fig 5 IKK α is required for TNF α -induced NOTCH1 activation

Top: WT MEFs were treated with or without TNF α (20 ng/ml) for 24 h, and cell lysates were subjected to western blot analysis for NICD expression.

Bottom: IKK α and IKK β were transfected into IKK α ^{-/-} MEFs and IKK β ^{-/-} MEFs, respectively. After treatment with TNF α (20 ng/ml) for 24 h, cell lysates were subjected to western blotting. Lysates from MEFs without TNF α treatment served as control. α -Tubulin was used as loading control.

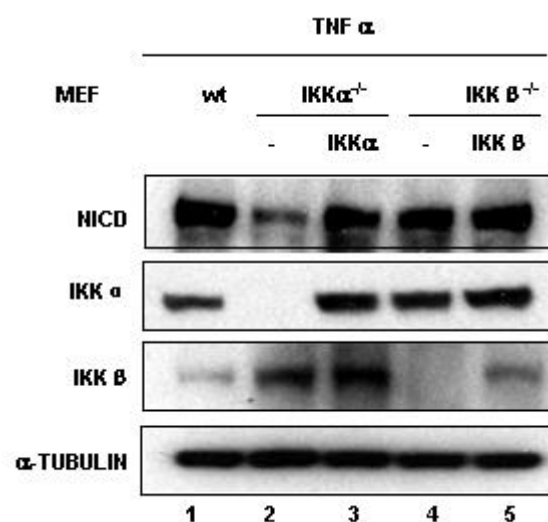
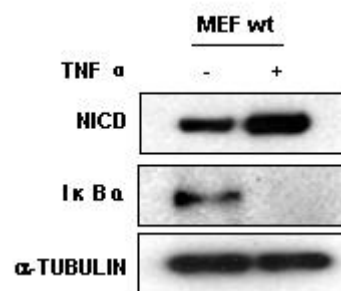
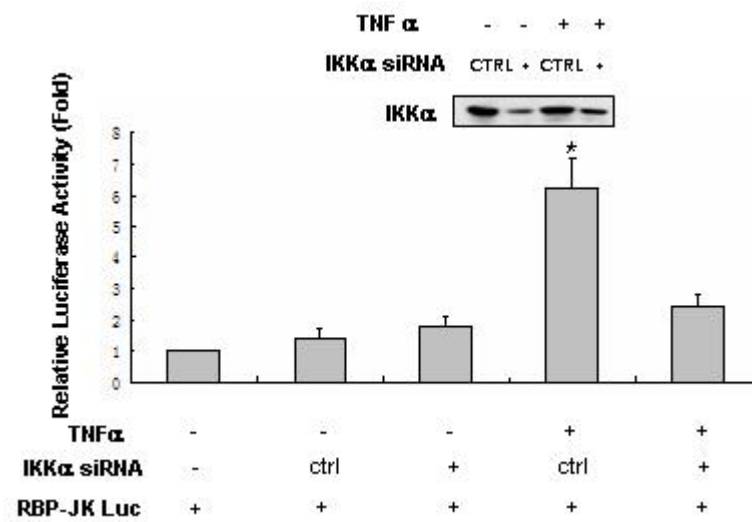


Fig 6 NOTCH1 activity was blocked by IKK α knocking down

RBP-Jk Luc and IKK α siRNA were transfected into Hep3B cells for 48 h. After transfection, WT and IKK α -knockdown Hep3B cells were treated with TNF α for 24 h. Cell lysates were subjected to western blotting and luciferase reporter assay. Cells were transfected with nontargeting siRNA as control. Error bars represent SD ($n = 3$). * indicates, $P < 0.05$.



CHAPTER 5 NUMB suppression involves in TNF α induced NOTCH1 activation

After we know TNF α /IKK α regulates NOTCH1 activation, we are trying to know how what is the regulator in TNF α /IKK α induced NOTCH1 activation. As NUMB is known to antagonist NOTCH1 activation through degradation membrane-bounded NOTCH1 intracellular domain, we first tested whether NUMB expression level changed in the process of TNF α /IKK α -mediated NOTCH1 activation. We found that TNF α treatment upregulated NICD expression and at the same time, NUMB expression level decreased in both Huh-7 and Hep3B in HCC cell lines (Figure 7A). Consistent with our results that only IKK α , but not IKK β , involves in TNF α /IKK α regulates NOTCH1 activation, ectopic expression of IKK α but not IKK β increased NICD expression and diminished NUMB expression as well (Figure 7B). In addition, blocking IKK α expression by siRNA knocking down recovered TNF α -mediated NUMB downregulation and subsequent impaired TNF α -mediated NOTCH1 activation (Figure 8A, compare lanes 2 and 4). Knockdown of both IKK α and NUMB restored NICD expression (Figure 8A, compare lanes 4 and 6), suggesting that TNF α /IKK α -mediated NOTCH1 activation involves NUMB downregulation. MEFs and IKK $\alpha^{-/-}$ MEFs were used to further analyzed the relationships of IKK α , NUMB, and NICD. Higher NUMB expression and consistently lower NICD expression were found in IKK $\alpha^{-/-}$ MEFs than in WT MEFs (Figure 8B). Rescue IKK α by overexpressing IKK α in IKK $\alpha^{-/-}$ MEFs leads to

decreasing of NUMB expression and upregulation of NICD expression accordingly (Figure 8B).

Fig 7 IKK α , but not IKK β downregulates NUMB expression

(A) Hep3B and Huh-7 cells were treated with TNF α (20 ng/ml) for 24 h. Endogenous NICD and NUMB expression levels were examined by western blotting. α -Tubulin was used as loading control.

(B) Hep3B cells were transfected with IKK α and IKK β . Endogenous NICD and NUMB expression was analyzed by western blotting. α -Tubulin was used as loading control.

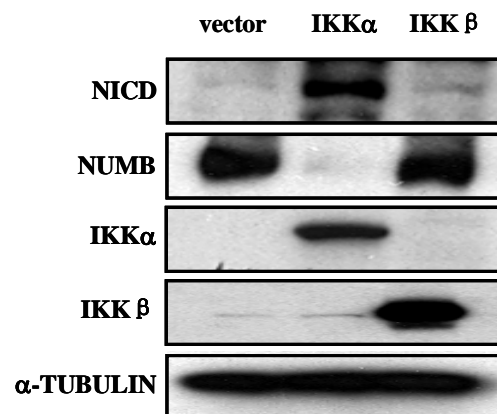
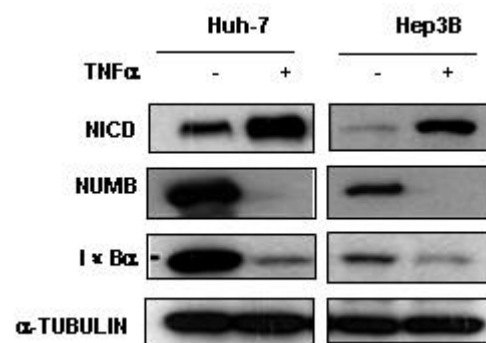
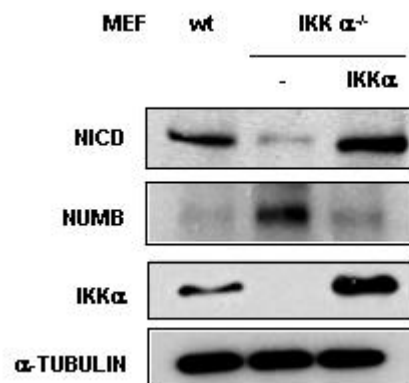
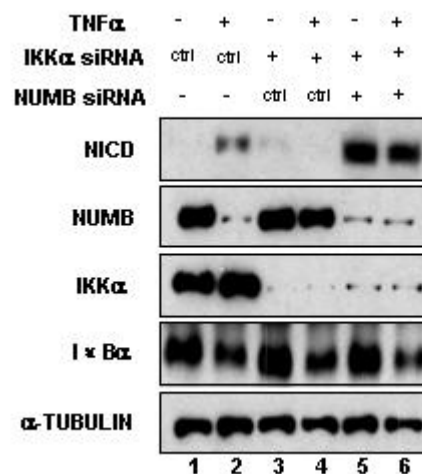


Fig 8 Knocking down IKK α impaired TNF α -induced NOTCH1 activation and increased NUMB expression

(A) Knockdown of IKK α or double knockdown of IKK α and NUMB by siRNA in Hep3B cells. Nontargeting siRNA was used as control. After 24 h of TNF α treatment, cell lysates (30 μ g) were subjected to western blotting to detect endogenous NICD and NUMB expression. α -Tubulin was used as loading control.

(B) Western blot analysis of endogenous NICD and NUMB expression in WT MEFs and IKK α ^{-/-} MEFs with or without reconstituted IKK α . α -Tubulin was used as loading control.



CHAPTER 6 IKK α kinase activity is a key factor for TNF α induced NUMB suppression and consequent NOTCH1 activation

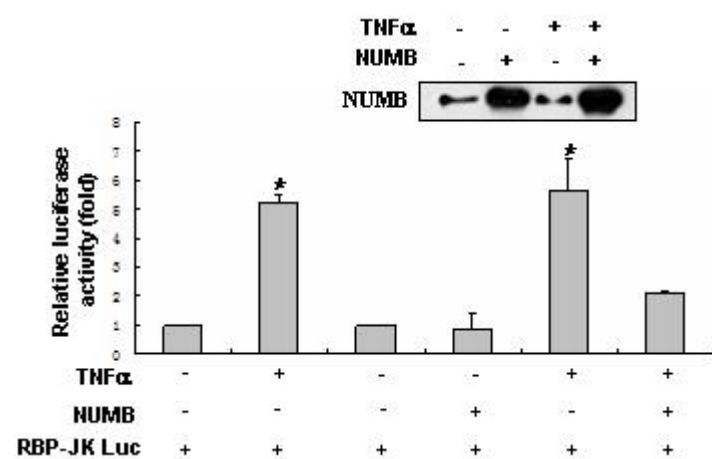
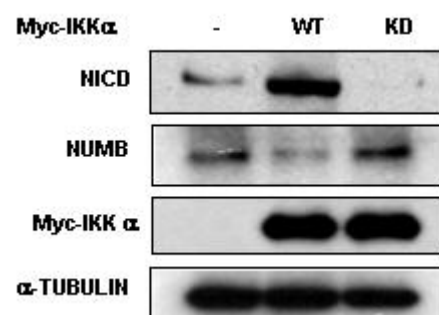
IKK α is a well-known downstream kinase of TNF α signalling, the next question naturally comes out “ does IKK α trigger NUMB/NOTCH1 signalling via its kinase activities” Kinase-dead (KD)IKK α mutant, which does not have any kinase activities, were introduced into hepatocyte cells. We found not similar to wide type IKK α , kinase-dead (KD) IKK α mutant could not downregulate NUMB expression (Figure 9A), indicating that TNF α /IKK α induce NUMB downregulation and NOTCH1 activation via IKK α kinase activities. . In addition, RBP-Jk luciferase assay showed that overexpression of NUMB could block TNF α -induced NOTCH1 activation (Figure 9B). To answer does IKK α -induced NOTCH1 activation functionally affects the NOTCH1, we detected transcriptional change of some NOTCH1 downstream genes. RNA levels of c-Myc, p21, p27, Hes6, Hey1, and Hey2 were measured via real-time PCR with IKK α or NUMB expression. The mRNA levels of NOTCH1 downstream targets enhanced with IKK α but were decreased in the presence of NUMB (Figure 9C). Together, these results indicate that TNF α triggered IKK α kinase activity could activate NOTCH1 through NUMB suppression.

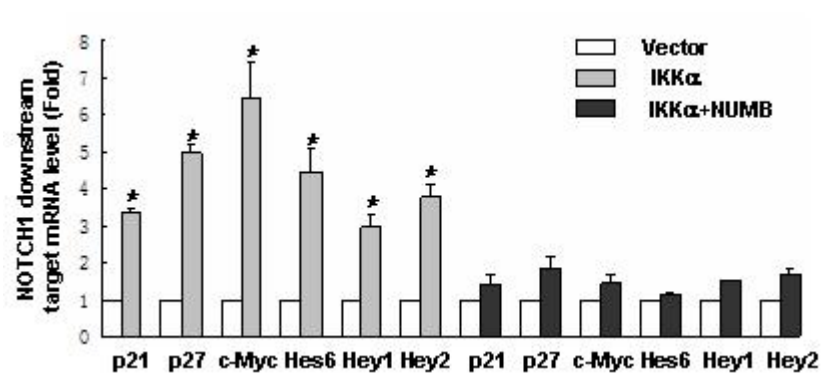
Fig 9 IKK α functionally effects NOTCH1 activities and suppression NUMB

(A) Hep3B cells were transfected with WT IKK α or KD IKK α . Endogenous NICD and NUMB expression was analyzed by western blotting. α -Tubulin was used as loading control.

(B) RBP-Jk Luc and NUMB were transfected into Hep3B cells for 48 h. After transfection, Hep3B cells were treated with TNF α (20ng/ml) for 24 h. Cell lysates were subjected to the luciferase reporter assay. NUMB expression was analyzed by western blotting with 10 μ g of total lysates. Error bars represent SD ($n = 3$). * indicates statistical significance ($P < 0.05$).

(C) Hep3B cells were infected with retrovirus expressing IKK α or IKK α and NUMB. RNA extracts were purified from the cell lysates and quantitated with real-time PCR. The NOTCH1 target gene mRNA levels were normalized to the mRNA levels of target genes in vector-infected cells. Error bars represent SD ($n = 3$). * indicates statistical significance ($P < 0.05$).





CHAPTER 7 NUMB was transcriptionally regulated by TNF α /IKK α via FOXA2

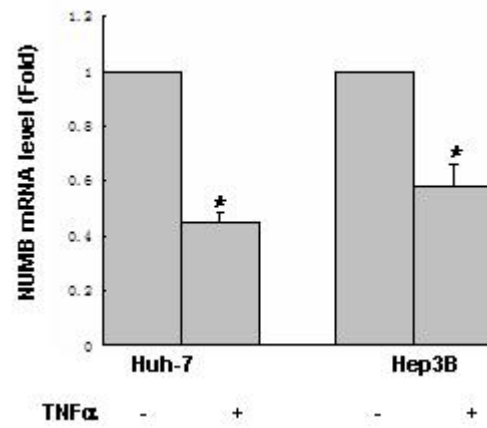
Since we know NUMB expression could be regulated by TNF α /IKK α , we further investigate the underneath molecular mechanism. We first checked the status NUMB mRNA using quantitative PCR (qPCR) after TNF α treatment in both Huh-7 and Hep3B cells. We found that mRNA of NUMB is decreased with TNF α treatment compared those without TNF α treatment (Figure 10A). Next, NUMB promoter was analysis to investigate possible candidates of transcriptional regulators on NUMB promoter activity. PROMO 8.3 program analysis results revealed five types of hepatocyte nuclear factor (HNF) binding sites richly clustered on the NUMB promoter (Figure 10B). HNFs are well known transcriptional factors involving in a wide spectrum of cell process in multiple human cancers, we further investigated whether and which HNFs associates with TNF α /IKK α mediated NUMB transcription regulation. Only FOXA2 (HNF3 β)-upregulated NUMB mRNA level was reduced with IKK α occurrence, indicating that FOXA2-activated NUMB transcription can be inhibited by IKK α (Figure 10C). Collectively, these results indicate that FOXA2, as a transcriptional factor of NUMB promoter, will decrease its transcriptional abilities on NUMB promoter with TNF α , therefore downregulate NUMB expression and activation of NOTCH1.

Fig 10 NUMB was transcriptionally regulated by TNF α /IKK α via FOXA2

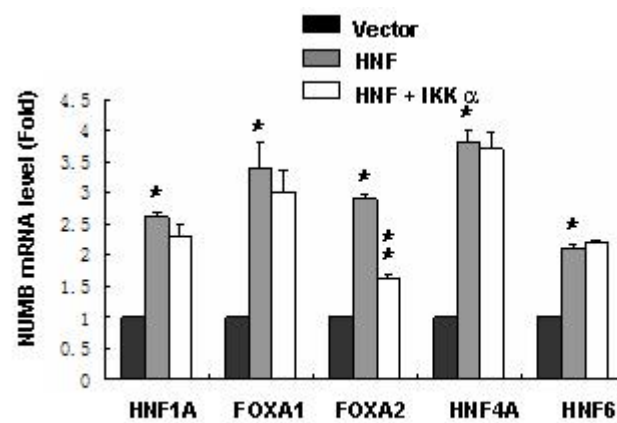
(A) Hep3B and Huh-7 cells were treated with TNF α (20 ng/ml) for 24 h. NUMB mRNA level was detected with qPCR. Error bars represent SD ($n = 3$). * indicates statistical significance ($P < 0.05$).

(B) The TRANSFAC 7.0 and PROMO 8.3 programs were used to predict transcriptional factors that bind to the NUMB promoter. Five HNFs were identified from the prediction.

(C) Each of the five indicated HNFs was transfected alone or together with IKK α into Hep3B cells. NUMB mRNA level was detected with real-time PCR. Error bars represent SD ($n = 3$). * indicates statistical significance ($P < 0.05$).



All predicted hepatocyte nuclear factors on Numb promoter				
HNF1A	HNF3α (FOXA1)	HNF3 β (FOXA2)	HNF4A	HNF6



CHAPTER 8 IKK α Interacts with and phosphorylates FOXA2 in vitro

Based on previous findings, the next coming out question goes to how FOXA2 activity was inhibited by IKK α . Since the kinase activity of IKK α involves in NUMB suppression and NOTCH1 activation, we first tested the interaction between IKK α and FOXA2 *in vivo*. Reciprocal co-immunoprecipitation showed that IKK α physically associated with FOXA2 (Figure 11A). The same protocol were used to detect the interaction between endogenous IKK α and FOXA2 using specific antibodies against IKK α and FOXA2 (Figure 11B). In addition, In vitro GST or His pull-down assays further supported only IKK α , but not IKK β , could binds with FOXA2 (Figure11C). Based on the physical association between IKK α and FOXA2, we examined whether IKK α could phosphorylate FOXA2. GST-FOXA2 was phosphorylated by purified recombinant IKK α in in vitro kinase assay. (Figure 11D, compare lanes 1 and 2). To further confirm and identified phosphorylation sites on FOXA2 by IKK α , we analyzed the kinase assay samples by mass spectrometry (MS) and found that FOXA2 was phosphorylated by IKK α at S107 and S111 (Figure 11E). Mutation of these two sites from serine to alanine abolished IKK α -mediated FOXA2 phosphorylation (Figure11D, lane 5). Taken together, these data demonstrate that IKK α interacts with and phosphorylates FOXA2 at S107/111 in vitro.

Fig 11 IKK α Interacts with and phosphorylates FOXA2 in vitro

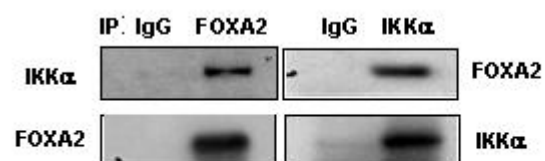
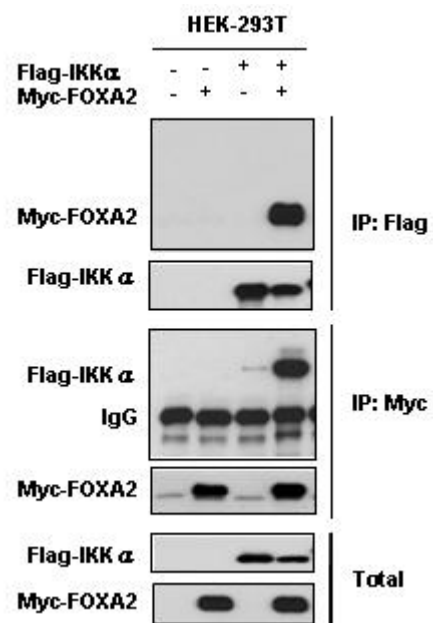
(A) IKK α and FOXA2 were transfected into 293T cells and analyzed by reciprocal co-immunoprecipitation and immunoblotting using indicated antibodies.

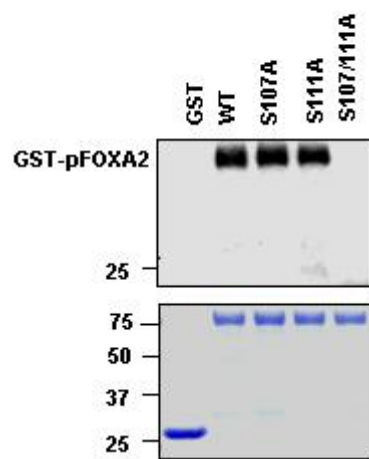
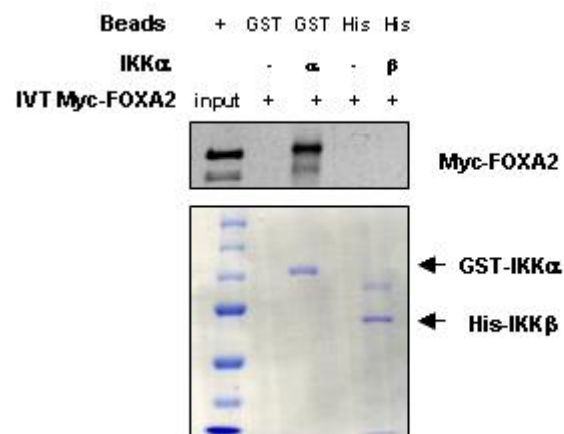
(B) Lysates of Hep3B cells were analyzed by reciprocal co-immunoprecipitation and immunoblotting using indicated antibodies.

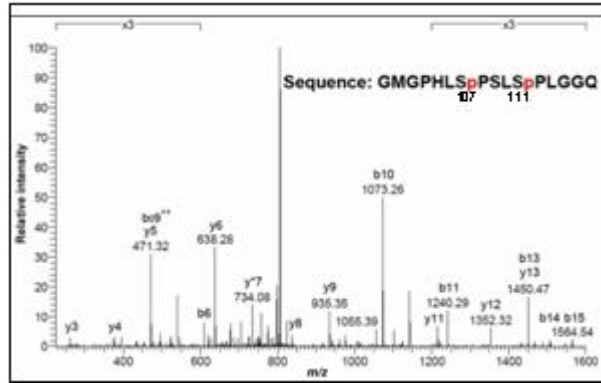
(C) In vitro transcribed FOXA2 proteins (labeled with S35) were incubated with commercially available recombinant His-IKK β and GST-IKK α and then pulled down with His/GST beads. IVT: in vitro transcription and translation. S35 concentration in cell lysates was determined by film exposure.

(D) FOXA2 phosphorylation was identified by in vitro kinase assay. GST-FOXA2 was pulled down by GST beads from Hep3B cells and incubated with commercially available IKK α for 30 min.

(E) Mass spectrum of FOXA2 phosphorylation sites by IKK α using samples (without isotope) from (D).







CHAPTER 9 IKK α Interacts with and phosphorylates FOXA2 in vivo

Although we identified two phosphorylation sites on FOXA2 in vitro, we need to further identify if IKK α could phosphorylate FOXA2 on these two sites in vivo. endogenous FOXA2 were isolated from TNF α treated Hep3B cells to perform MS analysis. These two phosphorylation sites were included in other 12 phosphorylation sites on FOXA2 with TNF α treatment (Figure 12A). For further investigation, we synthesized peptide including 107/111 phosphorylation sites and injected to mouse to raise specified mouse polyclonal antibodies against pFOXA2 at S107/111. The western immunoblotting assay showed that the antibody recognized specifically pFOXA2 (S107/111) phosphorylated by WT IKK α but not by KD IKK α (Figure 12B). The pFOXA2 antibody also recognized phosphorylated WT FOXA2 but not the double mutant with both serine residues mutated to alanine (FOXA2-SSAA), further supporting its specificity (Figure 12C). In addition, the pFOXA2 (S107/111) antibody detected pFOXA2 only in the presence of IKK α in an in vitro kinase assay (Figure 12D). Time course assays to study the effect of TNF α showed that levels of pFOXA2 (S107/111) were high at 10–30 min and declined thereafter (Figure 12E), consistent with the kinetics of TNF α -induced IKK α activation as measured by degradation of I κ B α .

Fig 12 IKK α Interacts with and phosphorylates FOXA2 in vivo

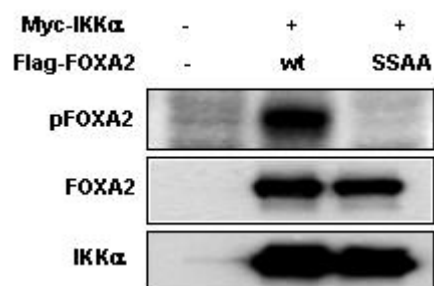
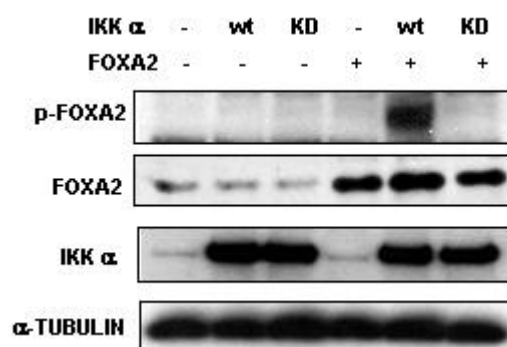
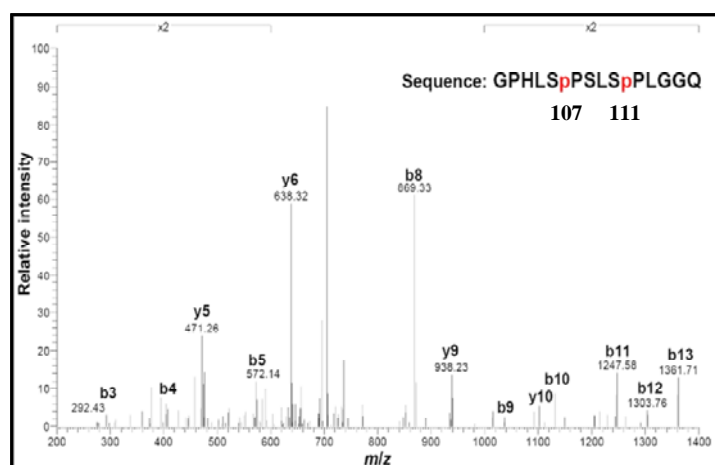
(A) Hep3B cells were treated with TNF α for 24 h, and cell lysates were analyzed by MS to identify in vivo FOXA2 phosphorylation sites.

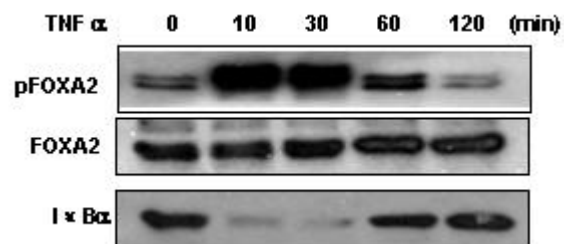
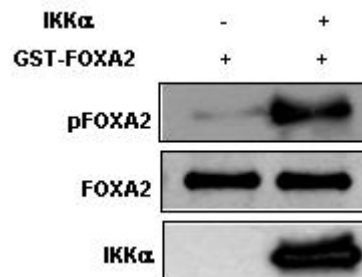
(B) Hep3B cells were co-transfected with FOXA2 and WT IKK α or KD IKK α . pFOXA2 expression was detected using an antibody specific to pFOXA2 (S107/111). Cell lysates (10 μ g) were loaded for western blotting.

(C) IKK α was co-transfected with WT FOXA2 or FOXA2-SSAA. pFOXA2 (S107/111) was detected using an antibody specific to pFOXA2 (S107/111). Total FOXA2 expression and IKK α expression are also shown.

(D) Western blot analysis of pFOXA2 with an antibody specific to pFOXA2 (S107/111) using samples (without isotope) from (D). Total FOXA2 expression and IKK α expression are also shown.

(E) Hep3B cells were treated with TNF α and harvested at 10, 30, 60, and 120 min after treatment. Cell lysates (40 μ g) were used for western blot analysis of pFOXA2 with an antibody specific to pFOXA2 (S107/111). Total FOXA2 expression and IKK α expression are also shown.



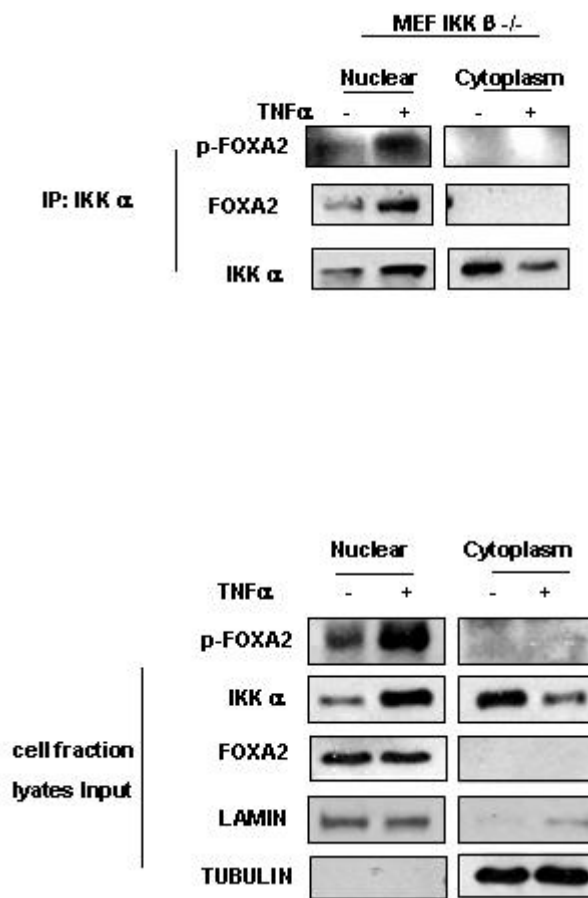


CHAPTER 10 IKK α phosphorylates FOXA2 in nucleus

Previous reports showed that response to TNF α , IKK α would translates from cytoplasm to nucleus and regulate its downstream target transcriptionally or translationally. To identify the subcellular location of FOXA2, IKK α before and after TNF α treatment, we perform cell fraction to identify the expression level of both proteins in different cell parts. We treated IKK $\beta^{-/-}$ MEFs with TNF α and isolated the nuclear and cytoplasmic fractions from the cells for immunoprecipitation by the IKK α antibody. We found that stimulation of IKK $\beta^{-/-}$ MEFs with TNF α increased nuclear accumulation of IKK α , the amount of FOXA2 bound to IKK α , and phosphorylation of FOXA2 (S107/111). However, we did not detect either pFOXA2 or FOXA2 expression in the cytoplasm after TNF α treatment, suggesting that TNF α increased the nuclear accumulation of IKK α which phosphorylates FOXA2 in nucleus. (Figure 13)

Fig 13 IKK α phosphorylates FOXA2 in nucleus

IKK $\beta^{-/-}$ MEFs were treated with TNF α for 45 min. Cell lysates were subjected to cell fractionation. After immunoprecipitation by the IKK α antibody, the nuclear and cytoplasmic fractions were subjected to western blot analysis with indicated antibodies. Lamin and tubulin were used as cell fractionation controls.



CHAPTER 11 FOXA2 Phosphorylation by IKK α Suppresses FOXA2 transactivation Activity

Since upon TNF α stimulation, IKK α phosphorylates FOXA2 and leads to NUMB suppression, it is possible that FOXA2 lost its function as a transcriptional factor after being phosphorylated by IKK α , therefore leads to the suppression of NUMB and consequently NOTCH1 pathway enhancement. To test this hypothesis, we constructed a plasmid containing FOXA2-responsive elements (FRE) to drive the luciferase reporter, which could mimic FOXA2 downstream gene promoter activities and monitor the FOXA2 transactivation activity in general. After we introduced it to 293T cells, we found that WT IKK α could suppress FOXA2 transactivation activity, but the activity would not be affected by KD IKK α (Figure 14A). The similar results represents in luciferase reporter assay used a plasmid containing a 1kb key promoter of NUMB which drives luciferase reporter gene to detect NUMB promoter activities with or without IKK α effects (Figure 14B).

To test whether IKK α suppression activities on FOXA2 comes from the two specific phosphorylation sites on FOXA2 (S107/111), we double mutated the two critical Serine amino acids to either Alanine or Glutamine, which mimic the nonphosphorylated and phosphorylated state of FOXA2. The NUMB promoter driven luciferase reporter assay showed that FOXA2-SSEE could not activate NUMB promoter activities comparing to WT FOXA2, but the present of FOXA2-SSAA has the similar transactivation activities to WT FOXA2. (Figure 14C) In addition, Unlike WT FOXA2, FOXA2-SSEE and FOXA2-SSAA maintained their

transactivation activities regardless the status of IKK α , suggesting that phosphorylation of FOXA2 at S107/111 is the major factors to effect FOXA2 transactivation activity (Figures 14C and 14D). These results support that IKK α decreased FOXA2 transactivation activity by phosphorylating FOXA2 at S107/111. To detect whether the reduction transactivation activities of FOXA2 is the reason for the suppression of NUMB expression with TNF α treatment, we examined the NUMB mRNA level by transfecting various FOXA2 constructs expressing WT FOXA2, FOXA2-SSAA, FOXA2-SSEE, or vector control into Hep3B cells. As expected, FOXA2-SSAA, but not FOXA2-SSEE, enhanced NUMB mRNA level (Figure 14E). These results further support the notion that S107/111 phosphorylation inhibits FOXA2 transactivation activity.

Fig 14 FOXA2 Phosphorylation by IKK α Suppresses FOXA2 transactivation activity

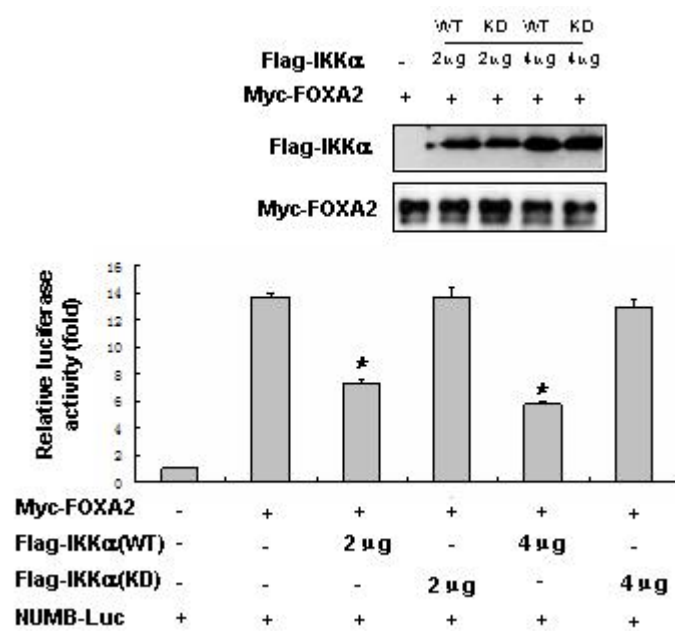
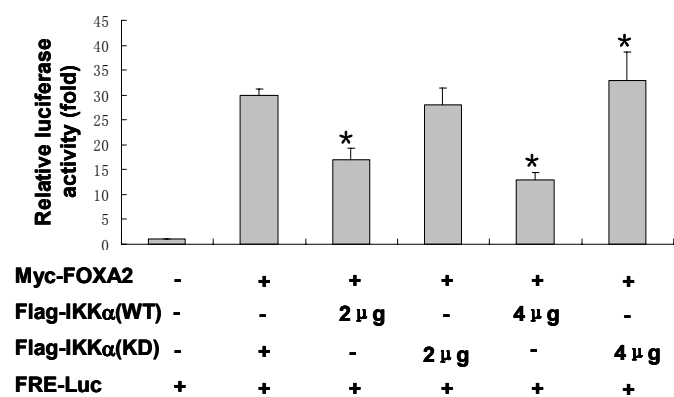
(A) Six repeated consensus binding sites of FOXA2 were constructed into Luc-driven reporter (FRE-Luc). NUMB promoter luciferase reporter and FOXA2 in the presence of different doses of WT IKK α or KD IKK α were transfected into 293T cells for 48 h. Cell lysates were subjected to luciferase reporter assays after transfection. The luciferase activities were normalized to mock cells with NUMB promoter luciferase reporter. Error bars represent SD ($n = 3$). * indicates statistical significance ($P < 0.05$).

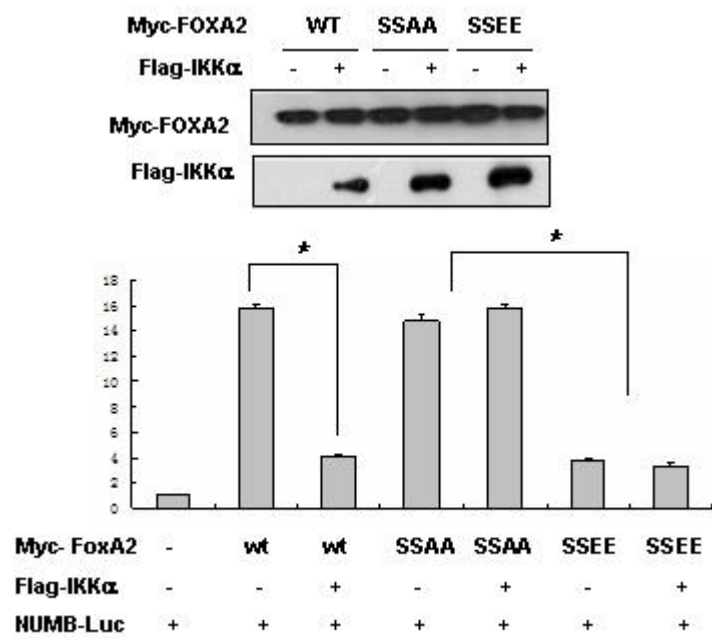
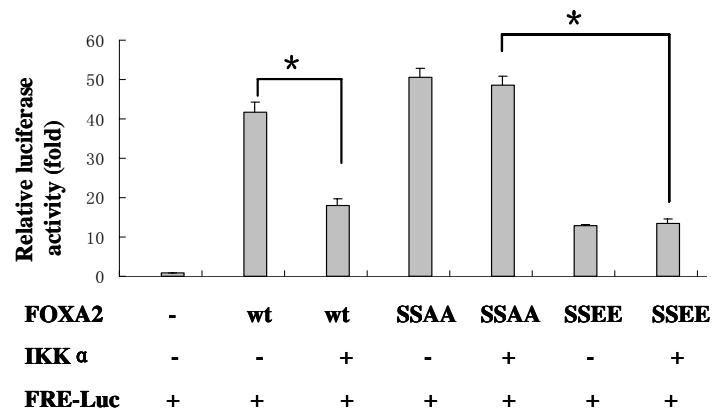
(B) NUMB promoter luciferase reporter and FOXA2 in the presence of different doses of WT IKK α or KD IKK α were transfected into 293T cells for 48 h. Cell lysates were subjected to luciferase reporter assays after transfection. Expression levels of WT IKK α , KD IKK α , and FOXA2 were analyzed by western blotting. Error bars represent SD ($n = 3$). * indicates statistical significance ($P < 0.05$).

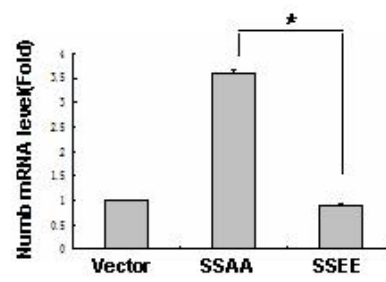
(C) Co-transfection of NUMB-promoter Luc and WT FOXA2, FOXA2-SSAA, or FOXA2-SSEE plus the indicated Flag-tagged IKK α in 293T cells for 48 h. Lysates of 293T cells were subjected to luciferase assays. Error bars represent SD ($n = 3$). * indicates statistical significance ($P < 0.05$). Expression levels of IKK α , WT FOXA2, and FOXA2 mutants were detected by western blot analysis.

(D) Co-transfection of FRE-Luc and WT FOXA2, FOXA2-SSAA, or FOXA2-SSEE plus the indicated Flag-tagged IKK α in 293T cells for 48 h. Lysates of 293T were subjected to luciferase assays. Error bars represent SD ($n = 5$). * indicates statistical significance ($P < 0.05$).

(E) Hep3B cells were infected with retrovirus expressing FOXA2-SSAA or FOXA2-SSEE. RNA extracts from the cells were isolated and subjected to real-time RT-PCR for NUMB mRNA expression. Error bars represent SD ($n = 3$). * indicates statistical significance ($P < 0.05$).







CHAPTER 12 FOXA2 Phosphorylation by IKK α decreases FOXA2 DNA

binding activities

Since we know that FOXA2 transactivation activities decrease after being phosphorylated by IKK α , the further question naturally comes to the underneath molecular mechanism involving in the inhibition activities. The DNA binding ability is a crucial factor to influent transactivation activities of transcriptional factors, we first examined the difference in the binding ability of FOXA2 to the NUMB promoter before and after TNF α treatment. We performed a chromatin immunoprecipitation (ChIP) assay using primers spanning the putative FOXA2 binding sites (see primer sequences in the Experimental Procedures section) in the NUMB promoter from Hep3B cell lysates with or without TNF α treatment. We found that binding of FOXA2 to the NUMB promoter was decreased by TNF α (Figure 15A, to left of arrow). In addition, we compared the binding ability of WT FOXA2, FOXA2-SSAA, and FOXA2-SSEE to the NUMB promoter by ChIP and found that WT FOXA2 and FOXA2-SSAA, but not FOXA2-SSEE, bound to the NUMB promoter (Figure 15B), suggesting that FOXA2 phosphorylation by IKK α suppressed FOXA2 transactivation activity on NUMB by decreasing FOXA2 accessibility to its promoter.

We further tested the function of FOXA2-SSAA and FOXA2-SSEE in FOXA2-knockdown Hep3B cells. The western immunoblotting assay showed that both WT FOXA2 and FOXA2-SSAA stimulated NUMB protein level (Figure 15C). WT FOXA2 expression could suppressed NUMB enhancement with TNF α

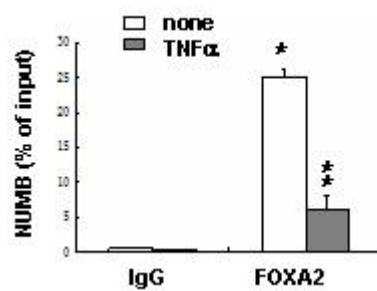
stimulation, (Figure 15C, compare lanes 2 and 6) but FOXA2-SSAA has no effect on FOXA2 –induced NUMB enhancement (compare lanes 3 and 7). Moreover, FOXA2-SSEE has no response to TNF α treatment (lane 8), suggesting that phosphorylation of FOXA2 on S107/111 is the major reason for suppression the FOXA2 associated NUMB expression. The results of monitoring NOTCH1 activities by RBP-Jk luciferase reporter assay showed the FOXA2-associated NOTCH1 suppression could be released by mimic phosphorylated FOXA2-SSEE (Figure 15D). Collectively, our results indicate that S107/111 phosphorylation of FOXA2 by IKK α limit the FOXA2 DNA binding ability, leads to less FOXA2 transactivation activities on NUMB promoter, therefore suppress NUMB promoter activities, NUMB mRNA level and protein expression, and triggers NOTCH1 signalling as a results.

Fig 15 FOXA2 Phosphorylation by IKK α decreases FOXA2 DNA binding activities

(A) Hep3B cells were treated with TNF α for 24 h, and cell lysates were harvested and subjected to primary ChIP assay using the FOXA2 antibody. The purified DNA from the ChIP assay was subjected to real-time PCR using NUMB primers (to left of arrow). Error bars represent SD ($n = 3$). * indicates statistical significance ($P < 0.05$).

(B) Endogenous FOXA2 in Hep3B cells was knocked down by shRNA against the 5'-UTR. After shRNA knockdown of FOXA2, we transfected WT FOXA2, FOXA2-SSAA, or FOXA2-SSEE into FOXA2^{-/-} Hep3B cells, followed by TNF α treatment for 24 h. Cell lysates were analyzed by western blotting using indicated antibodies.

(C) RBP-Jk Luc and WT FOXA2, FOXA2-SSAA, or FOXA2-SSEE were co-transfected into Hep3B cells for 24 h, followed by TNF α treatment for 24 h. Cell lysates were analyzed by luciferase reporter assay. Error bars represent SD ($n = 3$). * indicates statistical significance ($P < 0.05$).



CHAPTER 13 FOXA2 Phosphorylation by IKK α De-Represses FOXA2-Mediated Repression of Cell Growth In Vitro

Previous reports showed that NUMB and NOTCH1 pathway involves in cell proliferation in many type of cancers, we next to test if IKK α -induced FOXA2 phosphorylation associates with cell proliferation and growth in HCC cells. We examined the function of non-phosphorylation FOXA2, phosphorylation FOXA2 and WT FOXA2 in cell growth by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. We found that both FOXA2-SSAA and WT FOXA2 would inhibit cell growth compared to control or FOXA2-SSEE cells (Figure 16A). In addition, WT IKK α , but not KD IKK α , showed the ability to recovered Hep3B cells growth suppression from WT FOXA2 expression, presumably as a result of phosphorylation of WT FOXA2 by IKK α , which leads to loss function of FOXA2 in terms of inhibition of cell growth. (Figure 16D). Knocking down NUMB reversed the cell growth inhibition by WT FOXA2 and FOXA2-SSAA, supporting our proposed suppressive role of NUMB in the TNF α /IKK α pathway (Figure 16B). To verify that NOTCH1 activation is the main reason leading to cell growth in this TNF α /IKK α triggered pathway, we knocked down NOTCH1 in Hep3B cells to monitor cell growth in MTT assay. The results showed that Hep3B cell growth was inhibited after NOTCH1 knockingdown regardless of FOXA2 phosphorylation status, demonstrating that NOTCH1 signaling activation was the major reason for the TNF α /IKK α induced cell growth (Figure 16C). We also use two NOTCH1 pathway inhibitors, LY-411575 and

γ -secretase inhibitor (GSI) to block NOTCH1 pathway in different liver cancer cell lines to detect the influence on cell growth by counting cell numbers. The results showed that after NOTCH1 inhibitors treatment, the cell growth of all liver cancer cell lines decreased, further supporting that NOTCH1 activation plays an important role in liver cancer cell growth (Figure 16E). Consistent with MTT results, FOXA2-SSAA inhibited cell colony formation in an anchorage-independent growth assay (Figure 19F upper). Without NUMB expression would significantly increase colony formation no matter what the status of FOXA2. (Figure 16F bottom). When NOTCH1 was knocked down, colony formation was diminished which is consistent to cell growth (Figure 16G bottom).

To verify the status of DNA synthesis under these conditions, we also performed a bromodeoxyuridine (BrdU) incorporation assay. We found the similar results to MTT assay and anchorage-independent growth assay. (Fig 16 H, I)

Figure 16 FOXA2 Blocks IKK α -Induced Cell Proliferation and Tumor Growth In Vitro

(A, B, and C). Hep3B cells were infected with retrovirus expressing WT FOXA2, FOXA2-SSAA, or FOXA2-SSEE in the presence of (A) control shRNA, (B) NUMB shRNA, or (C) NOTCH1 shRNA. Cells (2.5×10^3) were plated in 96-well plates, and cell growth was determined by MTT assay each day for 4 days.

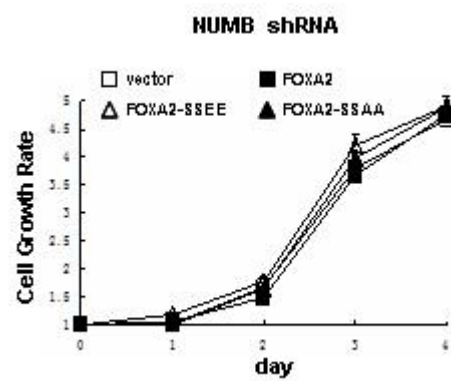
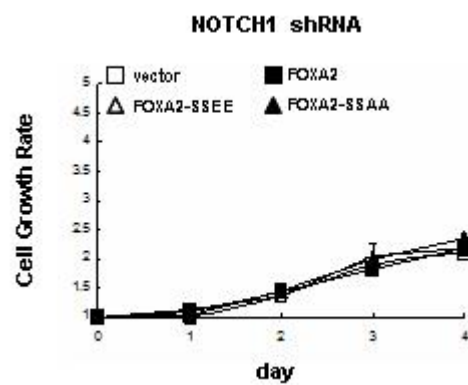
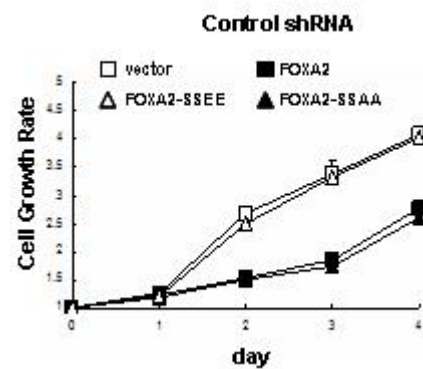
(D) WT IKK α or KD IKK α was co-transfected with GFP-tagged WT FOXA2, FOXA2-SSAA, or FOXA2-SSEE into Hep3B cells. After 48 h, GFP-positive cells were sorted by flow cytometry. Sorted cells were seeded in 96-well plates at 5000/well and subjected to MTT assay. Error bars represent SD ($n = 3$). * indicates statistical significance ($P < 0.05$).

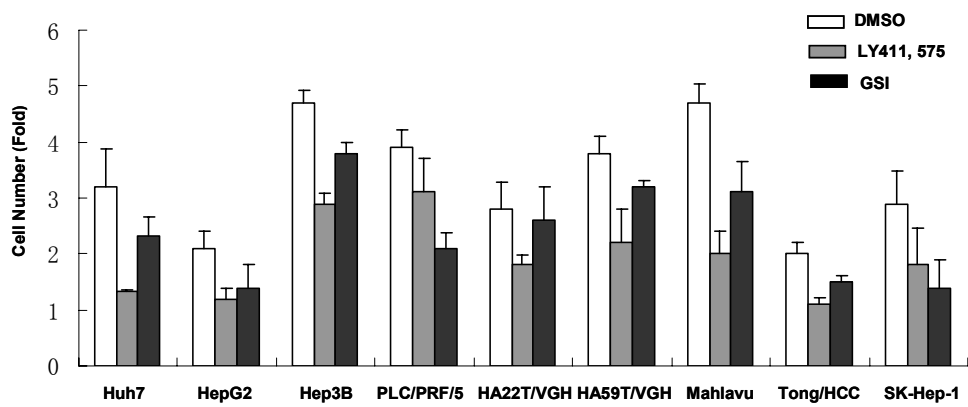
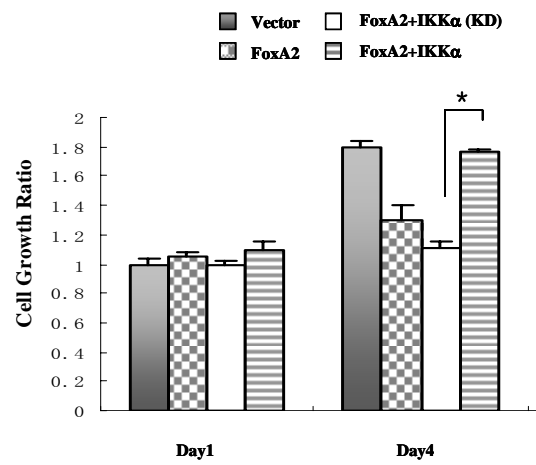
(E) Huh-7, HepG2, Hep3B, PLC/PRF/5, HA22T/VGH, HA59T/VGH, Mahlavu, Tong/HCC, and SK-Hep-1 cells were serum-starved for 24 h and then treated with LY-411575 (250 μ M), GSI (10 μ M), or DMSO for 48 h. After treatment, cells were counted with a Beckman Coulter cell counter. Error bars represent SD ($n = 5$).

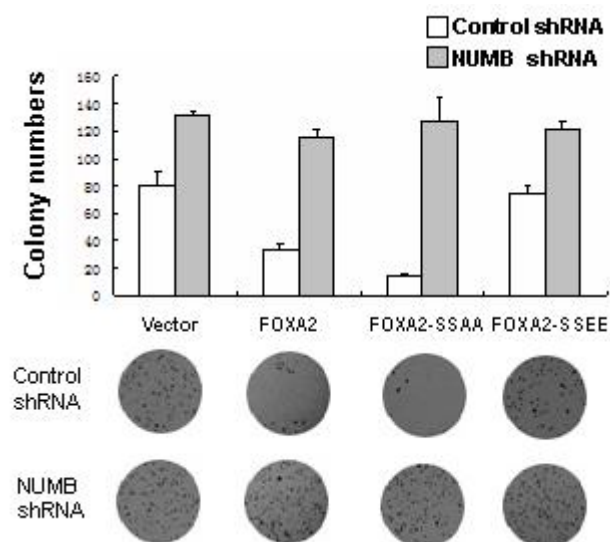
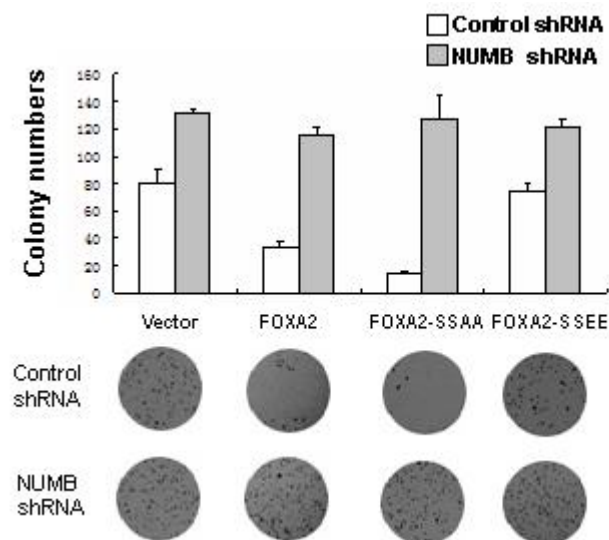
(F and G). Anchorage-independent growth assay of Hep3B cells infected with retrovirus expressing WT FOXA2, FOXA2-SSAA, or FOXA2-SSEE in the presence of (F) NUMB shRNA or (G) NOTCH1 shRNA, compared with control shRNA. Colony numbers (mean \pm SD) in week 3 are shown ($n = 6$). * indicates statistical significance ($P < 0.05$, Student's t test). Representative images from the anchorage-independent growth assay are shown below.

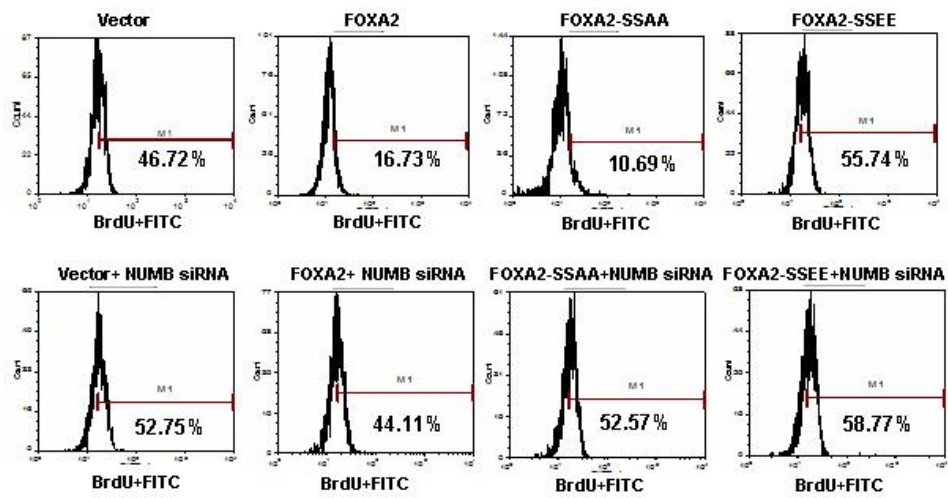
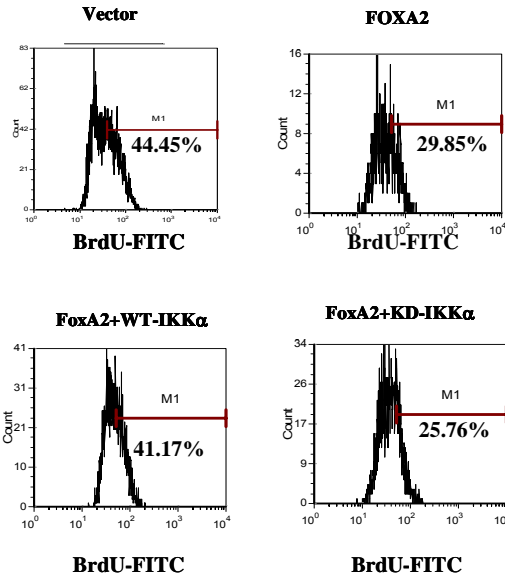
(H) KD IKK α inhibited Hep3B cell proliferation. WT FOXA2, FOXA2-SSAA, or FOXA2-SSEE and WT IKK α or KD IKK α were co-transfected into Hep3B cells. After 48 h, cells were subjected to BrdU assay and analyzed by flow cytometry.

(I) Hep3B cells were infected with retrovirus expressing WT FOXA2, FOXA2-SSAA, or FOXA2-SSEE either without (top) or with (bottom) NUMB shRNA. Infected cells were subjected to BrdU assay and analyzed by flow cytometry.









CHAPTER 14 FOXA2 Phosphorylation by IKK α De-Represses

FOXA2-Mediated Repression of Cell Growth In Vivo

The tumor promotion functions of IKK α -induced FOXA2 phosphorylation were further confirmed in mouse model. We injected Hep3B cells into three groups of nude mice using an orthotopic liver cancer animal model (Xiong, et al., 2010). We monitored the tumor volume (TV) of these mice for 6 weeks and analyzed tumor tissue samples by western blotting. To determine the role of IKK α in liver tumor growth, we injected the first group of mice with Hep3B cells infected with retrovirus expressing shRNA against IKK α . TV was much smaller in the mice harboring tumors (after transplantation) than in the control group, validating the role of IKK α in liver tumor growth (Figure 17A). Western blot analysis of these tumor tissue samples detected TNF α in tumors along with decreased FOXA2 phosphorylation, enhanced NUMB expression, and attenuated NICD expression (Figure 71B). The second group of mice was injected with cells infected with retrovirus expressing WT or mutant FOXA2, and transplantation was performed essentially as described for the first group. We found that WT FOXA2 and FOXA2-SSAA both inhibited tumor growth, whereas FOXA2-SSEE had no effect (Figure 17C, top). In addition, NUMB expression was increased with a decrease in NICD expression in the WT FOXA2 and FOXA2-SSAA tumors (Figure 17C, bottom). Together, these results suggest that FOXA2-SSAA functions in a dominant manner to inhibit tumor growth and support the importance of IKK α in promoting FOXA2-associated HCC development.

Previously, we showed that knockdown of NUMB increased cell growth rate and colony formation regardless of FOXA phosphorylation status. To demonstrate the role of NUMB in TNF α /IKK α -induced tumor growth in vivo, we knocked down NUMB by shRNA in Hep3B cells infected with retrovirus expressing WT FOXA2 and its mutants. Mice that received transplanted tumors harboring these NUMB-knockdown cells continued to show tumor growth even in the presence of FOXA2 and FOXA2-SSAA, which was probably due to restored NICD expression and is consistent with results from the in vitro assays (Figure 17D, bottom).

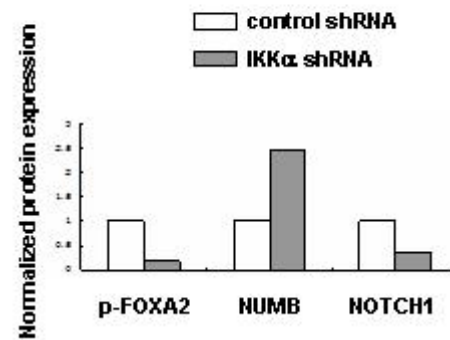
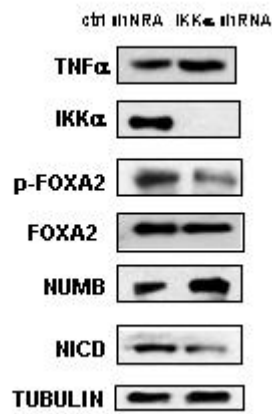
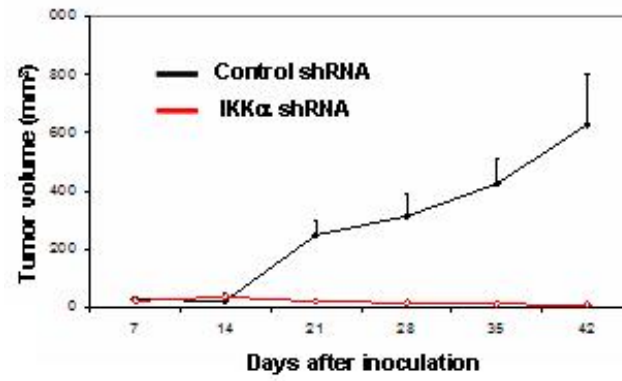
Figure 17. FOXA2 Blocks IKK α -Induced Cell Proliferation and Tumor Growth In Vivo

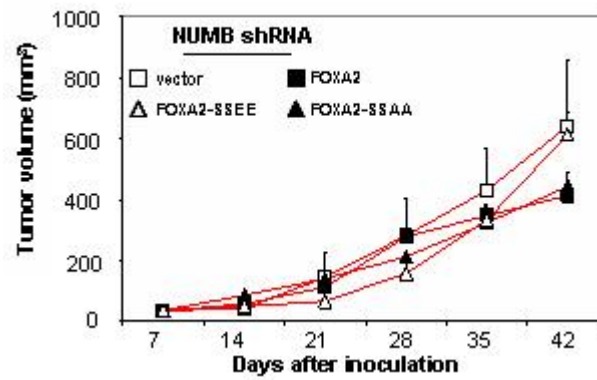
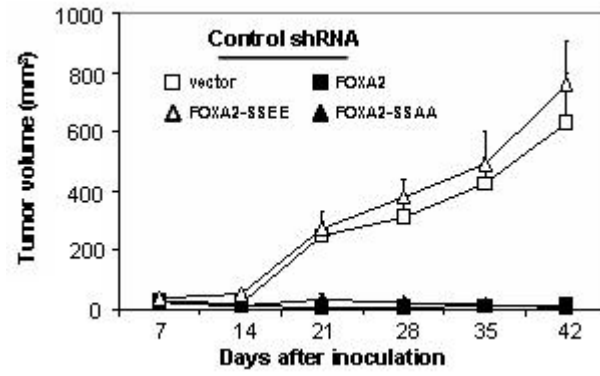
(A) Hep3B cells were infected with retrovirus expressing control or IKK α shRNA. Mice were injected subcutaneously with 1×10^6 cells. Error bars represent SD ($n = 5$). Please see the Experimental Procedures section for more detailed information.

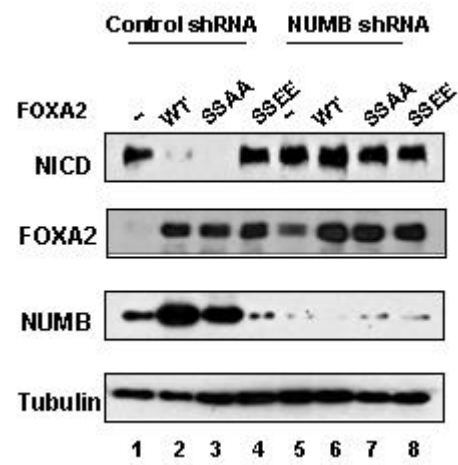
(B) Tumor tissues from mice that received transplants were analyzed 10 days after transplantation and subjected to western blot analysis for indicated antibodies. Right: Expression levels of pFOXA2, NUMB, and NOTCH1 quantified with ImageJ software.

(C) Hep3B cells were infected with retrovirus expressing WT FOXA2, FOXA2-SSAA, or FOXA2-SSEE in the presence of (top) control shRNA or (bottom) NUMB shRNA. Error bars represent SD ($n = 5$). Please see the Experimental Procedures section for more detailed information.

(D) Tumor tissues from (C) were harvested 10 days after transplantation and subjected to western blot analysis for NICD, FOXA2, and NUMB. α -Tubulin was included as control.







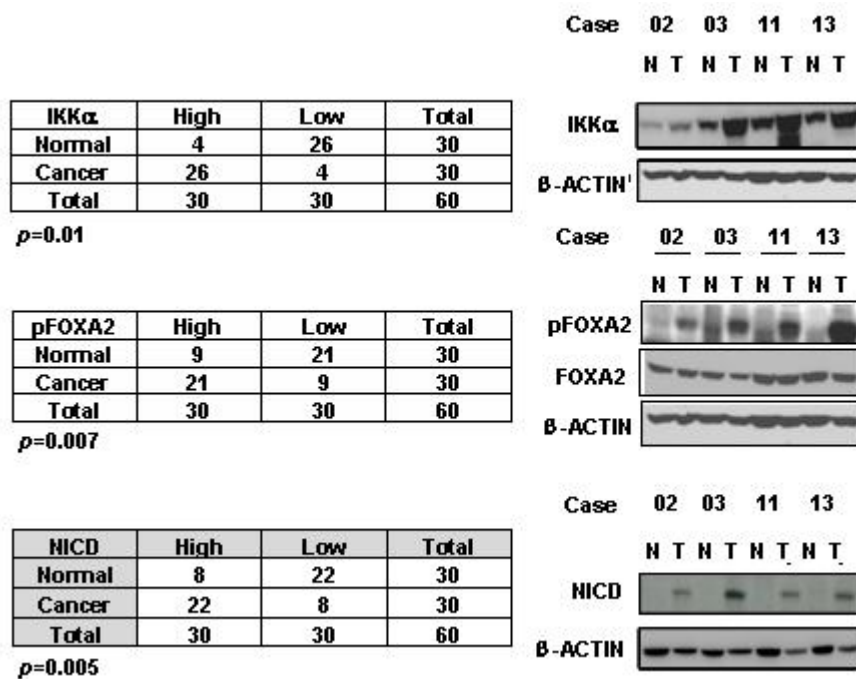
CHAPTER 15 FOXA2 Phosphorylation by IKK α play roles in liver cancer

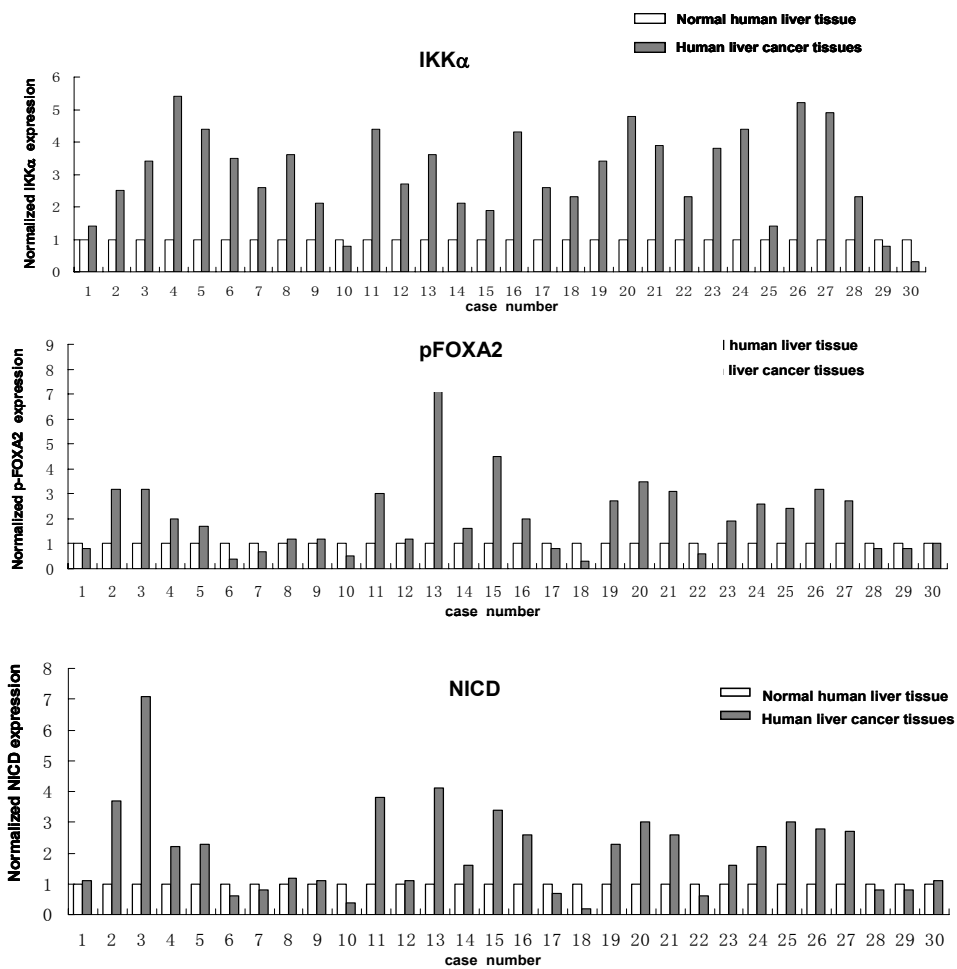
In addition, to further examine the aforementioned conclusion that FOXA2 phosphorylation by TNF α -induced IKK α plays a critical role in liver cancer, we examined the expression of IKK α , pFOXA2 (S107/111), and NICD in 30 freshly prepared human primary HCC tumor specimens and paired normal liver tissues by immunoblotting. We found that IKK α , pFOXA2 (S107/111), and NICD expression levels were significantly higher in tumor specimens than in the corresponding normal liver tissues ($P < 0.05$) (Figures 18A, 18B). In addition, pFOXA2 expression was positively correlated with expression of IKK α and NICD. These results strengthen the notion that IKK α regulates FOXA2 and activates NICD through phosphorylation at S107/111 and the physiological implication of pFOXA2 in IKK α -induced tumorigenesis (18C, 18D). Taken together, analyses of these clinical HCC tumor specimens support that FOXA2 phosphorylation by IKK α might be associated with HCC tumorigenesis.

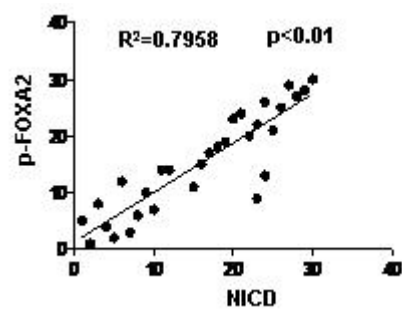
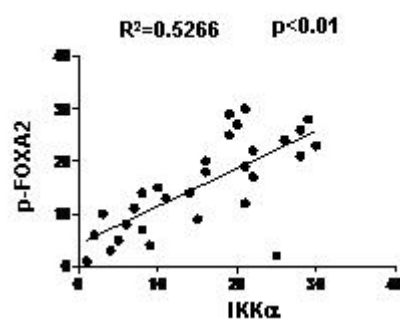
Fig 18 FOXA2 Phosphorylation by IKK α play roles in liver cancer

(A, B) Comparison of pFOXA2 (S107/111), IKK α , and NICD expression in tumors and their adjacent normal tissues. Protein expression was examined by immunoblotting 30 pairs (tumor and normal) of liver tissue samples and quantified with ImageJ software. Right: Four representative pairs (N, normal; T, tumor). Protein expression levels in tumor samples were normalized to those in paired normal tissues. Protein expression levels higher (or lower) than those in normal tissues were defined as “high” (or “low”). ($P < 0.01$, Pearson’s chi-square test.)

(C, D) Correlation analysis of pFOXA2 and IKK α /NICD expression levels in tumors ($R^2 > 0.5$, Spearman’s rank correlation test).





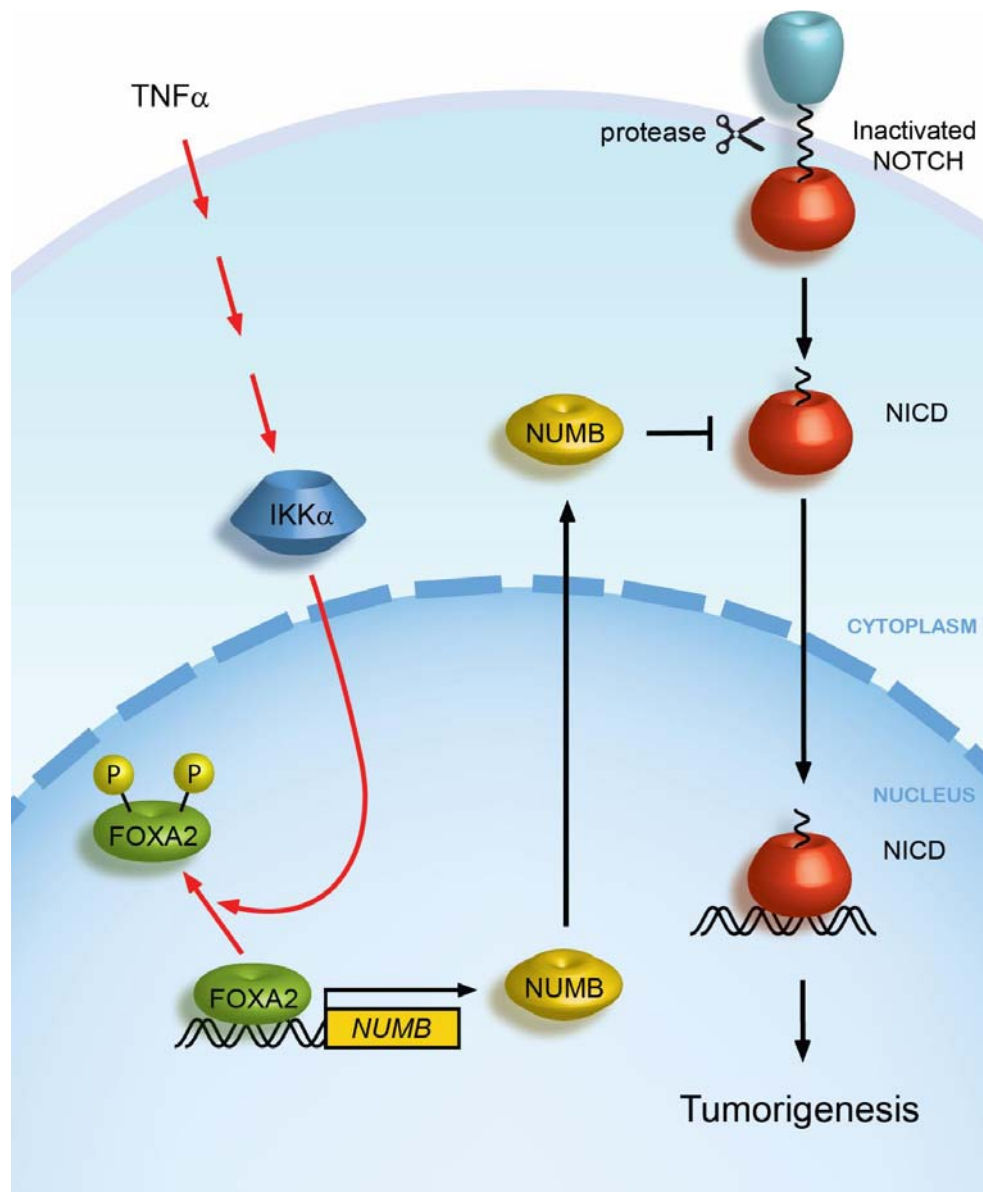


CHAPTER 16 Summary & Discussion

Summary

Here, we identified an axis in TNF α pathway involving activation of NOTCH1 signaling through suppression of FOXA2 transactivation activity by IKK α , leading to disruption of NUMB expression, which promotes NOTCH1-induced liver cell proliferation and growth. In our proposed model, TNF α -activated IKK α interacts with and phosphorylates FOXA2. We identified two IKK α -mediated FOXA2 phosphorylation sites, Ser107 and Ser111. Phosphorylation at these two sites decreased FOXA2 transactivation activities, reduced the expression of a FOXA2 downstream target gene, NUMB, and subsequently, increased tumor growth. Analysis of HCC patient tumor tissue samples suggests that phosphorylation of FOXA2 on Ser 107/111 residues could be a new marker for detecting inflammation-induced liver cancer.

Fig 19 Model of TNF α induced FOXA2/NOTCH1 pathway



Discussion

Inflammation, TNF α and Liver Cancer:

It is not surprise to see inflammation has a tight relationship to human cancers. In our daily life, food factors, stress, environmental pollution, bacteria and viruses could increase expression of chemokines and cytokines, including TNF α . The enhancement of chemokines and cytokines will further induce acute inflammation and chronic inflammation. Almost all acute inflammations are responded quickly by human innate immunity system. Chronic inflammation will turns to increase the potential possibilities of cancer in certain organs.

As one of the most important cytokines, TNF α has a large amount of activities in cell to cell communications and inflammation-associated human cancers. Dysregulation of TNF α signaling pathway contributes to the development of human cancers due to enhanced IKK complex activity and regulate NF- κ B downstream oncogenic target genes including cytokines, chemokines, enzymes, survival genes and some of angiogenesis factors. There are several mouse model were set up which could provide more evidence for the role of TNF α in cancers, especially in liver cancer.

The most direct evidence to show the tumor-promoting role of TNF α was similarly investigated in models of hepatic carcinogenesis. TNFRI $^{-/-}$ mice displayed reduced oval cell (hepatic stem cell) proliferation during the pre-neoplastic phase of liver carcinogenesis, correlating with fewer tumours than wild-type mice

In addition, one of the most famous liver cancer mouse models is

DEN-induced hepatocarcinogenesis model Dr. Michael Karin, who has previously set up the DEN system, indicated in some of his papers that the DEN-induced hepatocarcinogenesis model is “TNF-independent”. Data from these papers showed that upon DEN induction, the tumor multiplicity and maximum size of the lack of TNFR1 (TNF α receptor) mouse has no significant difference to those of wild type mouse, indicating TNF α signaling is not required for DEN-induced hepatocarcinogenesis.

Another model related to TNF α /IKK roles in liver cancer is the Mdr2-KO mouse model, It is an inflammation-associated model of HCC, which is directly linked to NF- κ B. The Mdr2-KO mouse system was engineered Dr. Yinon Ben-Neriah, who showed that NF- κ B functions as a tumor promoter in inflammation-associated liver cancer by anti-TNF α treatment or ectopic expression of an I κ B-super-repressor transgene (Pikarsky, 2004). To the best of our knowledge, an independent role of IKK complex unit in Mdr2-KO mouse has not been reported yet. It would be of interest to test our findings in this Mdr2-KO mouse system.

NOTCH1 and Cancers

We demonstrate that TNF α also stimulates the NOTCH1 pathway in liver cancer cell lines. Previous studies have reported that NOTCH1 has two different roles, one that promotes and the other that suppresses tumorigenesis. Which of the two roles is going to be dominant is dependent on the cellular context and the crosstalk with other signal-transduction pathways.

Although NOTCH1 has been shown to play a role as an oncogene in mainly in leukemia and breast cancer, one report demonstrated inhibition of HCC tumor growth through NOTCH1 signaling by induction of cell cycle arrest and apoptosis¹¹⁴. Several groups that analyzed clinical samples of human HCC also indicate a positive role of NOTCH1 in liver cancer¹¹⁵⁻¹¹⁷. More recently, Ning et al. specifically showed downregulation of NOTCH1 signaling inhibited tumor growth in human HCC in both cell lines and mouse model¹¹⁸. These studies are in line with our findings that NOTCH1 is activated by TNF α through deregulation of FOXA2 transactivity to promote cell proliferation and growth.

NOTCH1 signaling plays an oncogenic role in a majority of solid tumors, and thus, NOTCH1 inhibition may represent a viable treatment for cancer. So far, the best-developed tool for NOTCH1 signaling inhibition is a small molecule inhibitor of γ -secretase. In clinical trial, γ -secretase inhibitor for cancer therapy faced challenges for its high cytotoxicity and non-specific targeting⁴⁸.

FOXA2 proteins and Cancers

Some FOX subfamilies such as FOXO, FOXM, FOXP, FOXC, and FOXA have been linked to tumorigenesis and the progression of certain cancers. For instance, the loss of FOXA2 activity may increase EMT in both lung cancer and pancreatic cancer¹¹⁹⁻¹²⁰. IN addition, FOXA2 proteins are found to be a key regulator in lung inflammation³⁸. In our model, we showed that a new FOX family member plays important role in inflammation-induced liver cancer. FOXA2 is a

transcriptional factor that binds to the NUMB promoter in both genome-wide screen and software analysis. FOXA2 recently We showed that phosphorylation of FOXA2 by IKK α resulted in the loss of FOXA2 transactivation activity and decreased its binding capacity to the NUMB promoters, and therefore, activates NOTCH1 pathway. The crystal structure of FOXA2 DNA binding domain was published in 1993; however, the crystal structure of full-length of FOXA2 is not yet available. The phosphorylation sites that we identified (S109/111) are located in domain between TAD and WHD. The results from the CHIP assay suggested that phosphorylation on Ser 109/111 decreased the ability of FOXA2 to associate with the NUMB promoter. In addition, FOXA2 is known to associate with some promoter-associated factors such as TBP (TATA Box binding protein), HNF6, and the other two FOXA family members, FOXA1 and FOXA3. At this moment, it is not clear and would require further investigation to determine how phosphorylation of Ser 109/111 affects DNA-binding activity and/or its interaction with other transcription factors.

In summary, the identification of FOXA2 as a downstream substrate of IKK α links the TNF α and NOTCH1 signaling pathways and provides an important new starting point for uncovering the molecular basis of TNF α -mediated human tumor growth and identifying potential targets for cancer therapy. Inhibition of FOXA2 phosphorylation or activation of NUMB could have important clinical implications for the treatment or prevention of cancer.

FUTURE DIRECTION

We previously found that phosphorylation of FOXA2 by IKK α induces tumorigenesis through activating NOTCH pathway in a cell culture system and an orthotopic mouse model, but the *in vivo* role of these phosphorylations in tumorigenesis is still nebulous. In recent years, the use of knock-in technology to clarify the role of phosphorylation of specific kinase targets *in vivo* has emerged as a valuable tool for assessing the organism-wide importance of these signaling events⁴⁹⁻⁵¹. Thus, we will apply this strategy to engineer two phosphorylation mutants in the *FoxA2* genetic locus (*FoxA2*^{2A/2A} and *FoxA2*^{2E/2E} knock-in alleles) to clarify the importance of IKK α /FoxA2 signaling pathway in tumor progression.

Aim 1: Generate and characterize *FoxA2*^{2A/2A} and *FoxA2*^{2E/2E} knock-in mice.

Rationale: We previously found that phosphorylation of FOXA2 by IKK α induces tumorigenesis through activating NOTCH pathway in a cell culture system and an orthotopic mouse model^[43], but the *in vivo* role of these phosphorylations in metabolism and tumorigenesis is still nebulous. In recent years, the use of knock-in technology to clarify the role of phosphorylation of specific kinase targets *in vivo* has emerged as a valuable tool for assessing the organism-wide importance of these signaling events^[21-23]. In this aim, we will apply this strategy to *FoxA2* and will engineer two mutants from the *FoxA2* genomic locus (*FoxA2*^{2A/2A} and *FoxA2*^{2E/2E} knock-in alleles) to clarify the importance of IKK α /FoxA2 signaling pathway in tumor progression.

Experimental design

a. Mouse embryonic stem cell targeting. The knock-in targeting construct contains *FoxA2* exons 1-3 and the neomycin selection cassette. The approach is outlined in Figure 2 and described briefly here. The targeting construct will be amenable to site-directed mutagenesis of the IKK α phosphorylation sites for Ser107 and Ser111, which will be mutated to encode alanin (A) or glutamic acid (E), and for the insertion of a 2-kb floxed neomycin-resistance (Neo) cassette which allows selection in both bacteria(on kanamycin) and in embryonic stem(ES) cells (on neomycin/G418), into a unique XhoI site. The targeting construct will be provided to The University of Texas M. D. Anderson Cancer Center Genetically Engineered Mouse Facility (a core institutional resource) for ES cell targeting and neomycin selection. We will screen Neo-positive clones for proper integration of the Neo cassette and the two point mutations by Southern blot analysis with 5' and 3' probes directed to the *FoxA2* transgene in transgenic progenies. To remove the floxed Neo cassette, *FoxA2*^{+2A} or *FoxA2*^{+2D} ES cells will be transfected with pMC-CrePuro, which encodes for *Streptomyces alboniger* puromycin-N-acetyl-transferase and Cre recombinase, and selected in medium containing puromycin. Selected clones will be picked up and subjected to polymerase chain reaction (PCR) analysis using primers P1 (5'-CAAACAAAACCAACTAACC-3') and P2 (5'-GTAATTCTTCCTGTCCCTG-3'). These primers result in a 324-bp product from the wild-type allele and a 413-bp product from the targeted allele after the neomycin cassette has been excised (Figure 20). After several independent strains of

transgenic mice expressing either FoxA2-2A or FoxA2-2D have been generated, we will choose two independent lines for our further studies.

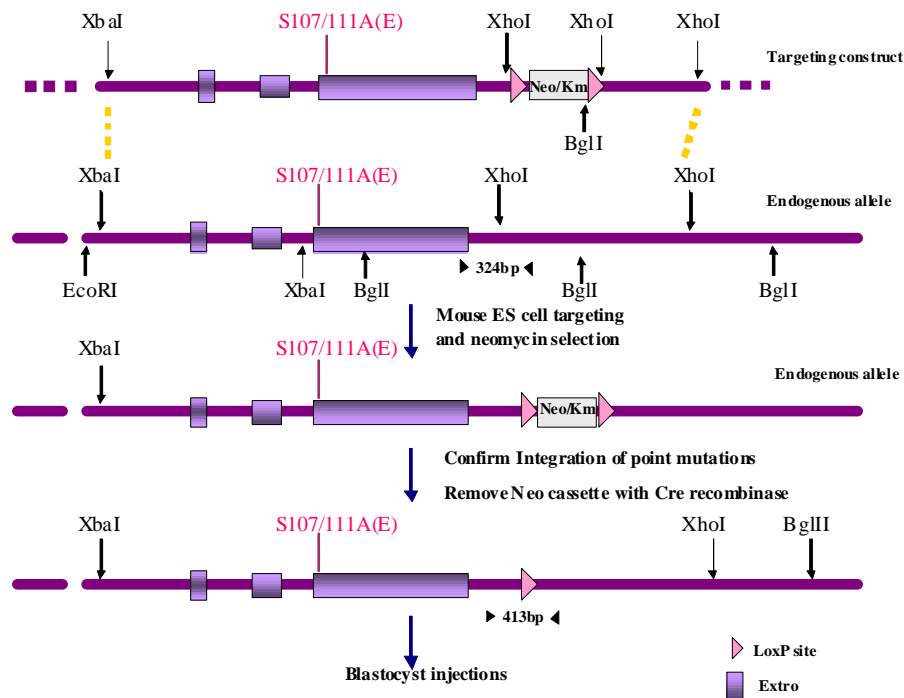


Fig 20 A diagram of knock-in strategy of the *FoxA2*^{2A/2A} and *FoxA2*^{2E/2E} allele

b. Generation of chimeric and *FoxA2*^{2A/2A} and *FoxA2*^{2E/2E} mice. Heterozygous knock-in *FoxA2*^{+2A} and *FoxA2*^{+2D} ES cell clones will be microinjected into C57BL/6J blastocysts, which will be then re-implanted into recipient female mice with the assistance of the M. D. Anderson Cancer Center Genetically Engineered Mouse Facility. Chimeric mice with a high degree of ES-cell contribution will be identified by coat color and then crossed to 129/SvJae mice. This allows germline transmission of the knock-in allele to be identified by the grey coat of the resulting pups. Multiple chimeric offspring will be obtained and bred with wild-type C57BL/6J mice to

produce F1 animals. Genotyping of *FoxA2*^{+/^{2A}} and *FoxA2*^{+/^{2D}} mice will be carried out by PCR of genomic DNA isolated from tails or embryonic membranes. Primers P1 and P2, originally used to screen cells for the excision of the neomycin cassette, will be used to genotype the mice. Homozygous knock-in *FoxA2*^{2A/2A} and *FoxA2*^{2E/2E} mice will be generated by crossing *FoxA2*^{+/^{2A}} mice with *FoxA2*^{+/^{2A}} mice and *FoxA2*^{+/^{2D}} mice with *FoxA2*^{+/^{2D}} mice, and the resulting pups will be genotyped by PCR.

c. Phenotypic analyses of *FoxA2*^{2A/2A} and *FoxA2*^{2E/2E} mice. Once a knock-in line is established, we will first assess any developmental defects in the *FoxA2*^{2A/2A} and *FoxA2*^{2E/2E} mice by assessing the genotypes of offspring from the various crosses. If embryonic lethality is observed for either of these genotypes, we will determine the developmental stage at which the mice die and assess the defects leading to mortality. Viability of *FoxA2*^{2A/2A} and *FoxA2*^{2E/2E} mice will show that this mutant *FoxA2* allele is functional *in vivo*, as *FoxA2*^{-/-} death at E10-11. If no lethality is observed for either *FoxA2*^{2A/2A} or *FoxA2*^{2E/2E} knock-in mice, we will use *FoxA2*^{2A/2A} and *FoxA2*^{2E/2E} knock-in mice for further phenotypic characterization in which cohorts of mice (20 of each genotype, 10 males and 10 females) will be killed at 6 and 12 months of age and sent to the M. D. Anderson Department of Veterinary Medicine and Surgery for full necropsy. Necropsy analysis will include examination and sectioning of brain, intestines, heart, lungs, thymus, kidneys, spleen, skeletal muscle and liver, and organs will be cut into dorsoventral sections for histologic

examination by standard H&E staining. We will also keep a cohort of *FoxA2*^{2A/2A} and *FoxA2*^{2E/2E} littermates (20 of each genotype, 10 males and 10 females) for survival and longevity studies.

Expected outcomes: Our previous *in vitro* study demonstrated that FoxA2 mutants lacking IKK α phosphorylation sites can dominantly impair FoxA2 downstream target gene activation. Because FoxA2 target genes are essential for early development in mammals, it is possible that both *FoxA2*^{2A/2A} and *FoxA2*^{2E/2E} mice will exhibit early developmental defects and embryonic lethality. It remains to be seen if this kind of regulation for FoxA2 is critical during mammalian development. If embryonic lethality is seen for either *FoxA2*^{2A/2A} or *FoxA2*^{2E/2E} mice, we will characterize the cause of death in detail. Otherwise, we expect that heterozygous *FoxA2*^{2E/2E} mice will develop polyps or intestinal lesions due to suppressed FOXA2 activity. A mild inflammation which is associated with tumors may occur.

Possible experimental problems and alternative approaches: FoxA2 tumor related function may affect tumor origination or tumor development or both. If phosphorylation of FoxA2 by IKK α promoting but not initiating the tumor development, we may not see tumor in previous mouse model. If it is the case, we will alter to cross *Apc*^{716/+} mice (a mouse model of liver tumorigenesis) with our knock-in mouse to see if *FoxA2*^{2A/2A} inhibit and *FoxA2*^{2E/2E} activate tumor progression in *Apc*^{716/+} mice.

Aim 2: Investigate FOXA2 downstream target gene inactivation, cell proliferation and tumor development in *FoxA2*^{2A/2A} and *FoxA2*^{2E/2E} knock-in mice.

Rationale: Because the TNF α /IKK α signaling pathway, which regulates FOXA2 downstream target genes inactivation through the inhibition of FOXA2 and culminates in cell growth, has been studied only *in vitro* [43], it remains unclear whether this pathway affects FOXA2 downstream target gene inactivation, cell metabolism, cell proliferation and tumor development *in vivo*. Thus, we propose to determine whether *FoxA2*^{2E/2E} mice (in which FOXA2 is inactivated) show abnormal target gene activation, metabolism disorder, cell proliferation, and tumor development relative to wild-type mice or *FoxA2*^{2A/2A} mice (in which FOXA2 is constitutively activated).

Experimental design: Different organs (including brain, intestine, heart, lungs, thymus, kidneys, spleen, skeletal muscle and liver) of *FoxA2*^{+/+}, *FoxA2*^{2A/2A} and *FoxA2*^{2E/2E} mice will be dissected for assessment of typical FOXA2 downstream target genes and NOTCH pathway marker (like *c-myc*, *hes-1*) by immunostaining. The offspring of 20 litters (a target of at least 90 mice in total) will also be analyzed for tumor development. Briefly, a cohort of *FoxA2*^{2A/2A} and *FoxA2*^{2E/2E} littermates (20 of each genotype, 10 males and 10 females) will be killed at 12 and 18 months of age and sent to the M. D. Anderson Department of Veterinary Medicine and Surgery for full necropsy and tumor examination.

Expected outcomes: We expect to see that organs from *FoxA2*^{2E/2E} mice (with inactivated FoxA2) will show higher expression of NOTCH pathway marker, like Hes-1, Hey-6, c-Myc and less expression in FOXA2 target genes, like Numb, Gcg, than *FoxA2*^{+/+} or *FoxA2*^{2A/2A} mice. Moreover, we may find that *FoxA2*^{2E/2E} mice have a greater potential to develop various types of colorectal cancers compared with the *FoxA2*^{+/+} and *FoxA2*^{2A/2A} mice. If using combination model of *FoxA2*^{2E/2E} × *Apc*^{716/+}, we expect to see the tumor growth in *FoxA2*^{2E/2E} × *Apc*^{716/+} mice will be larger than that in *Apc*^{716/+} parental mice.

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VITA

Mo Liu received the degree of Bachelor of Science with a major in Virology from Wuhan University, China in 2002 and the degree of Master of Science with a major of biomedical sciences from Wuhan University (mentor: Dr. Ying Zhu) in 2006. In August of 2006, she entered Graduate School of Biomedical Sciences, the University of Texas Health Science Center at Houston to pursue her doctoral degree in Dr. Mien-Chie Hung's lab in M. D. Anderson Cancer Center. During her Ph.D. career, she received Research Assistant Fellowship (2006~2007), Andrew Sowell-Huggins Scholarship Award (2012) and Chinese Government Award for Outstanding Self-financed Students Abroad (2012).