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IKKα Mediated NOTCH1 Activation Promotes Tumorigenesis via FOXA2 Suppression

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IKKα Mediated NOTCH1 Activation Promotes Tumorigenesis via FOXA2 Suppression

A

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and
The University of Texas
M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences
In Partial Fulfillment
Of the Requirements
For the Degree of

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by
Mo Liu, M.S.

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IKKα Mediated NOTCH1 Activation Promotes Tumorigenesisvia FOXA2 Suppression

Publication No. _____________

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Abstract

Proinflammatory cytokine TNFα plays critical roles in promoting malignant cell proliferation, angiogenesis, and tumor metastasis in many cancers. However, the mechanism of TNFα-mediated tumor development remains unclear. Here, we show that IKKα, an important downstream kinase of TNFα, 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 IKKa, 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 IKKα and activated NOTCH1 expression in tumor tissues. Therefore, dysregulation of NUMB-mediated suppression of NOTCH1 by TNFα/IKKα-associated FOXA2 inhibition likely contributes to inflammationmediated cancer pathogenesis. Here, we report a TNFα/IKKα/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 world[1]. 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 disease[4-6]. 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]. This extrinsic pathways converge resulting in the activation of transcription factors, like nuclear factor-κB (NF-κB), signal transducer and activator of transcription 3 (STAT3) and hypoxia-inducible factor 1α (HIF1α), in tumour cells. These transcription factors coordinate the production of inflammatory mediators, including cytokines and chemokines. The proinflammtory cytokines and chemokines, such as tumor necrosis factorα (TNFα), 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 development[14-16]. 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 TNFα 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 cells[19]. 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 tumorigenesis [20].

The classical signaling pathway responded to TNFα is NF-κB 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 IKKβ is on its activation loop. Active IKK phosphorylates IκBs on conserved serines, triggering their degradation through β-TRCP ubiquitin-proteasome proteolytic system and release of bound NF-κB dimers, which translocate to the nucleus to drive gene transcription which associates with tumorigenesis [21].

Accumulated evidences show that he low-dose, chronic TNFα production is a feature of many tumor cells[22]. For example, the tumor-promoting role of TNFα 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[23]. In addition, dysregulation of TNFα-IKK-NF-κB pathway is usually found in many human cancers. In Barrett's esophageal adenocarcinoma and hepatoma, TNFα-IKK-NF-κB pathway is upregulated[24,25]; Mutation of CYLD induced constitutive activation of NF-κB occurs in cylindromatosis patients[26-28]; couple of mutations or loss-fuction IκB are dected in couple of cancer types, including colon cacner and breast cancer[29-33]. These pathological findings indicate the clinical relevance of TNFα signaling pathway in human cancer development.

1.3 Independent Role of IKK subunits to NF-κB pathway

IKKα and IKKβ are major downstream serine/threonine kinases which have multiple functions dependent and independent on the NF-κB pathway. Previous study show that NF-κB-independent activities induced by TNFα[34-40], For example, independent IKKβ positively regulate inflammation linked tumorgenesis and angiogenesis in several cancer types[41-43].

It has also been reported that IKKα can be activated by TNFα treatment independently of IKKβ. For example, two Nature papers, published side-by-side, showed that IKKα, in response to TNFα, 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 IKKα is able to phosphorylate CBP and switches CBP binding preference from p53 to NF-κB upon TNFα stimulation [46]. These TNFα-induced IKKα activities are independent of IKKβ. There are several reports that strongly support the idea that IKKα is able to contribute to tumor development independently of NF-κB. For instance, the nuclear function of IKKα has also been implicated in prostate cancer metastasis by inhibiting the expression of the tumor suppressor, Maspin, in mouse model[47]. Moreover, IKKα is able to phosphorylate and regulate ER, AIB1, CYCLIN D1, and β-CATENIN in cell proliferation[48-52]. Briefly, IKKα 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 IKKα has the unique ability in regulating versatile physiological and pathological functions, which are independent of NF-κB.

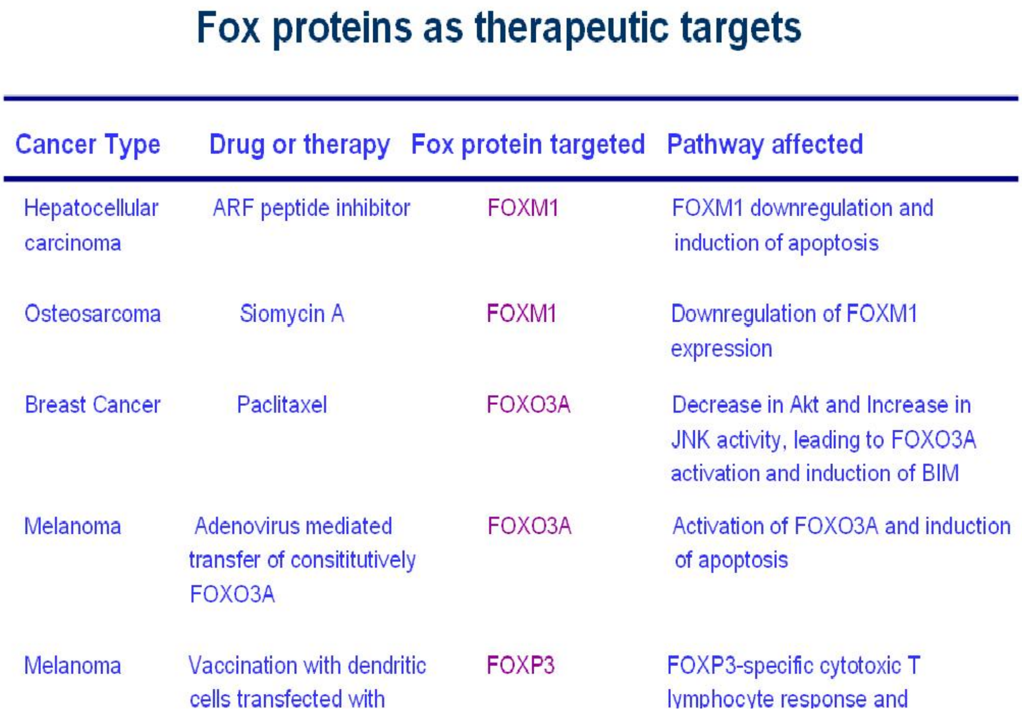
1.4 Forkhead Box Protein (Fox) and Cancer

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 phosphorylation[55]. 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 events[58-62]. 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 metastasis[65]. Except FoxA, several Fox subfamilies, such as FoxO, FoxP and FoxM are verified to link to tumorigenesis and the progression of certain cancers[66-70].

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 IκB kinase (IKK) phosphorylate FoxO3A to induce its nuclear export and degradation. Deregulation of these kinases normally happen in tumors and contribute to tumorgenesis[71-74]. For example, disruption of IKK affects chromatin remodeling and NFκB signaling. Moreover, IKK has been shown to physically bind to and phosphorylate FoxO3A in tumor, and cytoplasmic FoxO3A correlates with the expression of IKKβ in many tumors and associates with poor prognosis in breast cancer[72]. 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 basis[81-85]. 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 targets[88]. Breast carcinoma chemotherapeutic drugs pacliaxel activates JNK, which modulate the stability of FoxO3A by phosphorylation DBD of FoxO3A so that blocks tumorgenetic cascades regulated by FoxO3A[89,90]. FoxA1 as a marker for luminal subtype A breast cancer is also a good therapeutic candidate[91]. (Fig1)



Fox proteins as therapeutic targets

Cancer Type	Drug or therapy	Fox protein targeted	Pathway affected
Hepatocellular carcinoma	ARF peptide inhibitor	FOXM1	FOXM1 downregulation and induction of apoptosis
Osteosarcoma	Siomycin A	FOXM1	Downregulation of FOXM1 expression
Breast Cancer	Paclitaxel	FOXO3A	Decrease in Akt and Increase in JNK activity, leading to FOXO3A activation and induction of BIM
Melanoma	Adenovirus mediated transfer of consititutively FOXO3A	FOXO3A	Activation of FOXO3A 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.FOXM1 are used in HCC and Osteosarcoma; FOXO3A 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 melano*gaster in 1914 is the first time NOTCH gene get noticed [92]. 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 NOTCH4 [93]. 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 family[94-96].

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 tumorigenesis[97]. 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 well[98].

Several processes, including proteolysis, glycosylation, ubiquitylation and phosphorylation[99], control Notch activation. Ligands overexpression, abnormally activated receptors or dysregulation of negative regulators may lead to aberrant activation of the Notch pathway[100] Some of these circumstance are deregulated in cancers, resulting in aberrant Notch signaling[101-103].

NUMB and NUMB-like proteins function as signalling inhibitors for Notch by targeting the membrane-bound Notch for degradation following activation[104]. Loss of NUMB has been detected in over 50% percent of breast carcinoma[105], and possibly results in the stabilization and hyperactivation of Notch. In addition, NUMB binds to p53 and MDM2 to prevent ubiquitylation of p53[106]. 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 actitives in multiple cancer types, Notch has been proved as a tumor suppressor in come cancer types as well. This tumor suppressor activities is worked as a result of crosstalk with other signaling pathways, which decrease cell proliferation, increase apoptosis or cellular differentiation[107]. For example, NOTCH could block WNT signaling to suppress tumorgenesis thereby driving cells toward a more differentiated phenotype[108] .

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 therapies[109].

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, TNFα tumor promoting effects were found in several genetic mouse model of inflammation-related carcinogenesis through activating the key inflammatory transcriptional regulator NF-κB. Contrarily, hepatocyte-specific deletion of IKKβ gene or ablation of IKKγ/NEMO leads to the spontaneous development of HCC[110-113]. These phenomena indicate that other cascades, other than traditional NF-κB, are involved in TNFα induced liver cancer. Based on our previous research that IKKα/IKKβ can independently regulate downstream cascades to lead tumorgenesis and angiogenesis by phosphorylating Fox family, an interesting question is rising, whether IKKα/IKKβ 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 TNFα-mediated tumorgenesis, the previous study showed that controversial roles of NF-κB in TNFα-mediated liver cancer in several genetic mouse models, thus it is still not clear whether IKKα/β has a role independent to NF-κB in the TNFα-mediated tumorgenesis. 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-κB-independent pathways involved in TNFα-mediated NOTCH1 production and tumorigenesis. We hypothesize that TNFα 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 firbroblasts (*Ikkα*$^{-/-}$, *Ikkβ*$^{-/-}$) 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), IKKα (sc-7606, Santa Cruz), IKKβ (sc-8014, Santa Cruz), IκBα (9242S, Cell Signaling Technology), TNFα (Santa Cruz Biotechnology, SC-8301), IκBα (Santa Cruz Biotechnology, SC-371), IKKγ (Santa Cruz Biotechnology, SC-8330), RHEB (Santa Cruz Biotechnology, SC-6341), GAPDH (Santa Cruz Biotechnology, SC-20357), NOTCH1 (NICD) (sc-32745, Santa Cruz Biotechnology), pIκBα(S32/S36) (Cell Signaling Technology, 9246),NUMB (ab14140, Abcam), FOXA2 (ab60721, Abcam), p-IKKα(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.

TNFα was purchased from Sigma, and Bay (43-9006) and SCI34 were purchased from CalBiochem. Full-length recombinant GST-IKKα and His-IKKβ 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 IKKα 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 Na_2HPO_4, 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 TNFα (20ngl/ml), for 30 min or 24h.

2.5 In vitro pull-down assay

Recombinant IKKα proteins and IKKβ 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 μg of leupeptin/ml, 1 μg of aprotinin/ml, and 1 μg of pepstatin/ml) for 1 h at 4°C. Rabbit IgG antibody (rIgG) or anti-IKKβ 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 Na_2HPO_4 [pH 7.4], 150 mM NaCl , 1% Triton X-100) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 mM NaF, 2 mM sodium orthovanadate, 3μg/ml aprotinin, and 750μg/ml benzamidine). For immunoprecipitation, the samples were precleaned with either protein A or protein G agarose for 1 hr at 4 ^oC. Precleaned cell lysates were Proteins were immunoprecipitated by indicated antibodies for overnight at 4 ^oC, incubated with either protein A or protein G agarose for 3 hr at 4 ^oC, 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 TNFα for 30 min. The cells were lysed and immunoprecipitated with either anti-IKKα 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 μCi [γ-^{32}P]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 IKKα *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′-AATTTCGATTTTCA-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-IKKα 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 Shool.. 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 ($1*1*1\ mm^3$), 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 3 length * $width^2$. Animal experiment protocol number is approved by 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. R2 > 0.5 was considered statistically correlated.

CHAPTER 3

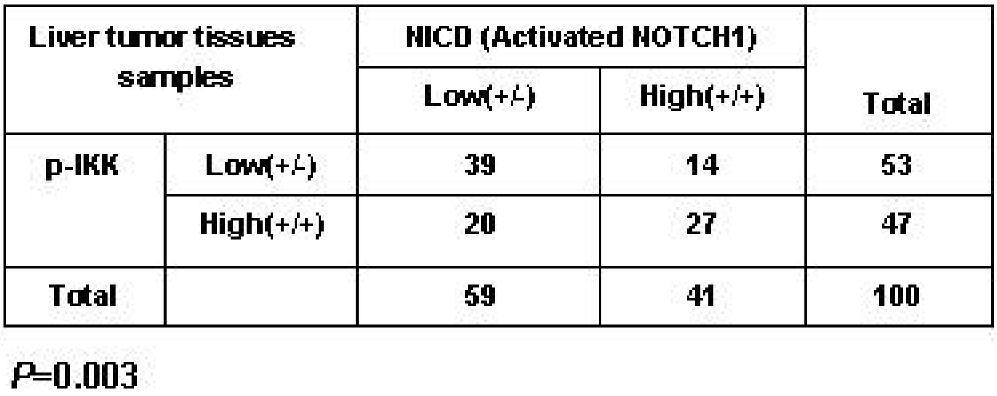
Phosphorylated IKK expression positively correlates with activated NOTCH1 expression and high HCC tumor grade

It is well known that both TNFα triggered signal pathways and NOTCH1 signling 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 TNFα, 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-IKKα/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

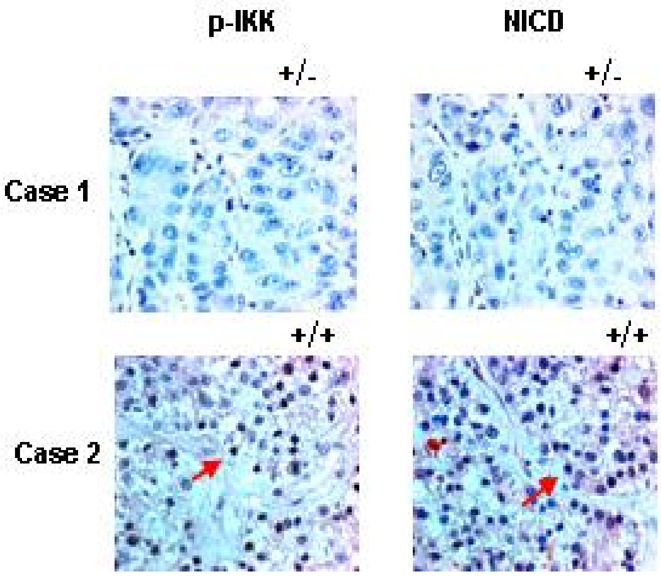
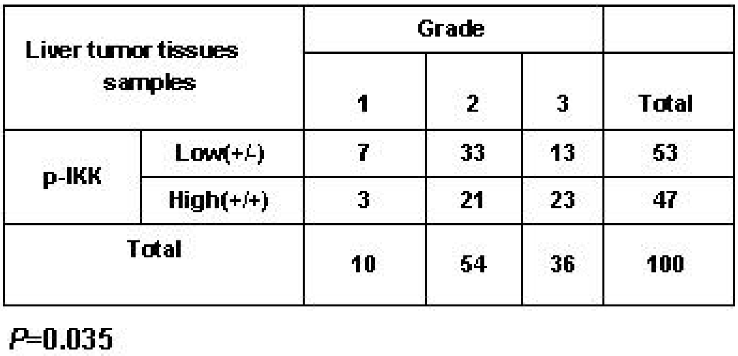


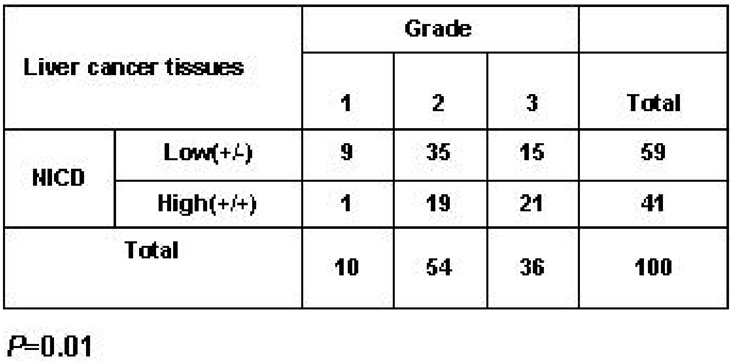
Fig 3 Clinic correlation between p-IKK/NICD and tumor grade

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

P=0.035



Liver cancer tissues		Grade			
		1	2	3	Total
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-IKKα/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 IKKα is required for TNFα-induced NOTCH1 activation

The clinical positive correlations between p-IKK and NICD raise a question in our mind: whether TNFα/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 TNFα (Figure 5). We found that after TNFα 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), IKKα$^{-/-}$, and IKKβ$^{-/-}$ MEFs were treated with TNFα and compared their NICD expression level by westernblotting assay. The NICD level in IKKα$^{-/-}$ MEFs was substantially lower than in WT MEFs (Figure 5, bottom; compare lanes 1 and 2). Re-expression of IKKα in the IKKα$^{-/-}$ MEFs rescued NICD expression (Figure 8, bottom; compare lanes 2 and 3). However, re-expression of IKKβ could not affect the NICD level in IKKβ$^{-/-}$ MEFs (Figure 5, bottom; compare lane 1 with lanes 4 and 5). Together, these results indicate that IKKα, but not IKKβ, is necessary components in TNFα-induced NICD expression, suggesting that TNFα may stimulate NOTCH1 activation through an IKKα-dependent pathway.

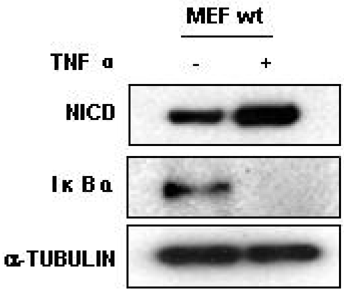
To further confirm that IKKα is required for TNFα-mediated increase of NICD expression, a RBP-Jk reporter luciferase assay, which could monitor the activity of NOTCH1 signal transduction pathways in cultured cells by showing activities of two NOTCH1 downstream enzymes, was used as a tool to detect NOTCH1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

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

Fig 5 IKKα is required for TNFα-induced NOTCH1 activation

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

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


MEF wt
TNF α
-
+
NICD
Iκ Bα
α-TUBULIN

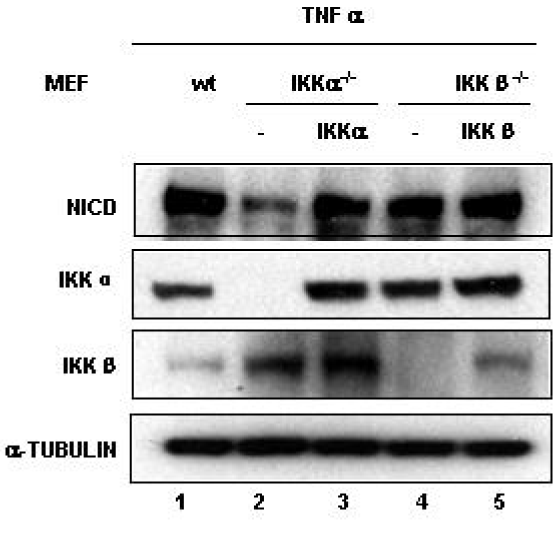
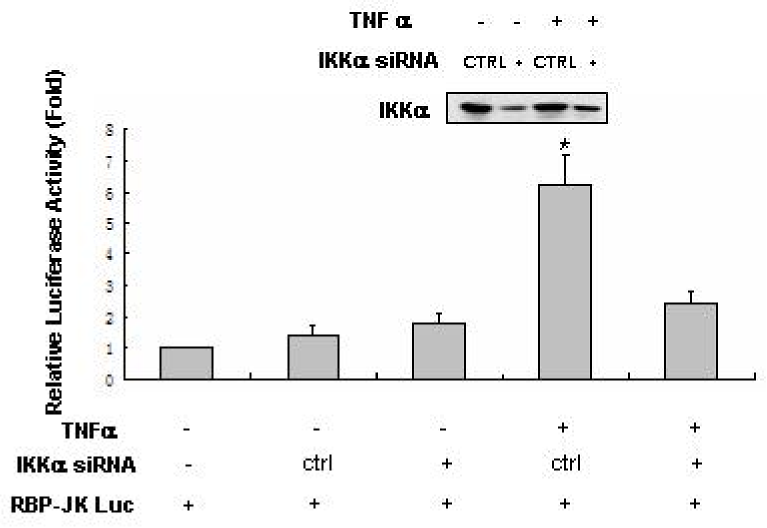

TNF α
MEF
wt
IKKα-/-
IKK β-/-
-
IKKα
-
IKK β
NICD
IKK α
IKK β
α-TUBULIN
1
2
3
4
5

Fig 6 NOTCH1 activity was blocked by IKKα knocking down

RBP-Jk Luc and IKKα siRNA were transfected into Hep3B cells for 48 h. After transfection, WT and IKKα-knockdown Hep3B cells were treated with TNFα 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 TNFα induced NOTCH1 activation

After we know TNFα/IKKα regulates NOTCH1 activation, we are trying to know how what is the regulator in TNFα/IKKα induced NOTCH1 activation. As NUMB is known to atagonist NOTCH1 activation through degradation membrane-bounded NOTCH1 intracellular domain, we first tested whether NUMB expression level changed in the process of TNFα/IKKα-mediated NOTCH1 activation. We found that TNFα 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 IKKα, but not IKKβ, involves in TNFα/IKKα regulates NOTCH1 activation, ectopic expression of IKKα but not IKKβ increased NICD expression and diminished NUMB expression as well (Figure 7B). In addition, blocking IKKα expression by siRNA knocking down recovered TNFα-mediated NUMB downregulation and subsequent impaired TNFα-mediated NOTCH1 activation (Figure 8A, compare lanes 2 and 4). Knockdown of both IKKα and NUMB restored NICD expression (Figure 8A, compare lanes 4 and 6), suggesting that TNFα/IKKα-mediated NOTCH1 activation involves NUMB downregulation. MEFs and IKKα$^{-/-}$ MEFs were used to further analyzed the relationships of IKKα, NUMB, and NICD. Higher NUMB expression and consistently lower NICD expression were found in IKKα$^{-/-}$ MEFs than in WT MEFs (Figure 8B). Rescure IKKα by overexpressing IKKα in IKKα$^{-/-}$ MEFs leads to decreasing of NUMB expression and upregulation of NICD expression accordingly (Figure 8B).

Fig 7 IKKα, but not IKKβ downregulates NUMB expression

(A) Hep3B and Huh-7 cells were treated with TNFα (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 IKKα and IKKβ. Endogenous NICD and NUMB expression was analyzed by western blotting. α-Tubulin was used as loading control.

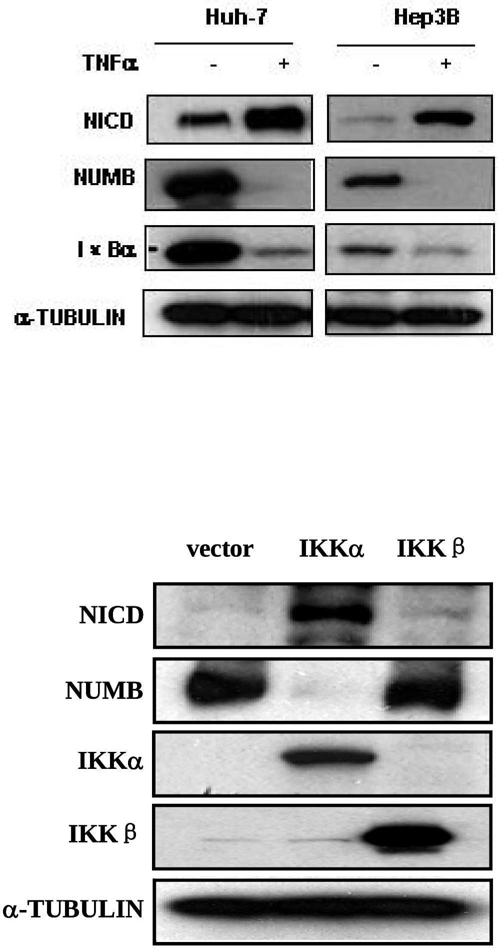
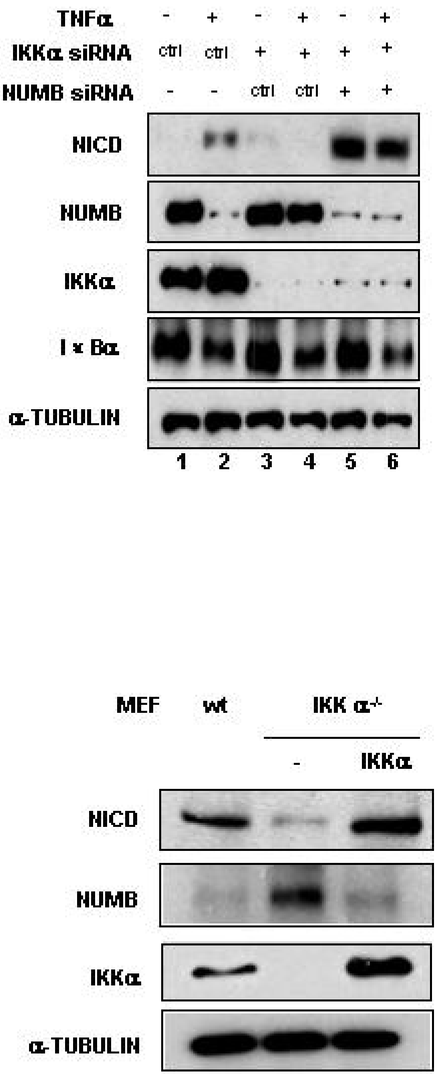

Huh-7
Hep3B
TNFα
-
+
-
+
NICD
NUMB
IκBα
α-TUBULIN
vector
IKKα
IKK β
NICD
NUMB
IKKα
IKK β
α-TUBULIN

Fig 8 Knocking down IKKα impaired TNFα-induced NOTCH1 activation and increased NUMB expression

(A) Knockdown of IKKα or double knockdown of IKKα and NUMB by siRNA in Hep3B cells. Nontargeting siRNA was used as control. After 24 h of TNFα treatment, cell lysates (30 μg) 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 IKKα$^{-/-}$ MEFs with or without reconstituted IKKα. α-Tubulin was used as loading control.


TNFα - + - + - +
IKKα siRNA ctrl ctrl + + + +
NUMB siRNA - - ctrl ctrl + +
NICD
NUMB
IKKα
IκBα
α-TUBULIN
1 2 3 4 5 6
MEF wt IKK α-/-
- IKKα
NICD
NUMB
IKKα
α-TUBULIN

CHAPTER 6 IKKα kinase activity is a key factor for TNFα induced NUMB suppression and consequent NOTCH1 activation

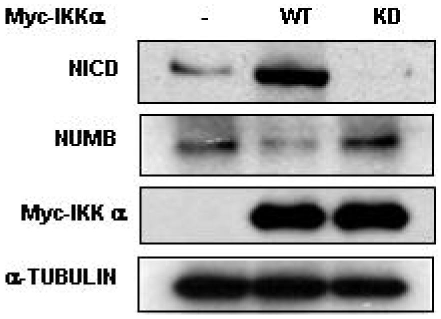
IKKα is a well-known downstream kinase of TNFα signalling, the next question naturally comes out " does IKKα trigger NUMB/NOTCH1 signalling via its kinase activites" Kinase-dead (KD)IKKα mutant, which does not have any kinase activites, were introduced into hepatocyte cells. We found not similar to wide type IKKα, kinase-dead (KD) IKKα mutant could not downregulate NUMB expression (Figure 9A), indicating that TNFα/IKKα induce NUMB downregulation and NOTCH1 activation via IKKα kinase activites. . In addition, RBP-Jk luciferase assay showed that overexpression of NUMB could block TNFα-induced NOTCH1 activation (Figure 9B). To answer does IKKα-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 IKKα or NUMB expression. The mRNA levels of NOTCH1 downstream targets enhanced with IKKα but were decreased in the presence of NUMB (Figure 9C). Together, these results indicate that TNFα triggered IKKα kinase activity could activate NOTCH1 through NUMB suppression.

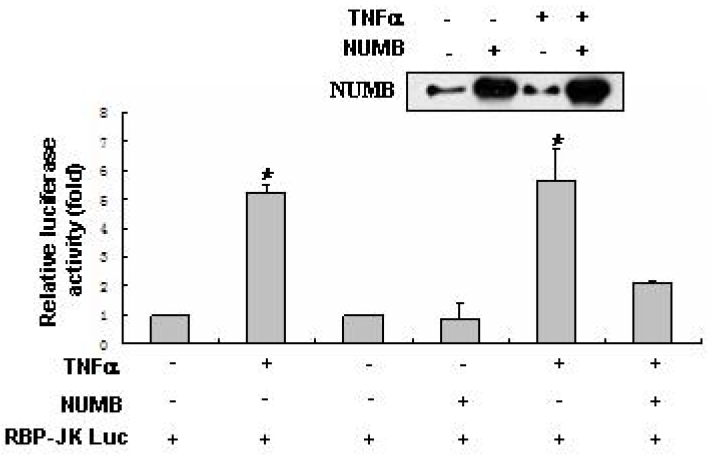
Fig 9 IKKα functionally effects NOTCH1 activities and suppression NUMB

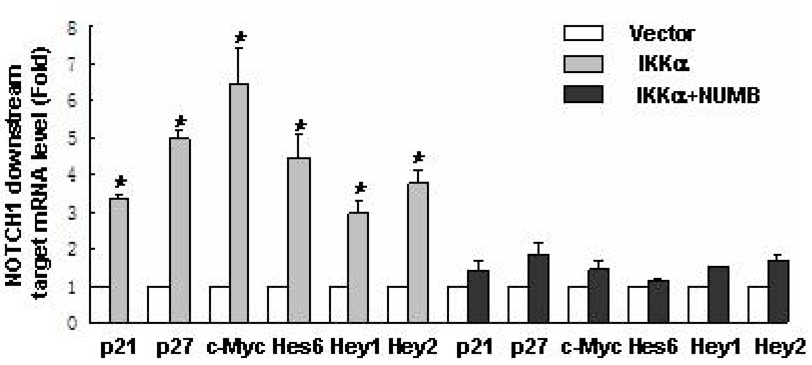
(A) Hep3B cells were transfected with WT IKKα or KD IKKα. Endogenous NICD and NUMB expression was analyzed by western blotting. α-Tubulin was used as loading control.

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

(C) Hep3B cells were infected with retrovirus expressing IKKα or IKKα 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 ($P < 0.05$).


Myc-IKKα
-
WT
KD
NICD
NUMB
Myc-IKK α
α-TUBULIN


TNFα - - + +
NUMB - + - +
NUMB
Relative luciferase activity (fold)
8
7
6
5
4
3
2
1
0
*
*
TNFα - + - - + +
NUMB - - - + - +
RBP-JK Luc + + + + + +


Vector
IKKα
IKKα+NUMB
NOTCH1 downstream target mRNA level (Fold)
0
1
2
3
4
5
6
7
8
*
*
*
*
*
*
p21
p27
c-Myc
Hes6
Hey1
Hey2
p21
p27
c-Myc
Hes6
Hey1
Hey2

CHAPTER 7 NUMB was transcriptionally regulated by TNFα/IKKα via FOXA2

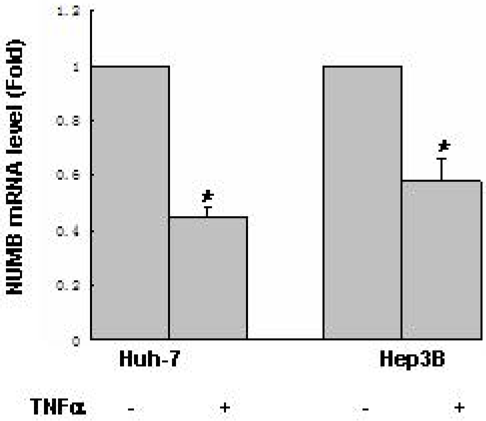
Since we know NUMB expression could be regulated by TNFα/IKKα, we further investigate the underneath molecular mechanism. We first checked the status NUMB mNRA using quantitative PCR (qPCR) after TNFα treatment in both Huh-7 and Hep3B cells. We found that mRNA of NUMB is decreased with TNFα treatment compared those without TNFα 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 TNFα/IKKα mediated NUMB transcription regulation. Only FOXA2 (HNF3β)-upregulated NUMB mRNA level was reduced with IKKα occurrence, indicating that FOXA2-activated NUMB transcription can be inhibited by IKKα (Figure 10C). Collectively, these results indicate that FOXA2, as a transcriptional factor of NUMB promoter, will decrease its transcriptional abilities on NUMB promoter with TNFα, therefore downregulate NUMB expression and activation of NOTCH1.

Fig 10 NUMB was transcriptionally regulated by TNFα/IKKα via FOXA2

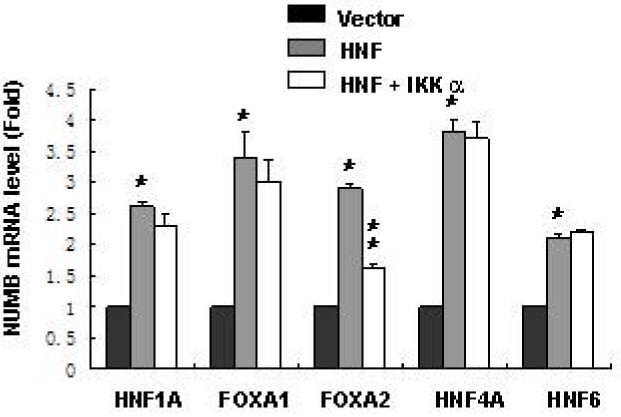
(A) Hep3B and Huh-7 cells were treated with TNFα (20 ng/ml) for 24 h. NUMB mRNA level was detected with qPCR. Error bars represent SD (*n* = 3). * indicates statistical significance (*P* < 0.05).

(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 IKKα 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 IKKα Interacts with and phosphorylates FOXA2 in vitro

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

Fig 11 IKKα Interacts with and phosphorylates FOXA2 in vitro

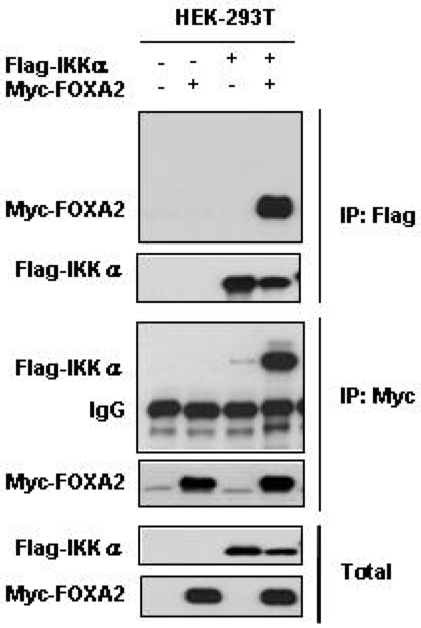
(A) IKKα and FOXA2 were transfected into 293T cells and analyzed by reciprocal co-immunoprecipitation and immunoblotting using indicated antibodies.

(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-IKKβ and GST-IKKα 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 IKKα for 30 min.

(E) Mass spectrum of FOXA2 phosphorylation sites by IKKα using samples (without isotope) from (D).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Flag-IKKα - - + +
Myc-FOXA2 - + - +
Myc-FOXA2
Flag-IKK α
IP: Flag
Flag-IKK α
IgG
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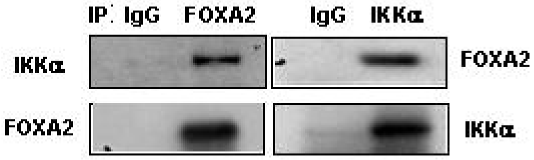
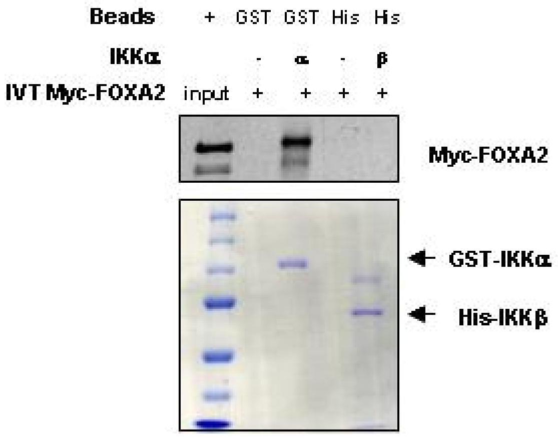
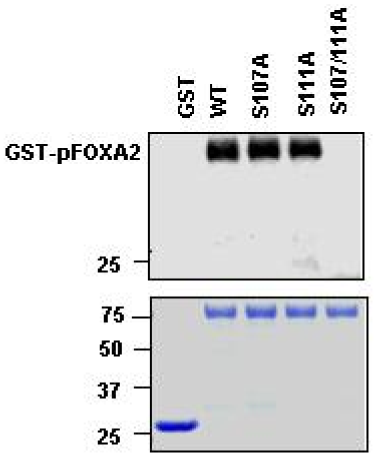
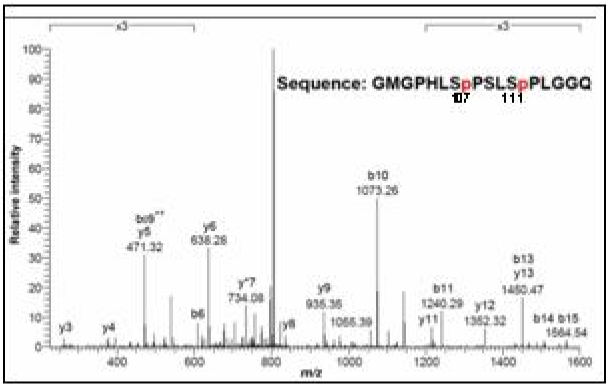
IP: Myc
Flag-IKK α
Myc-FOXA2
Total


IP: IgG FOXA2 IgG IKKα
IKKα
FOXA2
FOXA2
IKKα


Beads
+
GST
GST
His
His
IKKα
-
α
-
β
IVT Myc-FOXA2
input
+
+
+
+
Myc-FOXA2
GST-IKKα
His-IKKβ


GST
WT
S107A
S111A
S107/111A
GST-pFOXA2
25
75
50
37
25


x3
x3
Sequence: GMGPHLSpPSLSpPLGGQ
107
111
Relative intensity
100
90
80
70
60
50
40
30
20
10
0
b10
1073.26
bo9**
y5
471.32
y6
638.28
y*7
734.08
b6
y3
y4
y8
y9
935.36
1055.39
b11
1240.29
y11
y12
1352.32
b13
y13
1450.47
b14 b15
1564.54
400
600
800
1000
1200
1400
1600
m/z

CHAPTER 9 IKKα Interacts with and phosphorylates FOXA2 in vivo

Although we identified two phosphorylation sites on FOXA2 in vitro, we need to further identified IKKα could phosphorylates FOXA2 on these two sites in vivo. endogenous FOXA2 were isolated from TNFα treated Hep3B cells to perform MS analysis. These two phosphorylation sites were included in other 12 phosphorylate sites on FOXA2 with TNFα 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 IKKα but not by KD IKKα (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 IKKα in an in vitro kinase assay (Figure 12D). Time course assays to study the effect of TNFα showed that levels of pFOXA2 (S107/111) were high at 10–30 min and declined thereafter (Figure 12E), consistent with the kinetics of TNFα-induced IKKα activation as measured by degradation of IκBα.

Fig 12 IKKα Interacts with and phosphorylates FOXA2 in vivo

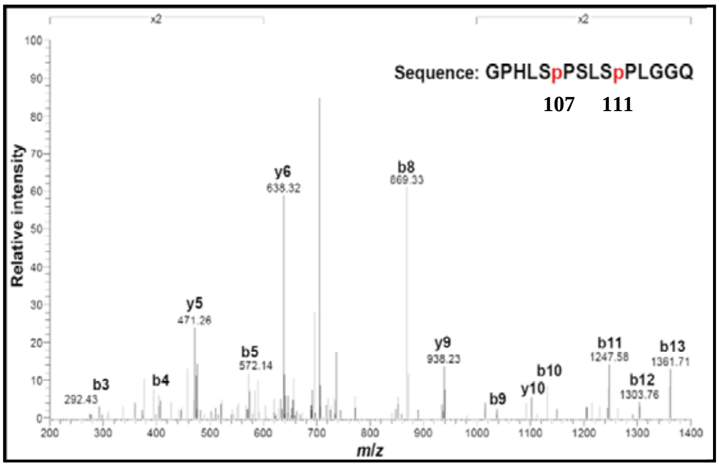
(A)Hep3B cells were treated with TNFα for 24 h, and cell lysates were analyzed by MS to identify in vivo FOXA2 phosphorylation sites.

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

(C) IKKα 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 IKKα 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 IKKα expression are also shown.

(E) Hep3B cells were treated with TNFα and harvested at 10, 30, 60, and 120 min after treatment. Cell lysates (40 μg) were used for western blot analysis of pFOXA2 with an antibody specific to pFOXA2 (S107/111). Total FOXA2 expression and IKKα expression are also shown.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x2
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107
111
Relative intensity
0
10
20
30
40
50
60
70
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100
b3
292.43
b4
y5
471.26
b5
572.14
y6
638.32
b8
869.33
y9
938.23
b9
y10
b10
b11
1247.58
b12
1303.76
b13
1361.71
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300
400
500
600
700
800
900
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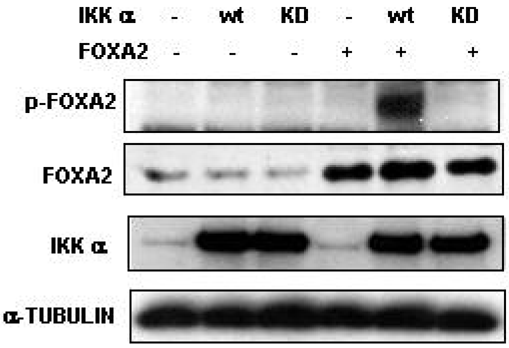
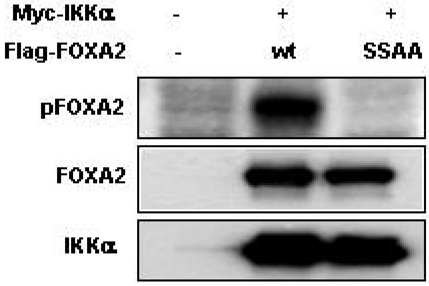
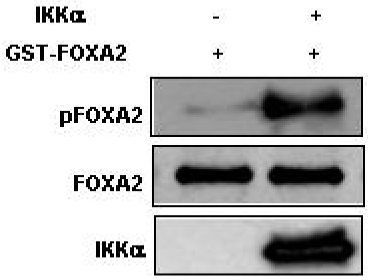
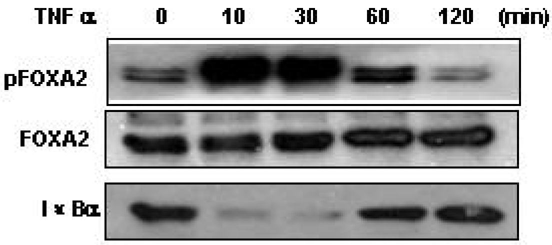
1200
1300
1400
m/z


IKK α
-
wt
KD
-
wt
KD
FOXA2
-
-
-
+
+
+
p-FOXA2
FOXA2
IKK α
α-TUBULIN


Myc-IKKα
-
+
+
Flag-FOXA2
-
wt
SSAA
pFOXA2
FOXA2
IKKα


IKKα
-
+
GST-FOXA2
+
+
pFOXA2
FOXA2
IKKα

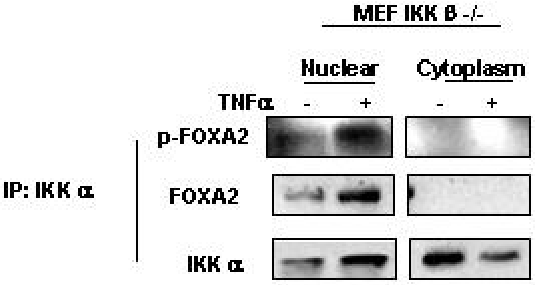

TNF α
0
10
30
60
120
(min)
pFOXA2
FOXA2
IκBα

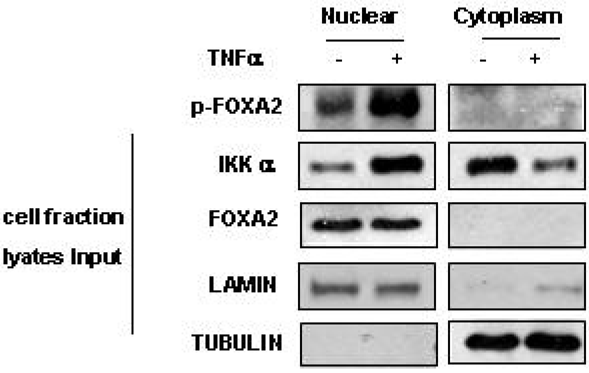
CHAPTER 10 IKKα phosphorylates FOXA2 in nucleus

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

Fig 13 IKKα phosphorylates FOXA2 in nucleus

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


MEF IKK β -/-
Nuclear
Cytoplasm
TNFα
-
+
-
+
p-FOXA2
IP: IKK α
FOXA2
IKK α


Nuclear
Cytoplasm
TNFα
-
+
-
+
p-FOXA2
IKK α
cell fraction
lyates Input
FOXA2
LAMIN
TUBULIN

CHAPTER 11 FOXA2 Phosphorylation by IKKα Suppresses FOXA2 transactivation Activity

Since upon TNFα stimulation, IKKα phosphorylates FOXA2 and leads to NUMB suppression, it is possible that FOXA2 lost its function as a transcriptional factor after being phosphorylated by IKKα, 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 activites and monitor the FOXA2 transactivation activity in general. After we introduced it to 293T cells, we found that WT IKKα could suppress FOXA2 transactivation activity, but the activity would not be affected by KD IKKα (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 IKKα effects (Figure 14B).

To test whether IKKα 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 drived 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 IKKα, 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 IKKα 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 TNFα 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 IKKα Suppresses FOXA2 transactivation activity

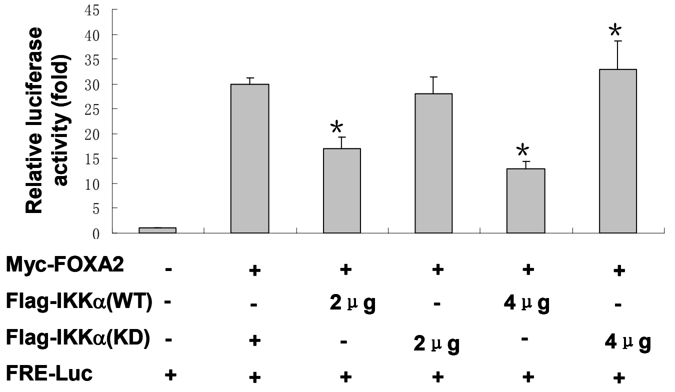
(A) Six repeated consensus binding sites of FOXA2 were constructed into Luc-driven reporter (FRE-Luc). NUMB promoter luciferase reporter and FOXA2 in the presence of different doses of WT IKKα or KD IKKα were transfected into 293T cells for 48 h. Cell lysates were subjected to luciferase reporter assays 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).

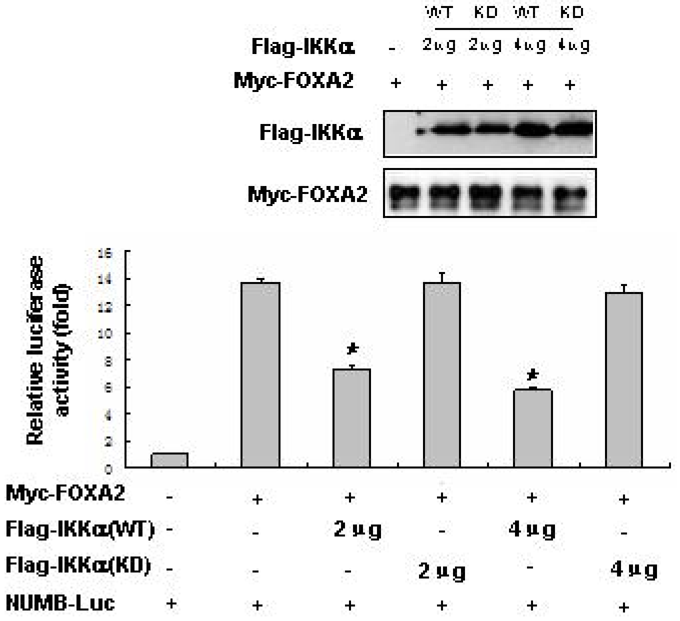
(B) NUMB promoter luciferase reporter and FOXA2 in the presence of different doses of WT IKKα or KD IKKα were transfected into 293T cells for 48 h. Cell lysates were subjected to luciferase reporter assays after transfection. Expression levels of WT IKKα, KD IKKα, and FOXA2 were analyzed by western blotting.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

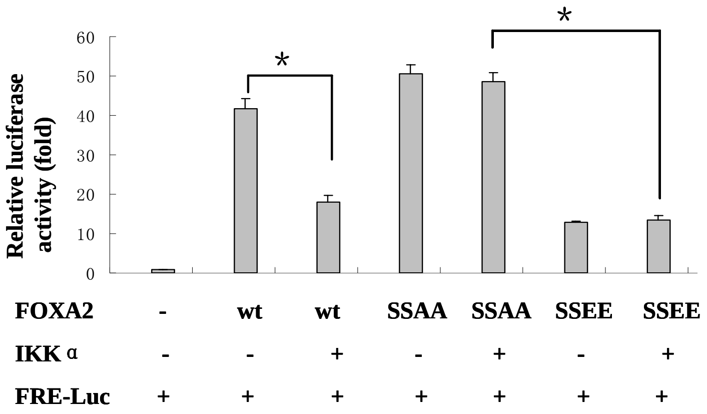
(C) Co-transfection of NUMB-promoter Luc and WT FOXA2, FOXA2-SSAA, or FOXA2-SSEE plus the indicated Flag-tagged IKKα in 293T cells for 48 h. Lysates of 293T cells were subjected to luciferase assays. Error bars represent SD (*n* = 3). * indicates statistical significance (*P* < 0.05). Expression levels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

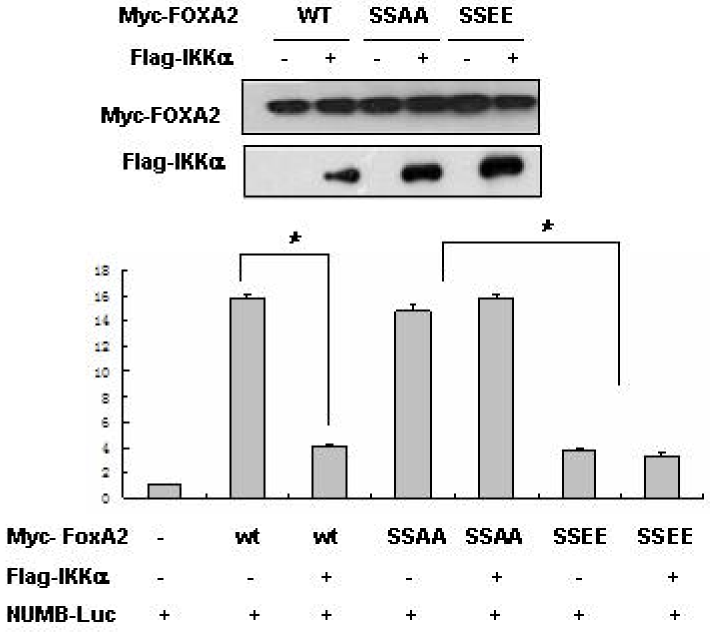
(D) Co-transfection of FRE-Luc and WT FOXA2, FOXA2-SSAA, or FOXA2-SSEE plus the indicated Flag-tagged IKKα in 293T cells for 48 h. Lysates of 293T were subjected to luciferase assays. Error bars represent SD (*n* = 5). * indicates statistical significance (*P* < 0.05).

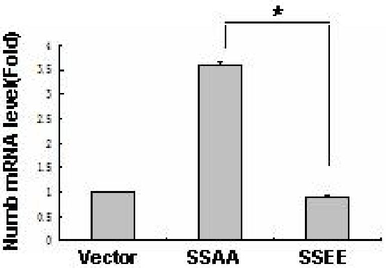
(E) Hep3B cells were infected with retrovirus expressing FOXA2-SSAA or FOXA2-SSEE. RNA extracts from the cells were isolated and subjected to real-time RT-PCR for NUMB mRNA expression. Error bars represent SD (*n* = 3). * indicates statistical significance (*P* < 0.05).


Relative luciferase activity (fold)
0
5
10
15
20
25
30
35
40
45
*
*
*
Myc-FOXA2 - + + + + +
Flag-IKKα(WT) - - 2μg - 4μg -
Flag-IKKα(KD) - + - 2μg - 4μg
FRE-Luc + + + + + +


WT KD WT KD
Flag-IKKα - 2μg 2μg 4μg 4μg
Myc-FOXA2 + + + + +
Flag-IKKα
Myc-FOXA2
Relative luciferase activity (fold)
0
2
4
6
8
10
12
14
16
*
*
Myc-FOXA2 - + + + + +
Flag-IKKα(WT) - - 2μg - 4μg -
Flag-IKKα(KD) - - - 2μg - 4μg
NUMB-Luc + + + + + +


Relative luciferase activity (fold)
60
50
40
30
20
10
0
*
*
FOXA2 - wt wt SSAA SSAA SSEE SSEE
IKK α - - + - + - +
FRE-Luc + + + + + + +


Myc-FOXA2
WT
SSAA
SSEE
Flag-IKKα - + - + - +
Myc-FOXA2
Flag-IKKα
*
*
18
16
14
12
10
8
6
4
2
0
Myc- FoxA2 - wt wt SSAA SSAA SSEE SSEE
Flag-IKKα - - + - + - +
NUMB-Luc + + + + + + +


*
Numb mRNA level(Fold)
4
3.5
3
2.5
2
1.5
1
0.5
0
Vector
SSAA
SSEE

CHAPTER 12 FOXA2 Phosphorylation by IKKα decreases FOXA2 DNA binding activities

Since we know that FOXA2 transactivation activities decrease after being phosphorylated by IKKα, 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 TNFα 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 TNFα treatment. We found that binding of FOXA2 to the NUMB promoter was decreased by TNFα (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 IKKα suppressed FOXA2 transactivation activity on NUMB by decreasing FOXA2 accessibility to its promoter.

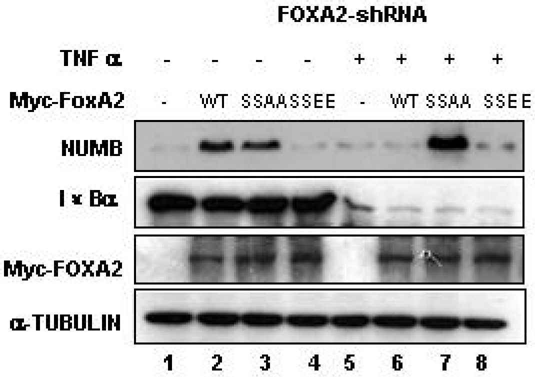
We further tested the function of FOXA2-SSAA and FOXA2-SSEE in FOXA2-knockdown Hep3B cells. The western immunoblotting assay showed that both WT FOXA2 and FOXA2-SSAA stimulated NUMB protein level (Figure 15C). WT FOXA2 expression could suppressed NUMB enhancement with TNFα stimulation, (Figure 15C, compare 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 TNFα 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 IKKα 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 IKKα decreases FOXA2 DNA binding activities

(A) Hep3B cells were treated with TNFα 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 TNFα 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 TNFα treatment for 24 h. Cell lysates were analyzed by luciferase reporter assay. Error bars represent SD (*n* = 3). * indicates statistical significance ($P < 0.05$).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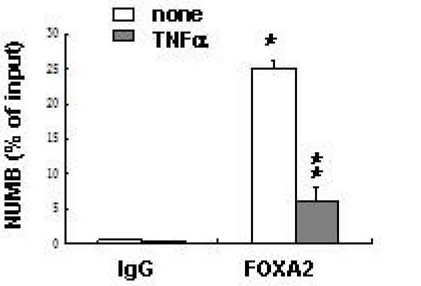
TNF α - - - - + + + +
Myc-FoxA2 - WT SSAA SSEE - WT SSAA SSEE
NUMB
I κ Bα
Myc-FOXA2
α-TUBULIN
1 2 3 4 5 6 7 8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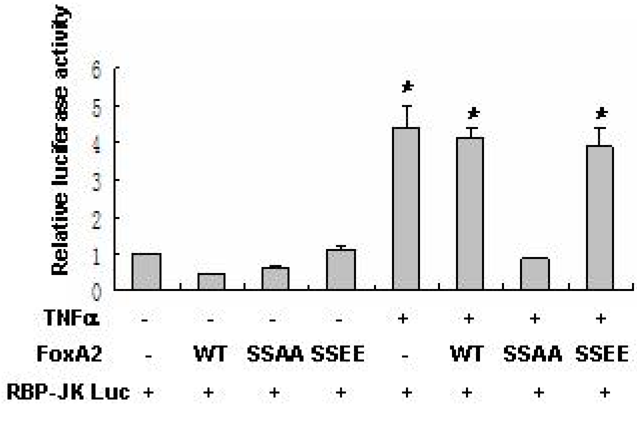
TNFα
NUMB (% of input)
30
25
20
15
10
5
0
*
**
IgG
FOXA2


Relative luciferase activity
6
5
4
3
2
1
0
*
*
*
TNFα - - - - + + + +
FoxA2 - WT SSAA SSEE - WT SSAA SSEE
RBP-JK Luc + + + + + + + +

CHAPTER 13 FOXA2 Phosphorylation by IKKα 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 IKKα-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 IKKα, but not KD IKKα, showed the ability to recovered Hep3B cells growth suppression from WT FOXA2 expression, presumably as a result of phosphorylation of WT FOXA2 by IKKα, 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 TNFα/IKKα pathway (Figure 16B). To verify that NOTCH1 activation is the main reason leading to cell growth in this TNFα/IKKα 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 TNFα/IKKα 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)

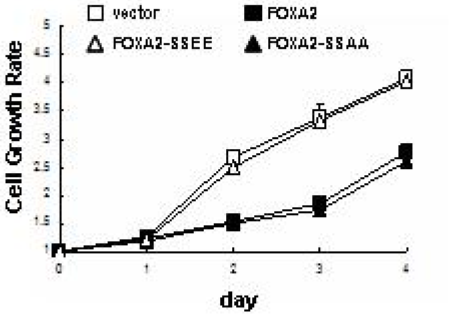
Figure 16 FOXA2 Blocks IKKα-Induced Cell Proliferation and Tumor Growth In Vitro

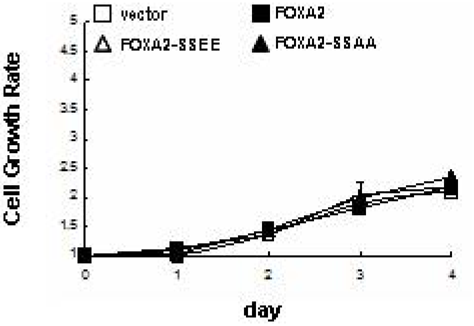
(A, B, and C). Hep3B cells were infected with retrovirus expressing WT FOXA2, FOXA2-SSAA, or FOXA2-SSEE in the presence of (A) control shRNA, (B) NUMB shRNA, or (C) NOTCH1 shRNA. Cells (2.5×10^3) were plated in 96-well plates, and cell growth was determined by MTT assay each day for 4 days.

(D) WT IKKα or KD IKKα 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 μM), GSI (10 μM), 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 ± 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 IKKα inhibited Hep3B cell proliferation. WT FOXA2, FOXA2-SSAA, or FOXA2-SSEE and WT IKKα or KD IKKα 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.


Control shRNA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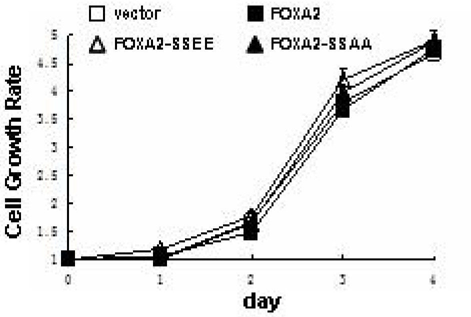
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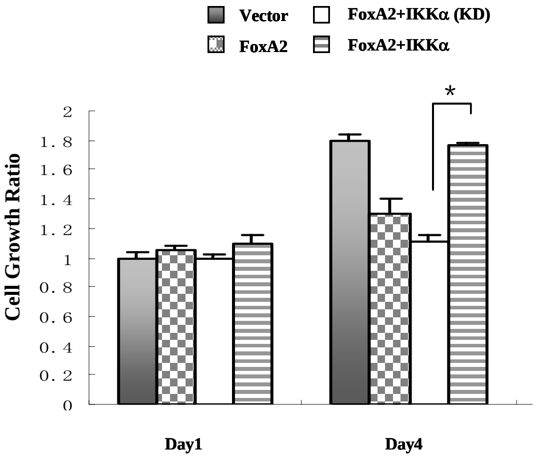
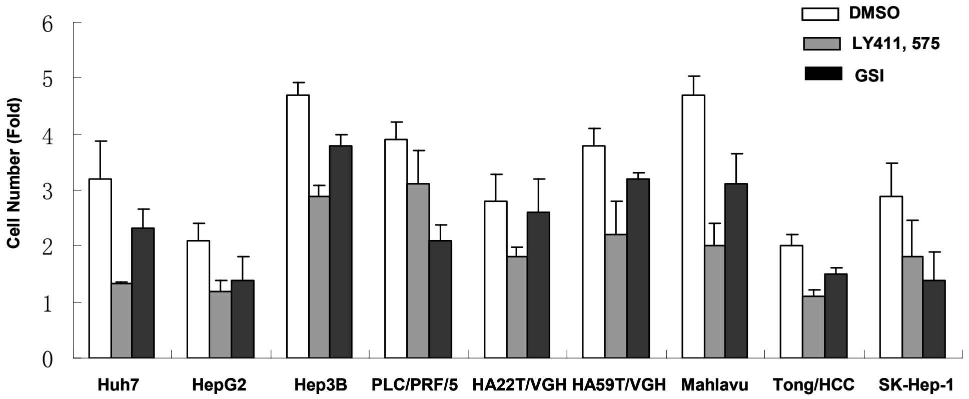
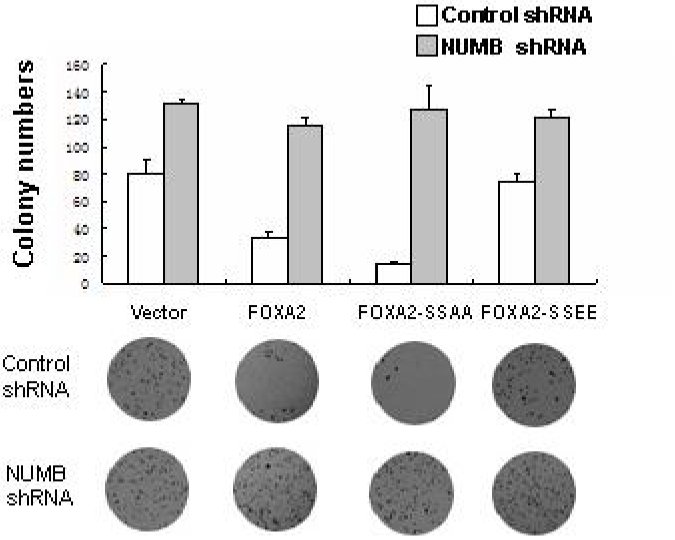
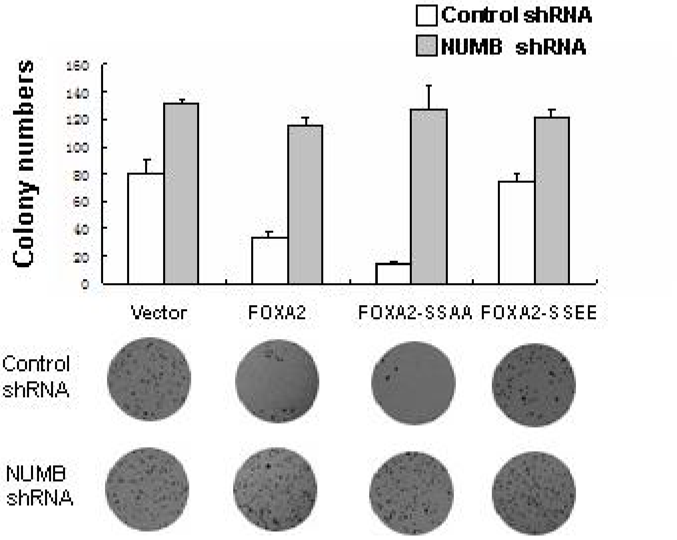

Vector
FoxA2
FoxA2+IKKα (KD)
FoxA2+IKKα
*
Cell Growth Ratio
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Day1
Day4


DMSO
LY411, 575
GSI
Cell Number (Fold)
6
5
4
3
2
1
0
Huh7
HepG2
Hep3B
PLC/PRF/5
HA22T/VGH
HA59T/VGH
Mahlavu
Tong/HCC
SK-Hep-1


Control shRNA
NUMB shRNA
Colony numbers
160
140
120
100
80
60
40
20
0
Vector
FOXA2
FOXA2-SSAA
FOXA2-SSEE
Control shRNA
NUMB shRNA


Control shRNA
NUMB shRNA
Colony numbers
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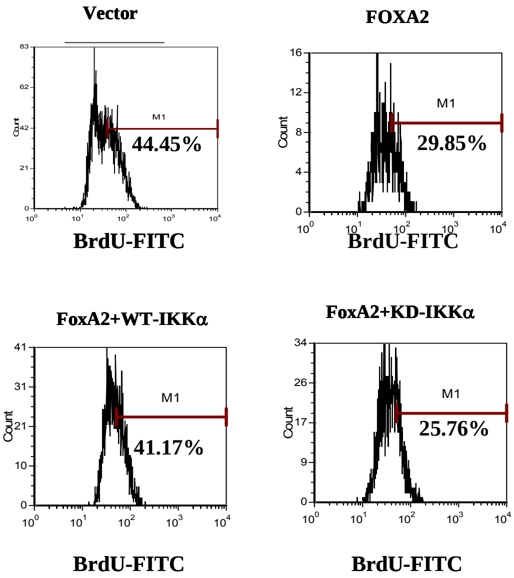
140
120
100
80
60
40
20
0
Vector
FOXA2
FOXA2-SSAA
FOXA2-SSEE
Control shRNA
NUMB shRNA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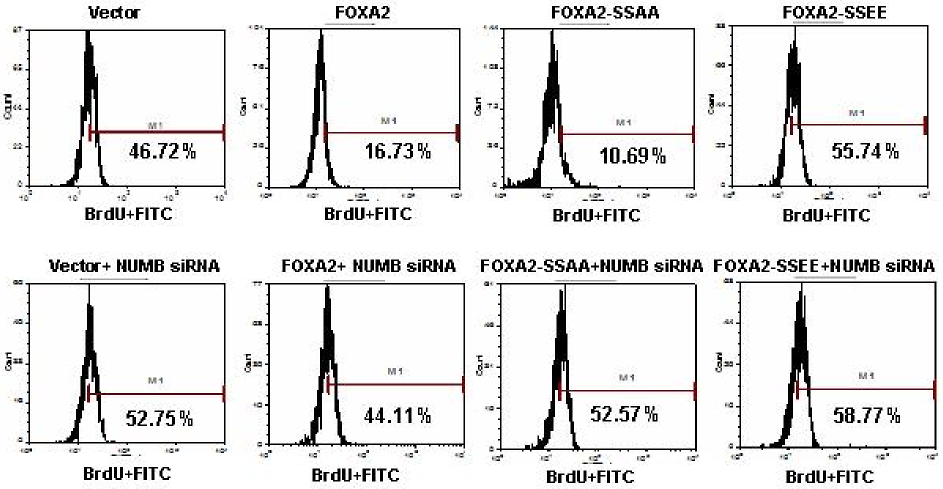
Count
M1
44.45%
29.85%
BrdU-FITC
BrdU-FITC
FoxA2+WT-IKKα
FoxA2+KD-IKKα
Count
M1
41.17%
25.76%
BrdU-FITC
BrdU-FITC


Vector
FOXA2
FOXA2-SSAA
FOXA2-SSEE
M1
46.72%
16.73%
10.69%
55.74%
BrdU+FITC
BrdU+FITC
BrdU+FITC
BrdU+FITC
Vector+ NUMB siRNA
FOXA2+ NUMB siRNA
FOXA2-SSAA+NUMB siRNA
FOXA2-SSEE+NUMB siRNA
M1
52.75%
44.11%
52.57%
58.77%
BrdU+FITC
BrdU+FITC
BrdU+FITC
BrdU+FITC

CHAPTER 14 FOXA2 Phosphorylation by IKKα De-Represses FOXA2-Mediated Repression of Cell Growth In Vivo

The tumor promotion functions of IKKα-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 IKKα in liver tumor growth, we injected the first group of mice with Hep3B cells infected with retrovirus expressing shRNA against IKKα. TV was much smaller in the mice harboring tumors (after transplantation) than in the control group, validating the role of IKKα in liver tumor growth (Figure 17A). Western blot analysis of these tumor tissue samples detected TNFα 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 IKKα 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 TNFα/IKKα-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).

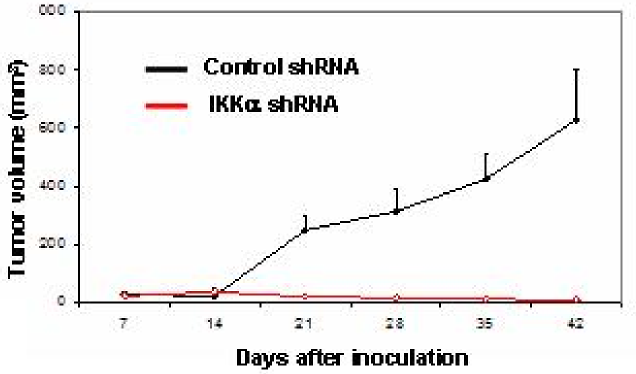
Figure 17. FOXA2 Blocks IKKα-Induced Cell Proliferation and Tumor Growth In Vivo

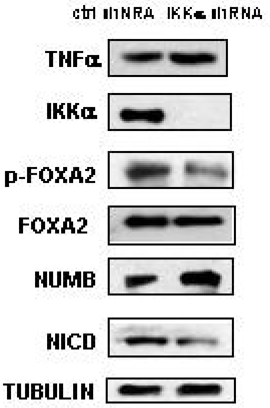
(A) Hep3B cells were infected with retrovirus expressing control or IKKα shRNA. Mice were injected subcutaneously with 1 × 10^6 cells. Error bars represent SD (*n* = 5). Please see the Experimental Procedures section for more detailed information.

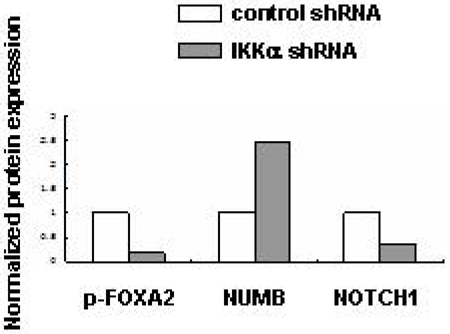
(B) Tumor tissues from mice that received transplants were analyzed 10 days after transplantation and subjected to western blot analysis for indicated antibodies. Right: Expression levels of pFOXA2, NUMB, and NOTCH1 quantified with ImageJ software.

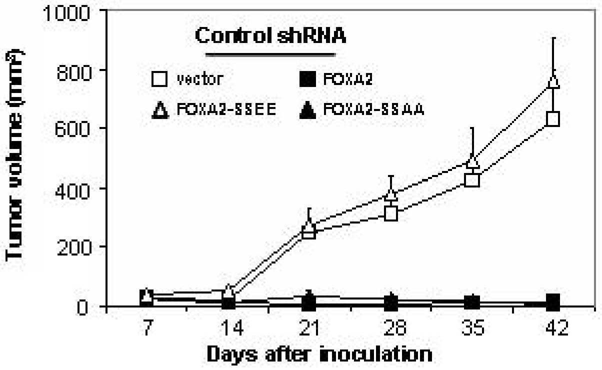
(C) Hep3B cells were infected with retrovirus expressing WT FOXA2, FOXA2-SSAA, or FOXA2-SSEE in the presence of (top) control shRNA or (bottom) NUMB shRNA. Error bars represent SD (*n* = 5). Please see the Experimental Procedures section for more detailed information.

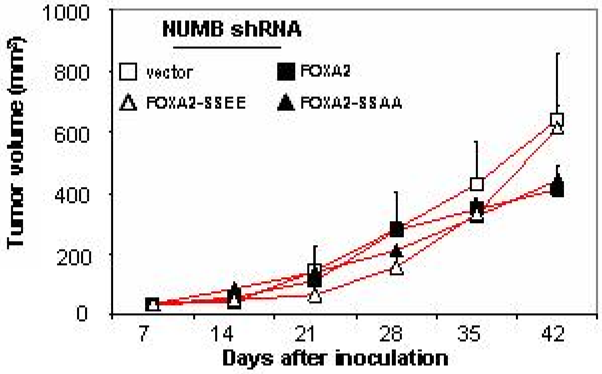
(D) Tumor issues from (C) were harvested 10 days after transplantation and subjected to western blot analysis for NICD, FOXA2, and NUMB. α-Tubulin was included as control.

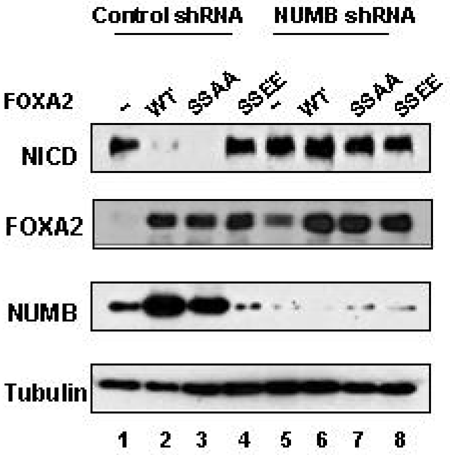

000
800
600
400
200
0
Tumor volume (mm³)
Control shRNA
IKKα shRNA
7
14
21
28
35
42
Days after inoculation


ctrl shNRA
IKKα shRNA
TNFα
IKKα
p-FOXA2
FOXA2
NUMB
NICD
TUBULIN


control shRNA
IKKα shRNA
Normalized protein expression
3
2.5
2
1.5
1
0.5
0
p-FOXA2
NUMB
NOTCH1


Control shRNA
□ vector
■ FOXA2
△ FOXA2-SSEE
▲ FOXA2-SSAA
Tumor volume (mm³)
1000
800
600
400
200
0
7
14
21
28
35
42
Days after inoculation


NUMB shRNA
□ vector
■ FOXA2
△ FOXA2-SSEE
▲ FOXA2-SSAA
Tumor volume (mm³)
1000
800
600
400
200
0
7
14
21
28
35
42
Days after inoculation


Control shRNA
NUMB shRNA
FOXA2
-
WT
SSAA
SSEE
-
WT
SSAA
SSEE
NICD
FOXA2
NUMB
Tubulin
1
2
3
4
5
6
7
8

CHAPTER 15 FOXA2 Phosphorylation by IKKα play roles in liver cancer

In addition, to further examine the aforementioned conclusion that FOXA2 phosphorylation by TNFα-induced IKKα plays a critical role in liver cancer, we examined the expression of IKKα, pFOXA2 (S107/111), and NICD in 30 freshly prepared human primary HCC tumor specimens and paired normal liver tissues by immunoblotting. We found that IKKα, 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 IKKα and NICD. These results strengthen the notion that IKKα regulates FOXA2 and activates NICD through phosphorylation at S107/111 and the physiological implication of pFOXA2 in IKKα-induced tumorigenesis (18C, 18D). Taken together, analyses of these clinical HCC tumor specimens support that FOXA2 phosphorylation by IKKα might be associated with HCC tumorigenesis.

Fig 18 FOXA2 Phosphorylation by IKKα play roles in liver cancer

(A, B) Comparison of pFOXA2 (S107/111), IKKα, 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 IKKα/NICD expression levels in tumors ($R^2 > 0.5$, Spearman’s rank correlation test).

IKKα	High	Low	Total
Normal	4	26	30
Cancer	26	4	30
Total	30	30	60

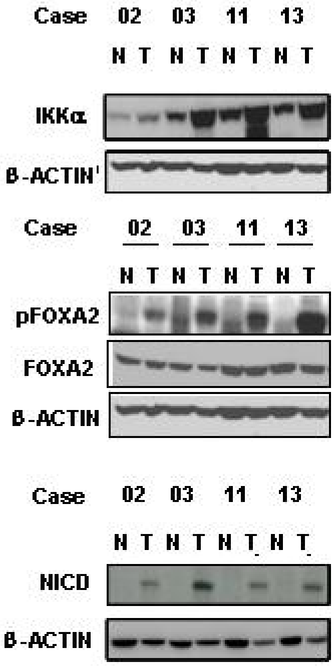
p=0.01

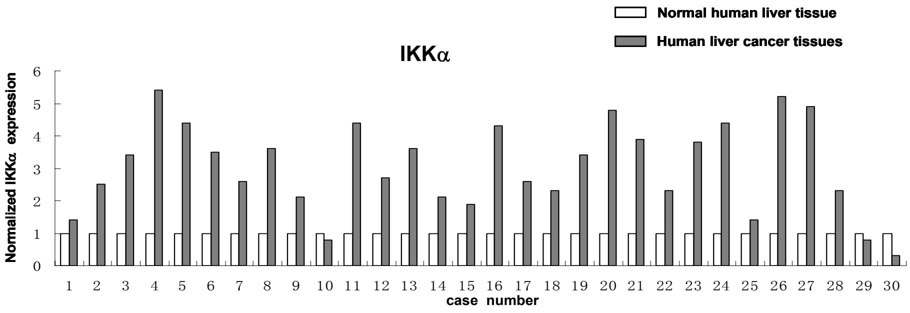
pFOXA2	High	Low	Total
Normal	9	21	30
Cancer	21	9	30
Total	30	30	60

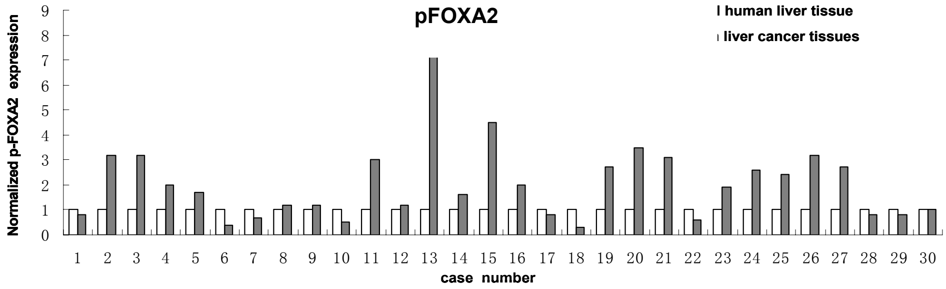
p=0.007

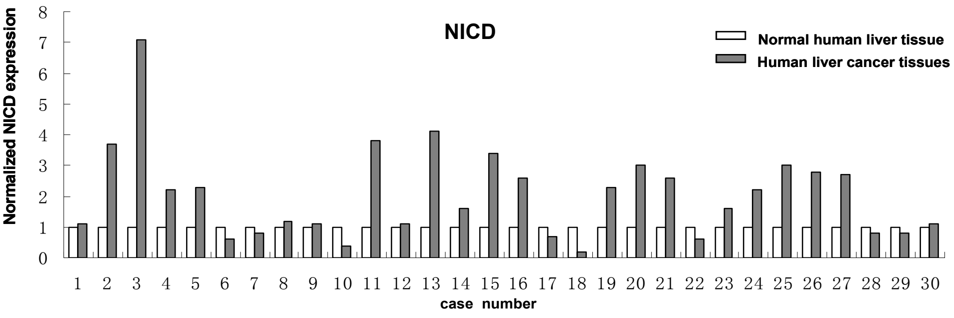
NICD	High	Low	Total
Normal	8	22	30
Cancer	22	8	30
Total	30	30	60

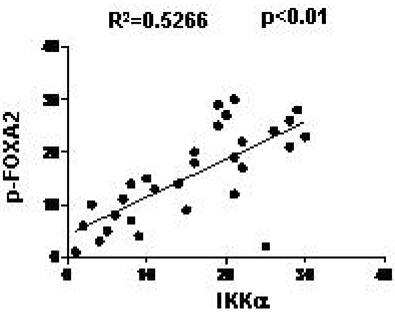
p=0.005

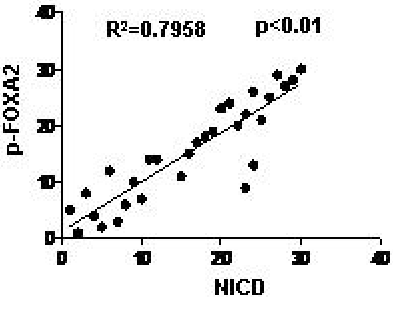

Case 02 03 11 13
N T N T N T N T
IKKα
β-ACTIN
Case 02 03 11 13
N T N T N T N T
pFOXA2
FOXA2
β-ACTIN
Case 02 03 11 13
N T N T N T N T
NICD
β-ACTIN


Normal human liver tissue
Human liver cancer tissues
IKKα
Normalized IKKα expression
0
1
2
3
4
5
6
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
case number


pFOXA2
l human liver tissue
ı liver cancer tissues
Normalized p-FOXA2 expression
0
1
2
3
4
5
6
7
8
9
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
case number


NICD
Normal human liver tissue
Human liver cancer tissues
Normalized NICD expression
0
1
2
3
4
5
6
7
8
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
case number


R²=0.5266
p<0.01
p-FOXA2
0
10
20
30
40
IKKα

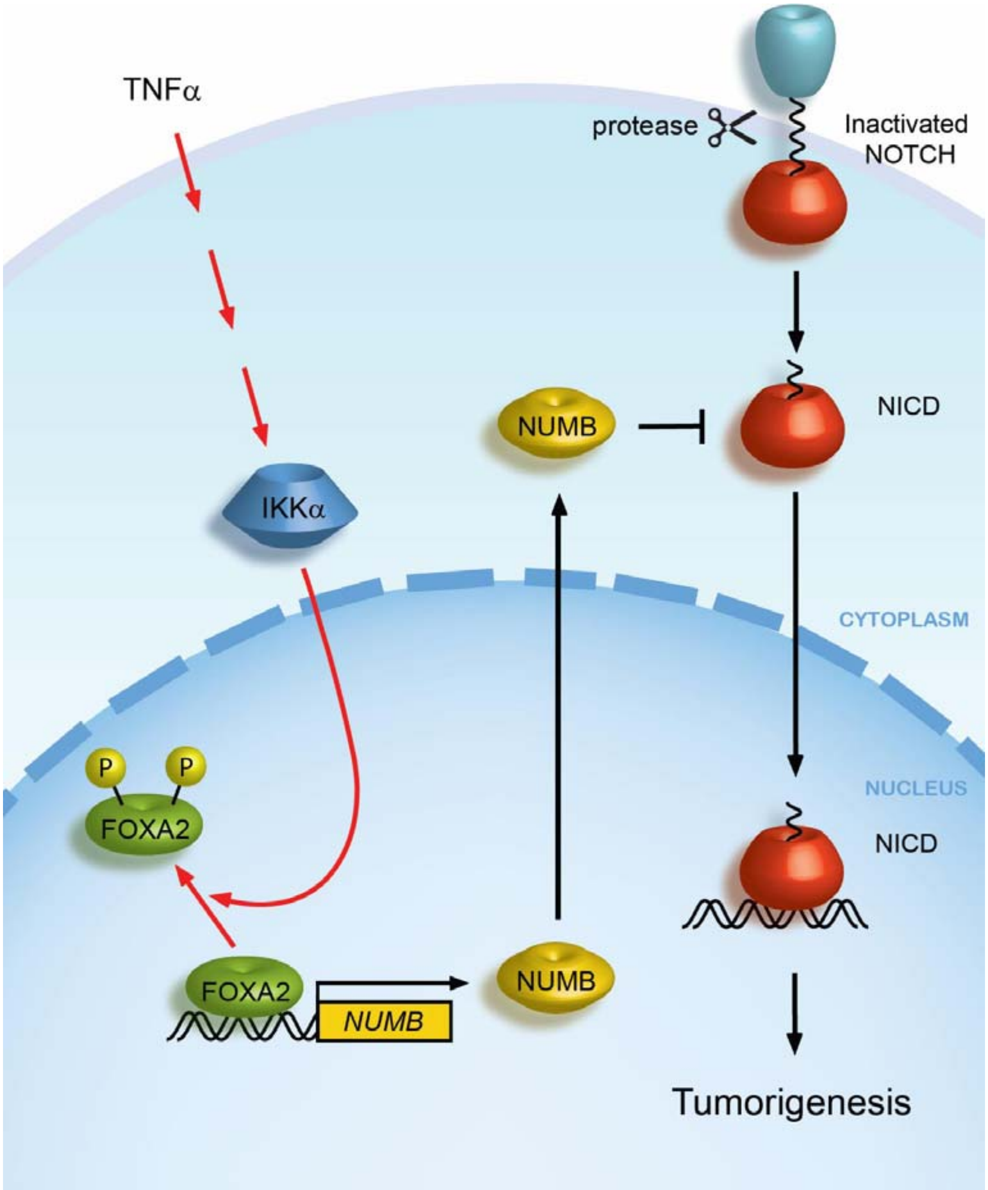

R²=0.7958
p<0.01
p-FOXA2
0
10
20
30
40
NICD

CHAPTER 16 Summary & Discussion

Summary

Here, we identified an axis in TNFα pathway involving activation of NOTCH1 signaling through suppression of FOXA2 transactivation activity by IKKα, leading to disruption of NUMB expression, which promotes NOTCH1-induced liver cell proliferation and growth. In our proposed model, TNFα-activated IKKα interacts with and phosphorylates FOXA2. We identified two IKKα-mediated FOXA2 phosphorylation sites, Ser107 and Ser111. Phosphorylation at these two sites decreased FOXA2 transactivaction 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 TNFα induced FOXA2/NOTCH1 pathway


TNFα
protease
Inactivated
NOTCH
NUMB
NICD
IKKα
CYTOPLASM
NUCLEUS
P
P
FOXA2
NICD
FOXA2
NUMB
NUMB
Tumorigenesis

Discussion

Inflammation, TNFα 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 TNFα. 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, TNFα has a large amount of activities in cell to cell communications and inflammation-associated human cancers. Dysregulation of TNFα signaling pathway contributes to the development of human cancers due to enhanced IKK complex activity and regulate NF-κB 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 TNFα in cancers, especially in liver cancer.

The most direct evidence to show the tumor-promoting role of TNFα 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 (TNFα receptor) mouse has no significant difference to those of wild type mouse, indicating TNFα signaling is not required for DEN-induced hepatocarcinogenesis.

Another model related to TNFα/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-κB. The Mdr2-KO mouse system was engineered Dr. Yinon Ben-Neriah, who showed that NF-κB functions as a tumor promoter in inflammation-associated liver cancer by anti-TNFα treatment or ectopic expression of an IκB-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 TNFα 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 apoptosis[114]. Several groups that analyzed clinical samples of human HCC also indicate a positive role of NOTCH1 in liver cancer[115-117]. More recently, Ning et al. specifically showed downregulation of NOTCH1 signaling inhibited tumor growth in human HCC in both cell lines and mouse model[118]. These studies are in line with our findings that NOTCH1 is activated by TNFα 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 targeting[48].

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 cancer[119-120]. IN addition, FOXA2 proteins are found to be a key regulator in lung inflammation[38]. 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 phosporylation of FOXA2 by IKKα 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 IKKα links the TNFα and NOTCH1 signaling pathways and provides an important new starting point for uncovering the molecular basis of TNFα-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 IKKα 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 events[49-51]. 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 IKKα/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 IKKα 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 IKKα/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 IKKα 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'-CAAAACAAAACCAACTAACC-3') and P2 (5'-GTAATTCTTTCCTGTCCCTG-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.

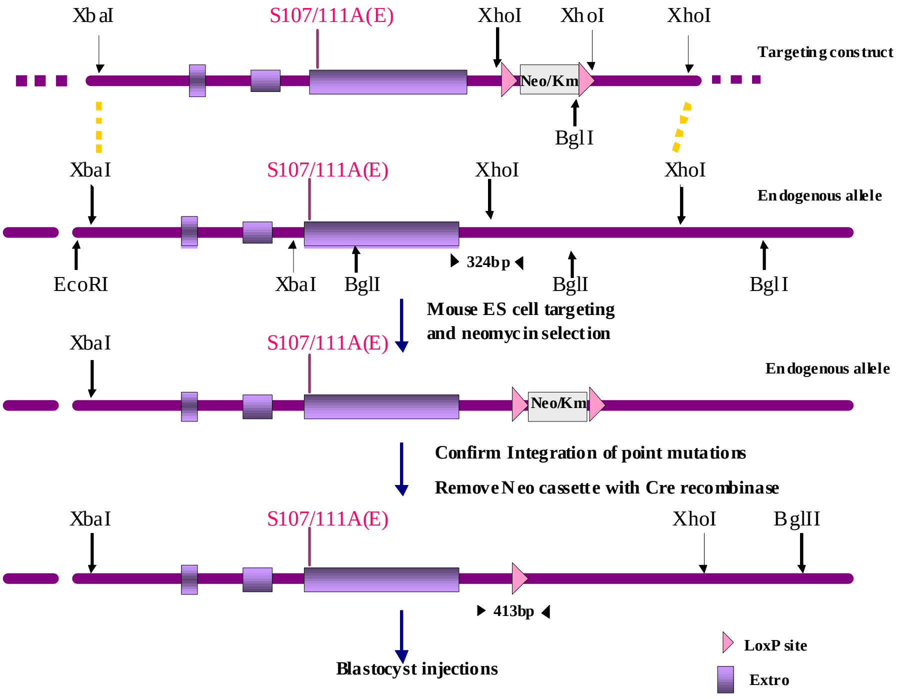

XbaI
S107/111A(E)
XhoI
XhoI
XhoI
Targeting construct
Neo/Km
BglI
XbaI
S107/111A(E)
XhoI
XhoI
Endogenous allele
EcoRI
XbaI
BglI
324bp
BglI
BglI
Mouse ES cell targeting
and neomycin selection
XbaI
S107/111A(E)
Endogenous allele
Neo/Km
Confirm Integration of point mutations
Remove Neo cassette with Cre recombinase
XbaI
S107/111A(E)
XhoI
BglII
413bp
Blastocyst injections
LoxP site
Extro

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 IKKα 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 IKKα 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 TNFα/IKKα 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.

BIBLIOGRAPH

1. Castello, G., Scala, S., Palmieri, G., Curley, S.A. & Izzo, F. HCV-related hepatocellular carcinoma: From chronic inflammation to cancer. *Clin Immunol* 134, 237-50. (2010)

2. Berasain, C., Castillo J, Perugorria MJ, Latasa MU, Prieto J, Avila MA.. Inflammation and liver cancer: new molecular links. *Ann N Y Acad Sci* 1155, 206-21 (2009).

3. Allavena, P., Garlanda, C., Borrello, M.G., Sica, A. & Mantovani, A. Pathways connecting inflammation and cancer. *Curr Opin Genet Dev* 18, 3-10 (2008).

4. Karin, M. & Greten, F.R. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol* 5, 749-59 (2005).

5. Pikarsky, E. Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, Gutkovich-Pyest E, Urieli-Shoval S, Galun E, Ben-Neriah Y NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature* 431, 461-6 (2004).

6. Lee, D.F. Kuo HP, Chen CT, Hsu JM, Chou CK, Wei Y, Sun HL, Li LY, Ping B, Huang WC, He X, Hung JY, Lai CC, Ding Q, Su JL, Yang JY, Sahin AA, Hortobagyi GN, Tsai FJ, Tsai CH, Hung MC. IKK beta suppression of TSC1 links inflammation and tumor angiogenesis via the mTOR pathway. *Cell* 130, 440-55 (2007).

7 Yen, C.J. Izzo JG, Lee DF, Guha S, Wei Y, Wu TT, Chen CT, Kuo HP, Hsu JM, Sun HL, Chou CK, Buttar NS, Wang KK, Huang P, Ajani J, Hung MC. Bile acid exposure up-regulates tuberous sclerosis complex 1/mammalian target of

rapamycin pathway in Barrett's-associated esophageal adenocarcinoma. *Cancer Res* 68, 2632-40 (2008).

8. Lee, S. Andrieu C, Saltel F, Destaing O, Auclair J, Pouchkine V, Michelon J, Salaun B, Kobayashi R, Jurdic P, Kieff ED, Sylla BS. IkappaB kinase beta phosphorylates Dok1 serines in response to TNF, IL-1, or gamma radiation. *Proc Natl Acad Sci U S A* 101, 17416-21 (2004).

9. Albanese, C. Wu K, D'Amico M, Jarrett C, Joyce D, Hughes J, Hulit J, Sakamaki T, Fu M, Ben-Ze'ev A, Bromberg JF, Lamberti C, Verma U, Gaynor RB, Byers SW, Pestell RG. IKKalpha regulates mitogenic signaling through transcriptional induction of cyclin D1 via Tcf. *Mol Biol Cell* 14, 585-99 (2003).

10. Carayol, N. & Wang, C.Y. IKKalpha stabilizes cytosolic beta-catenin by inhibiting both canonical and non-canonical degradation pathways. *Cell Signal* 18, 1941-6 (2006).

11. Lamberti, C. Lin KM, Yamamoto Y, Verma U, Verma IM, Byers S, Gaynor RB. Regulation of beta-catenin function by the IkappaB kinases. *J Biol Chem* 276, 42276-86 (2001).

12. Huang, W.C., Ju, T.K., Hung, M.C. & Chen, C.C. Phosphorylation of CBP by IKKalpha promotes cell growth by switching the binding preference of CBP from p53 to NF-kappaB. *Mol Cell* 26, 75-87 (2007).

13. Affara, N.I. & Coussens, L.M. IKKalpha at the crossroads of inflammation and metastasis. *Cell* 129, 25-6 (2007).

14. Luo, J.L. Tan W, Ricono JM, Korchynskyi O, Zhang M, Gonias SL, Cheresh DA,

Karin M. Nuclear cytokine-activated IKKalpha controls prostate cancer metastasis by repressing Maspin. *Nature* 446, 690-4 (2007).

15. Friedman, J.R. & Kaestner, K.H. The Foxa family of transcription factors in development and metabolism. *Cell Mol Life Sci* 63, 2317-28 (2006).

16. Epstein, D.J., McMahon, A.P. & Joyner, A.L. Regionalization of Sonic hedgehog transcription along the anteroposterior axis of the mouse central nervous system is regulated by Hnf3-dependent and -independent mechanisms. *Development* 126, 281-92 (1999).

17. Lee, C.S., Sund, N.J., Behr, R., Herrera, P.L. & Kaestner, K.H. Foxa2 is required for the differentiation of pancreatic alpha-cells. *Dev Biol* 278, 484-95 (2005).

18. Sasaki, H. & Hogan, B.L. HNF-3 beta as a regulator of floor plate development. *Cell* 76, 103-15 (1994).

19. Wolfrum, C. Shih DQ, Kuwajima S, Norris AW, Kahn CR, Stoffel M. Role of Foxa-2 in adipocyte metabolism and differentiation. *J Clin Invest* 112, 345-56 (2003).

20. Kaestner, K.H. The making of the liver: developmental competence in foregut endoderm and induction of the hepatogenic program. *Cell Cycle* 4, 1146-8 (2005).

21. Yang, F. Huang X, Yi T, Yen Y, Moore DD, Huang W. Spontaneous development of liver tumors in the absence of the bile acid receptor farnesoid X receptor. *Cancer Res* 67, 863-7 (2007).

22. Bochkis, I.M. Rubins NE, White P, Furth EE, Friedman JR, Kaestner KH.

Hepatocyte-specific ablation of Foxa2 alters bile acid homeostasis and results in endoplasmic reticulum stress. *Nat Med* 14, 828-36 (2008).

23. Hofmann, A.F. Bile Acids: The Good, the Bad, and the Ugly. *News Physiol Sci* 14, 24-29 (1999).

24. Lehner, F., Kulik, U., Klempnauer, J. & Borlak, J. The hepatocyte nuclear factor 6 (HNF6) and FOXA2 are key regulators in colorectal liver metastases. *Faseb J* 21, 1445-62 (2007).

25. Messeguer, X. Escudero R, Farré D, Núñez O, Martínez J, Albà MM. PROMO: detection of known transcription regulatory elements using species-tailored searches. *Bioinformatics* 18, 333-4 (2002).

26. Hsieh, J.J. Henkel T, Salmon P, Robey E, Peterson MG, Hayward SD.Truncated mammalian Notch1 activates CBF1/RBPJk-repressed genes by a mechanism resembling that of Epstein-Barr virus EBNA2. *Mol Cell Biol* 16, 952-9 (1996).

27. Kimble, J. & Crittenden, S.L. Controls of germline stem cells, entry into meiosis, and the sperm/oocyte decision in Caenorhabditis elegans. *Annu Rev Cell Dev Biol* 23, 405-33 (2007).

28. Tanigaki, K. & Honjo, T. Regulation of lymphocyte development by Notch signaling. *Nat Immunol* 8, 451-6 (2007).

29. Strizzi, L. Hardy KM, Seftor EA, Costa FF, Kirschmann DA, Seftor RE, Postovit LM, Hendrix MJ. Development and cancer: at the crossroads of Nodal and Notch signaling. *Cancer Res* 69, 7131-4 (2009).

30. Iso, T., Kedes, L. & Hamamori, Y. HES and HERP families: multiple effectors of the Notch signaling pathway. *J Cell Physiol* 194, 237-55 (2003).

31. Allman, D. Karnell FG, Punt JA, Bakkour S, Xu L, Myung P, Koretzky GA, Pui JC, Aster JC, Pear WS. Separation of Notch1 promoted lineage commitment and expansion/transformation in developing T cells. *J Exp Med* 194, 99-106 (2001).

32. Girard, L. Hanna Z, Beaulieu N, Hoemann CD, Simard C, Kozak CA, Jolicoeur P. Frequent provirus insertional mutagenesis of Notch1 in thymomas of MMTVD/myc transgenic mice suggests a collaboration of c-myc and Notch1 for oncogenesis. *Genes Dev* 10, 1930-44 (1996).

33. Gridley, T. Notch signaling and inherited disease syndromes. *Hum Mol Genet* 12 Spec No 1, R9-13 (2003).

34. Ruas, J.L., Lendahl, U. & Poellinger, L. Modulation of vascular gene expression by hypoxia. *Curr Opin Lipidol* 18, 508-14 (2007).

35. Weijzen, S. Rizzo P, Braid M, Vaishnav R, Jonkheer SM, Zlobin A, Osborne BA, Gottipati S, Aster JC, Hahn WC, Rudolf M, Siziopikou K, Kast WM, Miele L. Activation of Notch-1 signaling maintains the neoplastic phenotype in human Ras-transformed cells. *Nat Med* 8, 979-86 (2002).

36. Gulino, A., Di Marcotullio, L. & Screpanti, I. The multiple functions of Numb. *Exp Cell Res* 316, 900-6. (2004)

37. Shi, W. & Harris, A.L. Notch signaling in breast cancer and tumor angiogenesis: cross-talk and therapeutic potentials. *J Mammary Gland Biol Neoplasia* 11, 41-52 (2006).

38. Yamaguchi, N. Oyama T, Ito E, Satoh H, Azuma S, Hayashi M, Shimizu K, Honma R, Yanagisawa Y, Nishikawa A, Kawamura M, Imai J, Ohwada S, Tatsuta K, Inoue J, Semba K, Watanabe S. NOTCH3 signaling pathway plays crucial roles in the proliferation of ErbB2-negative human breast cancer cells. *Cancer Res* 68, 1881-8 (2008).

39. Mazzone, M. Selfors LM, Albeck J, Overholtzer M, Sale S, Carroll DL, Pandya D, Lu Y, Mills GB, Aster JC, Artavanis-Tsakonas S, Brugge JS. Dose-dependent induction of distinct phenotypic responses to Notch pathway activation in mammary epithelial cells. *Proc Natl Acad Sci U S A* 107, 5012-7.(1999)

40. Aldinucci, D., Gloghini, A., Pinto, A., De Filippi, R. & Carbone, A. The classical Hodgkin's lymphoma microenvironment and its role in promoting tumour growth and immune escape. *J Pathol* 221, 248-63. (2009)

41. Hayashi, Y. Wang W, Ninomiya T, Nagano H, Ohta K, Itoh H. Liver enriched transcription factors and differentiation of hepatocellular carcinoma. *Mol Pathol* 52, 19-24 (1999).

42. Xiong, W. Ren ZG, Qiu SJ, Sun HC, Wang L, Liu BB, Li QS, Zhang W, Zhu XD, Liu L, Wang WQ, Tang ZY. Residual hepatocellular carcinoma after oxaliplatin treatment has increased metastatic potential in a nude mouse model and is attenuated by Songyou Yin. *BMC Cancer* 10, 219 (2010)

43. Karin, M. The IkappaB kinase - a bridge between inflammation and cancer. *Cell Res* 18, 334-42 (2008).

44. Lee, D.F. & Hung, M.C. Advances in targeting IKK and IKK-related kinases for

cancer therapy. *Clin Cancer Res* 14, 5656-62 (2008).

45. Bray, S.J. Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol* 7, 678-89 (2006).

46. Inoki, K. Ouyang H, Zhu T, Lindvall C, Wang Y, Zhang X, Yang Q, Bennett C, Harada Y, Stankunas K, Wang CY, He X, MacDougald OA, You M, Williams BO, Guan KL. TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. *Cell* 126, 955-68 (2006).

47. Koch, U. & Radtke, F. Notch and cancer: a double-edged sword. *Cell Mol Life Sci* 64, 2746-62 (2007).

48. Pui, C.H. T cell acute lymphoblastic leukemia: NOTCHing the way toward a better treatment outcome. *Cancer Cell* 15, 85-7 (2009).

49. Zardawi, S.J. Zardawi I, McNeil CM, Millar EK, McLeod D, Morey AL, Crea P, Murphy NC, Pinese M, Lopez-Knowles E, Oakes SR, Ormandy CJ, Qiu MR, Hamilton A, Spillane A, Soon Lee C, Sutherland RL, Musgrove EA, O'Toole SA. High Notch1 protein expression is an early event in breast cancer development and is associated with the HER-2 molecular subtype. *Histopathology* 56, 286-96. (2000)

50. Qi, R. An H, Yu Y, Zhang M, Liu S, Xu H, Guo Z, Cheng T, Cao X. Notch1 signaling inhibits growth of human hepatocellular carcinoma through induction of cell cycle arrest and apoptosis. *Cancer Res* 63, 8323-9 (2003).

51. Ning, L., Wentworth, L., Chen, H. & Weber, S.M. Down-regulation of Notch1 signaling inhibits tumor growth in human hepatocellular carcinoma. *Am J Transl*

Res 1, 358-66 (2009).

52. Cantarini, M.C. de la Monte SM, Pang M, Tong M, D'Errico A, Trevisani F, Wands JR. Aspartyl-asparagyl beta hydroxylase over-expression in human hepatoma is linked to activation of insulin-like growth factor and notch signaling mechanisms. *Hepatology* 44, 446-57 (2006).

53. Gramantieri, L. Giovannini C, Lanzi A, Chieco P, Ravaioli M, Venturi A, Grazi GL, Bolondi L. Aberrant Notch3 and Notch4 expression in human hepatocellular carcinoma. *Liver Int* 27, 997-1007 (2007).

54. Yang, J.Y. Zong CS, Xia W, Yamaguchi H, Ding Q, Xie X, Lang JY, Lai CC, Chang CJ, Huang WC, Huang H, Kuo HP, Lee DF, Li LY, Lien HC, Cheng X, Chang KJ, Hsiao CD, Tsai FJ, Tsai CH, Sahin AA, Muller WJ, Mills GB, Yu D, Hortobagyi GN, Hung MC. ERK promotes tumorigenesis by inhibiting FOXO3a via MDM2-mediated degradation. *Nat Cell Biol* 10, 138-48 (2008).

55. Lei, H. & Quelle, F.W. FOXO transcription factors enforce cell cycle checkpoints and promote survival of hematopoietic cells after DNA damage. *Mol Cancer Res* 7, 1294-303 (2009).

56. Koon, H.B., Ippolito, G.C., Banham, A.H. & Tucker, P.W. FOXP1: a potential therapeutic target in cancer. *Expert Opin Ther Targets* 11, 955-65 (2007).

57. Gomez-Gutierrez, J.G. Souza V, Hao HY, Montes de Oca-Luna R, Dong YB, Zhou HS, McMasters KM. Adenovirus-mediated gene transfer of FKHRL1 triple mutant efficiently induces apoptosis in melanoma cells. *Cancer Biol Ther* 5, 875-83 (2006).

58. Wederell, E.D. Bilenky M, Cullum R, Thiessen N, Dagpinar M, Delaney A, Varhol R, Zhao Y, Zeng T, Bernier B, Ingham M, Hirst M, Robertson G, Marra MA, Jones S, Hoodless PA. Global analysis of in vivo Foxa2-binding sites in mouse adult liver using massively parallel sequencing. *Nucleic Acids Res* 36, 4549-64 (2008).

59. Momand, J., Jung, D., Wilczynski, S. & Niland, J. The MDM2 gene amplification database. Nucleic Acids Res 26, 3453-9 (1998).

60. Iwakuma, T. & Lozano, G. MDM2, an introduction. Mol Cancer Res 1, 993-1000 (2003).

61. Dei Tos, A. P. Doglioni C, Piccinin S, Sciot R, Furlanetto A, Boiocchi M, Dal Cin P, Maestro R, Fletcher CD, Tallini G. Coordinated expression and amplification of the MDM2, CDK4, and HMGI-C genes in atypical lipomatous tumours. J Pathol 190, 531-6 (2000).

62. Morgan, R. J., Newcomb, P. V., Hardwick, R. H. & Alderson, D. Amplification of cyclin D1 and MDM-2 in oesophageal carcinoma. Eur J Surg Oncol 25, 364-7 (1999).

63. Korkolopoulou, P. Christodoulou P, Kapralos P, Exarchakos M, Bisbiroula A, Hadjiyannakis M, Georgountzos C, Thomas-Tsagli E. The role of p53, MDM2 and c-erb B-2 oncoproteins, epidermal growth factor receptor and proliferation markers in the prognosis of urinary bladder cancer. Pathol Res Pract 193, 767-75 (1997).

64. Ikeguchi, M. Ueda T, Fukuda K, Yamaguchi K, Tsujitani S, Kaibara N. Expression of the murine double minute gene 2 oncoprotein in esophageal

squamous cell carcinoma as a novel marker for lack of response to chemoradiotreatment. Am J Clin Oncol 25, 454-9 (2002).

65. Arora, S., Mathew, R., Mathur, M., Chattopadhayay, T. K. & Ralhan, R. Alterations in MDM2 expression in esophageal squamous cell carcinoma: relationship with p53 status. Pathol Oncol Res 7, 203-8 (2001).

66. Bardeesy, N. Falkoff D, Petruzzi MJ, Nowak N, Zabel B, Adam M, Aguiar MC, Grundy P, Shows T, Pelletier J. Anaplastic Wilms' tumour, a subtype displaying poor prognosis, harbours p53 gene mutations. Nat Genet 7, 91-7 (1994).

67. Fontana, X. Ferrari P, Abbes M, Monticelli J, Namer M, Bussière F. Study of mdm2 gene amplification in primary breast tumors Bull Cancer 81, 587-92 (1994).

68. McCann, A. H. Kirley A, Carney DN, Corbally N, Magee HM, Keating G, Dervan PA. Amplification of the MDM2 gene in human breast cancer and its association with MDM2 and p53 protein status. Br J Cancer 71, 981-5 (1995).

69. Koga, T. Hashimoto S, Sugio K, Yoshino I, Nakagawa K, Yonemitsu Y, Sugimachi K, Sueishi K. Heterogeneous distribution of P53 immunoreactivity in human lung adenocarcinoma correlates with MDM2 protein expression, rather than with P53 gene mutation. Int J Cancer 95, 232-9 (2001).

70. Saito, H. Tsujitani S, Oka S, Ikeguchi M, Maeta M, Kaibara N. The expression of murine double minute 2 is a favorable prognostic marker in esophageal squamous cell carcinoma without p53 protein accumulation. Ann Surg Oncol 9, 450-6 (2002).

71. Lukas, J. Gao DQ, Keshmeshian M, Wen WH, Tsao-Wei D, Rosenberg S,

Press MF. Alternative and aberrant messenger RNA splicing of the mdm2 oncogene in invasive breast cancer. Cancer Res 61, 3212-9 (2001).

72. Osman, I. Drobnjak M, Fazzari M, Ferrara J, Scher HI, Cordon-Cardo C.Inactivation of the p53 pathway in prostate cancer: impact on tumor progression. Clin Cancer Res 5, 2082-8 (1999).

73. Kubbutat, M. H., Jones, S. N. & Vousden, K. H. Regulation of p53 stability by Mdm2. Nature 387, 299-303 (1997).

74. Haupt, Y., Maya, R., Kazaz, A. & Oren, M. Mdm2 promotes the rapid degradation of p53. Nature 387, 296-9 (1997).

75. Vassilev, L. T. Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z, Kong N, Kammlott U, Lukacs C, Klein C, Fotouhi N, Liu EA. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science 303, 844-8 (2004).

76. Swaroop, M. & Sun, Y. Mdm2 ligase dead mutants did not act in a dominant negative manner to re-activate p53, but promoted tumor cell growth. Anticancer Res 23, 3167-74 (2003).

77. Vargas, D. A., Takahashi, S. & Ronai, Z. Mdm2: A regulator of cell growth and death. Adv Cancer Res 89, 1-34 (2003).

78. Moore, L. Venkatachalam S, Vogel H, Watt JC, Wu CL, Steinman H, Jones SN, Donehower LA. Cooperativity of p19ARF, Mdm2, and p53 in murine tumorigenesis. Oncogene 22, 7831-7 (2003).

79. Steinman, H. A. Burstein E, Lengner C, Gosselin J, Pihan G, Duckett CS, Jones SN. An alternative splice form of Mdm2 induces p53-independent cell growth

and tumorigenesis. J Biol Chem 279, 4877-86 (2004).

80. Fridman, J. S. Hernando E, Hemann MT, de Stanchina E, Cordon-Cardo C, Lowe SW. Tumor promotion by Mdm2 splice variants unable to bind p53. Cancer Res 63, 5703-6 (2003).

81. Ladanyi, M. Cha C, Lewis R, Jhanwar SC, Huvos AG, Healey JH. MDM2 gene amplification in metastatic osteosarcoma. Cancer Res 53, 16-8 (1993).

82. Datta, M. W., Macri, E., Signoretti, S., Renshaw, A. A. & Loda, M. Transition from in situ to invasive testicular germ cell neoplasia is associated with the loss of p21 and gain of mdm-2 expression. Mod Pathol 14, 437-42 (2001).

83. Greer, E. L. & Brunet, A. FOXO transcription factors at the interface between longevity and tumor suppression. Oncogene 24, 7410-25 (2005).

84. Finnberg, N. & El-Deiry, W. S. Activating FOXO3a, NF-kappaB and p53 by targeting IKKs: an effective multi-faceted targeting of the tumor-cell phenotype? Cancer Biol Ther 3, 614-6 (2004).

85. Burgering, B. M. & Kops, G. J. Cell cycle and death control: long live Forkheads. Trends Biochem Sci 27, 352-60 (2002).

86. Tran, H., Brunet, A., Griffith, E. C. & Greenberg, M. E. The many forks in FOXO's road. Sci STKE 2003, RE5 (2003).

87. Dijkers, P. F. Medema RH, Pals C, Banerji L, Thomas NS, Lam EW, Burgering BM, Raaijmakers JA, Lammers JW, Koenderman L, Coffer PJ. Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1). Mol Cell Biol 20, 9138-48 (2000).

88. Schmidt, M. Fernandez de Mattos S, van der Horst A, Klompmaker R, Kops GJ, Lam EW, Burgering BM, Medema RH. Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D. Mol Cell Biol 22, 7842-52 (2002).

89. Yang, J. Y., Xia, W. & Hu, M. C. Ionizing radiation activates expression of FOXO3a, Fas ligand, and Bim, and induces cell apoptosis. Int J Oncol 29, 643-8 (2006).

90. Hu, M. C. Lee DF, Xia W, Golfman LS, Ou-Yang F, Yang JY, Zou Y, Bao S, Hanada N, Saso H, Kobayashi R, Hung MC. IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. Cell 117, 225-37 (2004).

91. Hu, M. C. & Hung, M. C. Role of IkappaB kinase in tumorigenesis. Future Oncol 1, 67-78 (2005).

92. Potente, M. Urbich C, Sasaki K, Hofmann WK, Heeschen C, Aicher A, Kollipara R, DePinho RA, Zeiher AM, Dimmeler S. Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization. J Clin Invest 115, 2382-92 (2005).

93. Birchmeier, W. & Behrens, J. Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. Biochim Biophys Acta 1198, 11-26 (1994).

94. Takeichi, M. Cadherins in cancer: implications for invasion and metastasis. Curr Opin Cell Biol 5, 806-11 (1993).

95. Christofori, G. & Semb, H. The role of the cell-adhesion molecule E-cadherin

as a tumour-suppressor gene. Trends Biochem Sci 24, 73-6 (1999).

96. Thiery, J. P. & Chopin, D. Epithelial cell plasticity in development and tumor progression. Cancer Metastasis Rev 18, 31-42 (1999).

97. Chan, J. K. & Wong, C. S. Loss of E-cadherin is the fundamental defect in diffuse-type gastric carcinoma and infiltrating lobular carcinoma of the breast. Adv Anat Pathol 8, 165-72 (2001).

98. Jiang, W. G. & Mansel, R. E. E-cadherin complex and its abnormalities in human breast cancer. Surg Oncol 9, 151-71 (2000).

99. Berx, G. Cleton-Jansen AM, Nollet F, de Leeuw WJ, van de Vijver M, Cornelisse C, van Roy F. E-cadherin is a tumour/invasion suppressor gene mutated in human lobular breast cancers. Embo J 14, 6107-15 (1995).

100.Bolos, V. Peinado H, Pérez-Moreno MA, Fraga MF, Esteller M, Cano A. The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors. J Cell Sci 116, 499-511 (2003).

102. Cheng, C. W. Wu PE, Yu JC, Huang CS, Yue CT, Wu CW, Shen CY. Mechanisms of inactivation of E-cadherin in breast carcinoma: modification of the two-hit hypothesis of tumor suppressor gene. Oncogene 20, 3814-23 (2001).

103. Leptin, M. twist and snail as positive and negative regulators during Drosophila mesoderm development. Genes Dev 5, 1568-76 (1991).

104. Nieto, M. A. The snail superfamily of zinc-finger transcription factors. Nat Rev Mol Cell Biol 3, 155-66 (2002).

105. Bond, G. L., Hu, W. & Levine, A. J. MDM2 is a central node in the p53 pathway: 12 years and counting. Curr Cancer Drug Targets 5, 3-8 (2005).

106. Zhou, B. P. Liao Y, Xia W, Zou Y, Spohn B, Hung MC. HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. Nat Cell Biol 3, 973-82 (2001).

107. Uchida, C. Miwa S, Kitagawa K, Hattori T, Isobe T, Otani S, Oda T, Sugimura H, Kamijo T, Ookawa K, Yasuda H, Kitagawa M. Enhanced Mdm2 activity inhibits pRB function via ubiquitin-dependent degradation. Embo J 24, 160-9 (2005).

108. Yang, J. Y. Zong CS, Xia W, Wei Y, Ali-Seyed M, Li Z, Broglio K, Berry DA, Hung MC. MDM2 Promotes Cell Motility and Invasiveness by Regulating E-Cadherin Degradation. Mol Cell Biol 26, 7269-82 (2006).

109. Ries, S. Biederer C, Woods D, Shifman O, Shirasawa S, Sasazuki T, McMahon M, Oren M, McCormick F. Opposing effects of Ras on p53: transcriptional activation of mdm2 and induction of p19ARF. Cell 103, 321-30 (2000).

110. Derksen, P. W. Liu X, Saridin F, van der Gulden H, Zevenhoven J, Evers B, van Beijnum JR, Griffioen AW, Vink J, Krimpenfort P, Peterse JL, Cardiff RD, Berns A, Jonkers J. Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. Cancer Cell 10, 437-49 (2006).

111. Kastan MB. Wild-type p53: tumors can’t stand it. Cell;128:837-40. (2007)

53. Paik J.H. Kollipara R, Chu G, Ji H, Xiao Y, Ding Z, Miao L, Tothova Z, Horner

JW, Carrasco DR, Jiang S, Gilliland DG, Chin L, Wong WH, Castrillon DH, DePinho RA.FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell. Jan 26; 128(2):309-23. (2007)

112. Vassilev LT. MDM2 inhibitors for cancer therapy. TrendsMol Med; 13:23-31. (2007)

113. Lau LM, Nugent JK, Zhao X, Irwin MS. HDM2 antagonist Nutlin-3 disrupts p73 binding and enhances p73 function. Oncogene;27:997-1003. (2008)

114. Ambrosini G, Sambol EB, Carvajal D, Vassilev LT, Singer S, Schwartz GK. Mouse double minute antagonist Nutlin-3a enhances chemotherapy-induced apoptosis in cancer cells with mutant p53 by activating E2F1. Oncogene;26:3473-81. (2007)

115 Balkwill, F. & Mantovani, A. Inflammation and cancer: back to Virchow? *Lancet* 357, 539-45 (2001).

116 Danese, S. & Mantovani, A. Inflammatory bowel disease and intestinal cancer: a paradigm of the Yin-Yang interplay between inflammation and cancer. *Oncogene* 29, 3313-23.(2006)

117. Shih Ie, M. & Wang, T.L. Notch signaling, gamma-secretase inhibitors, and cancer therapy. *Cancer Res* 67, 1879-82 (2007).

118. Yaguchi, S. Fukui Y, Koshimizu I, Yoshimi H, Matsuno T, Gouda H, Hirono S, Yamazaki K, Yamori T. Antitumor activity of ZSTK474, a new phosphatidylinositol 3-kinase inhibitor. *J Natl Cancer Inst* 98, 545-56 (2006).

119. LaRusch GA, JacksonMW, DunbarJD,Warren RS, Donner DB, Mayo LD.

Nutlin3 blocks vascular endothelial growth factor induction by preventing the interaction between hypoxia inducible factor 1a and Hdm2. Cancer Res;67:450-4. (2007)

120. Colaluca IN,Tosoni D, Nuciforo P, et al. NUMB controls p53 tumour suppressor activity. Nature; 451:76-80. (2008)

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