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College of Life Science
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IKK 調控組蛋白甲基轉移酶 G9a 促進癌症發展之探討

IKK Regulates Histone Methyltransferase G9a to Promote Cancer Progression

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Promote Cancer Progression

本論文係李啟恆君（R01B46019）在國立臺灣大學生化科學研究所完成之碩士學位論文，於民國 103 年 7 月 16 日承下列考試委員審查通過及口試及格，特此證明。

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（簽名）

（召集人）

（指導教授）

誌謝

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啟恆 2014 夏

中文摘要

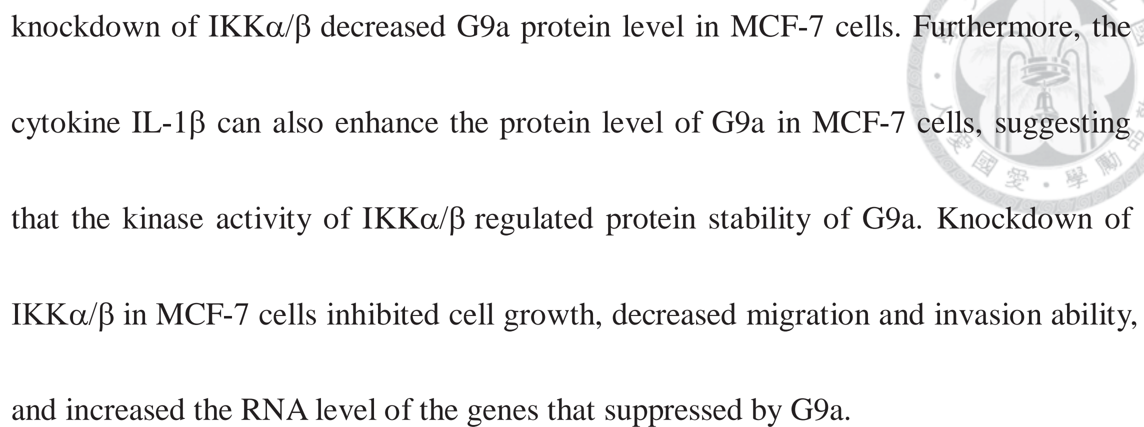
G9a 是哺乳動物的組蛋白甲基轉移酶，在不同癌症中常常會高度表現，使得抑癌基因靜默而無法轉錄，調控腫瘤發展的進程。由於轉譯後修飾對於調控蛋白的功能十分重要，而目前沒有文獻探討 G9a 的轉譯後修飾，因此我們利用生物資訊的軟體預測 G9a 可能的磷酸化位置，在其中發現可能具有 IKKα/β的磷酸化位置。

在本篇的研究中，我們在 293T 細胞和乳癌細胞 MCF-7、BT549 中利用免疫沉澱方法以及 GST pull down assay 證明了 G9a 可以在 in vivo 及 in vitro 的情況下跟 IKKα/β結合，在 *in vitro* kinase assay 的實驗中證明了 IKKα/β 確實可以磷酸化 G9a。為了進一步探討 IKKα/β對於 G9a 的調控，我們在 293T cell 過度表現 IKKα/β 後，發現 G9a 的蛋白質表現量會有明顯上升而 RNA 的量沒有太大改變，推斷 IKKα/β可能影響到 G9a 轉譯或者是蛋白質的穩定度。因此在 293T 細胞中加入 cycloheximide，可以發現過度表現 IKKα/β的細胞可以減緩 G9a 的降解速度，而在MCF-7 細胞中把IKKα/β剔除則會使 G9a 的表現量下降，另外當加入細胞激素 IL-1β後也可以看到 G9a 的蛋白質表現量有一定的上升，代表 IKKα/β的活性調控 G9a 的穩定度。在乳癌細胞 MCF-7 中剔除IKKα/β也會造成細胞的生長、轉移和侵襲的能力下降，而由 G9a 所抑制的基因則是有所上升。由於 G9a 在癌症發生過程中扮演重要的調控角色，本篇研究發現 IKKα/β可以藉由磷酸化 G9a 來調控它的功能來促進癌症發生，未來或許在 G9a 表現比較高的病人中，可以同時針對 IKK 跟 G9a 進行抑制，提高治療效果。

Abstract

G9a, also known as EHMT2, is a histone H3 lysine 9 methyltransferase, has been observed overexpression in various human cancers and contributes to the epigenetic silencing of tumor suppressor genes to promote cancer progression. Post-translational modification (PTM) is very important to regulate protein function. It has been shown that several epigenetic regulators were regulated by different PTMs. The post translational modification that regulates function of G9a had not been reported yet, therefore it is necessary to investigate the PTM of G9a. Interestingly, from the bioinformatics prediction, we found that G9a contains IKKα/β consensus phosphorylation site, suggesting a possible interaction between IKKα/β and G9a.

In this study, we used the co-immunoprecipitation in 293T cells, MCF-7 and BT549 breast cancer cells and GST pull down assay to prove that G9a interacted with IKKα and β *in vivo* and *in vitro*. By using *in vitro* kinase assay, we proved that G9a can be phosphorylated by both IKKα and β. To investigate the regulation of G9a by IKKα/β, we overexpressed IKKα/β in 293T cell, and found the protein expression level of G9a was increased but not the RNA level. It suggested that IKKα/β may regulate the translation activity or protein stability of G9a. For this reason, we added cycloheximide in 293T cells to examine the half-life of G9a. The cells with overexpression of IKKα/β can prolong the half-life of G9a, on the contrary,



knockdown of IKKα/β decreased G9a protein level in MCF-7 cells. Furthermore, the cytokine IL-1β can also enhance the protein level of G9a in MCF-7 cells, suggesting that the kinase activity of IKKα/β regulated protein stability of G9a. Knockdown of IKKα/β in MCF-7 cells inhibited cell growth, decreased migration and invasion ability, and increased the RNA level of the genes that suppressed by G9a.

G9a had played an important role in cancer progression, this study reveals that IKKα/β can phosphorylate G9a to promote cancer progression. Targeting IKK and G9a in the cancer patients with G9a overexpression may potentially enhance the therapeutic efficacy in the future.

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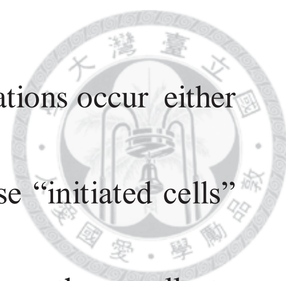
Chapter 1.

Introduction

1.1 Inflammation and Cancer Progression

Inflammation is the immune response to injury caused by microbial pathogen infection, wounding, chemical irritation or cell damage. Further, inflammation can be divided into acute inflammation and chronic inflammation. Acute inflammation attracts and activates different leukocytes, lymphocytes, neutrophils, and other inflammatory cell to infiltrate disrupted and damaged tissue by a great number of proinflammatory cytokines and chemokines to contribute tissue breakdown (1). To resolve acute inflammation, it requires a rapid clearance of inflammatory cells by inducing apoptosis and phagocytosis with neighboring macrophages, dendritic cells, and phagocytes (2). The phagocytosis of apoptotic cells also enhances the production of transforming growth factor-β to promote an anti-inflammatory response (3). However, if inflammation resolution is dysregulated, cellular response will change to the pattern of chronic inflammation. In chronic inflammation, inflammatory cells generate a great amount of growth factors, cytokines, and reactive oxygen and nitrogen species that may cause DNA damage and mutation (1). The sustained cell proliferation is induced by continued tissue damage with these microenvironments, and then chronic inflammation tends to neoplasia (4).

Carcinogenesis is a multi-step process with sequential stages of initiation, promotion, and progression. Carcinogen-triggered irreversible genetic alteration



accumulates in various cell types during normal life. These mutations occur either spontaneously or due to undetectable exposure to carcinogens. These "initiated cells" accumulate during life, and inflammation acts as promoter to cause these cells to accumulate more mutations through anti-apoptosis and ROS, promote proliferation and mechanisms and Darwinian-selection pressure (5). The inflammation microenvironment also drives initiated cells to malignant tumors. It may release angiogenic factors, such as vascular endothelial growth factor (VEGF), endothelin-2 to facilitate tumor growth (6, 7). It also can degrade the extracellular matrix and the basement membrane by releasing matrix metalloproteinases, to facilitate the invasion and migration of tumor cells (8). Moreover, Tumor associated macrophages release epidermal growth factor and other epidermal growth factor receptor family ligands to promote tumor cell proliferation and migration (6).

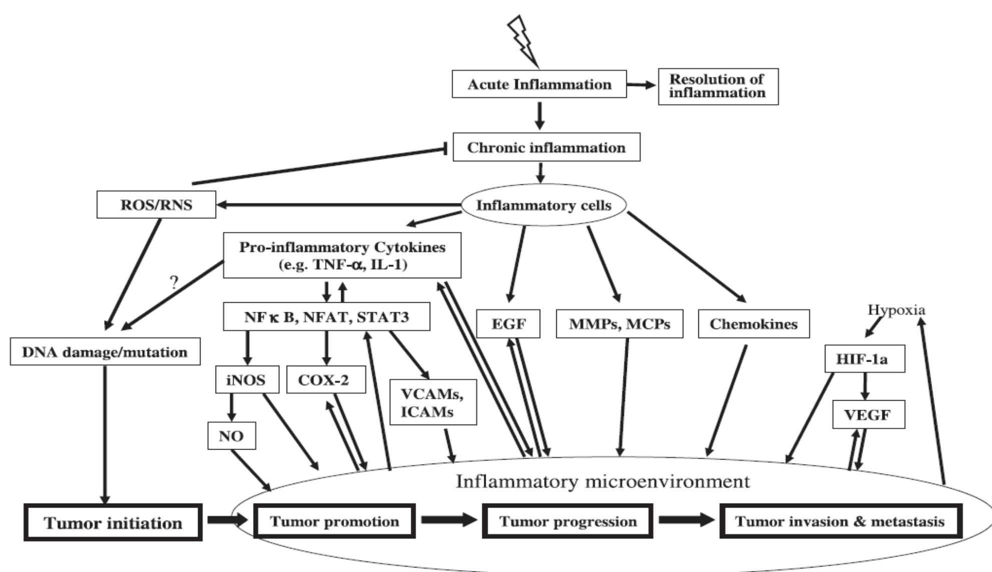
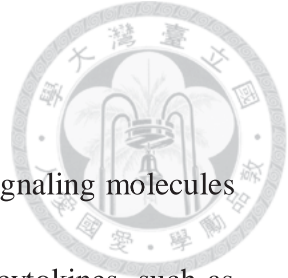


Figure I-1. Summary of mechanisms for inflammation in cancer development (9).



1.2 NF-κB Pathway and The Function of IKK

Nuclear factor-κB (NF-κB) is one of the major inflammation signaling molecules activated when cells are exposed to a variety of stimuli, including cytokines, such as tumor necrosis factor (TNF) and interleukin 1 (IL-1), ultraviolet (UV) radiation, stress, and pathogenic infection (10). In the canonical pathway, NF-κB dimers are bound to inhibitor of NF-κB (IκB) proteins, which sequester NF-κB complexes in the cytoplasm Upon activation, IκB is phosphorylated by the IκB kinase (IKK) complex, composed of three major components: IKKα, IKKβ, and NEMO (IKKγ). Phosphorylation of IκB leads to its degradation, and subsequently, nuclear transport of NF-κB proteins initiates the downstream transcription of anti-apoptotic genes that provide cell survival and cytokines that regulate the immune response, such as TNFα, IL-1, IL-6 and IL-8 (11). In acute inflammatory processes, where full activation of NF-κB is accompanied by a high activity of cytotoxic immune cells against cancer cells (12). However, NF-κB is constitutively activated in many types of cancer and can exert a variety of pro-tumorigenic functions.

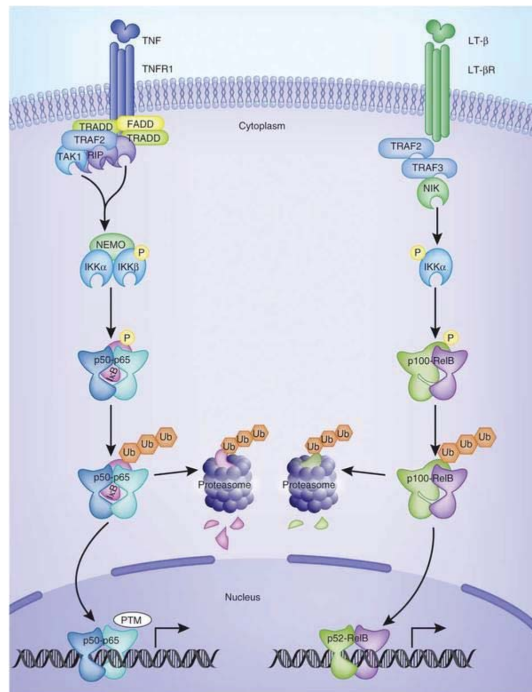
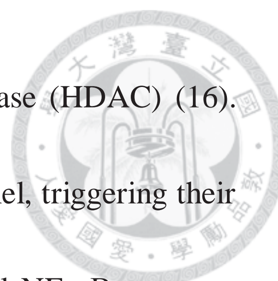


Figure I-2. Canonical and noncanonical pathways of NF-κB activation (13).

After stimulation of IL-1 or TNF, TAK1 complex were activated and phosphorylated S176 and S180 on IKKα, and S177 and S181 on IKKβ leading a conformation change in the activation loop to activate IKK complex (14). Also hypoxia stress activates IKKβ through decreased PHD-dependent hydroxylation repression of IKKβ (15). Then IKK phosphorylated IκB to promote the NF-κB dependent pathway. Different from their roles in the NF-κB pathways, both IKKα and IKKβ have been shown to phosphorylate many other substrates that are involved in immune functions, tumor suppression, cell proliferation, and chromatin remodeling. Because of its nuclear-localization signal, IKKα can target both cytosolic and nuclear proteins. In the nucleus, IKKα modulates gene expression through histone H3



modification and regulation of the recruitment of histone deacetylase (HDAC) (16). IKKα has also been suggested to directly phosphorylate p65 and c-Rel, triggering their turnover and removal from the promoter to terminate the canonical NF-κB response and limit inflammation (17). In addition, IKKα can phosphorylate β-catenin leading to its stabilization and induction of TCF-dependent expression of cyclin D1 (18, 19). In addition to IκB, IKKβ also phosphorylates many substrates. IKKβ disrupts TSC1/TSC2 complex function by phosphorylation of TSC1 and consequent mTOR activation promotes tumor progression and inflammation-mediated angiogenesis (20). Other pro-proliferative or antiapoptotic targets include the tumor suppressor Foxo3a, as well as p105 and Dok1, through which IKKβ affects MAPK activation (21, 22).

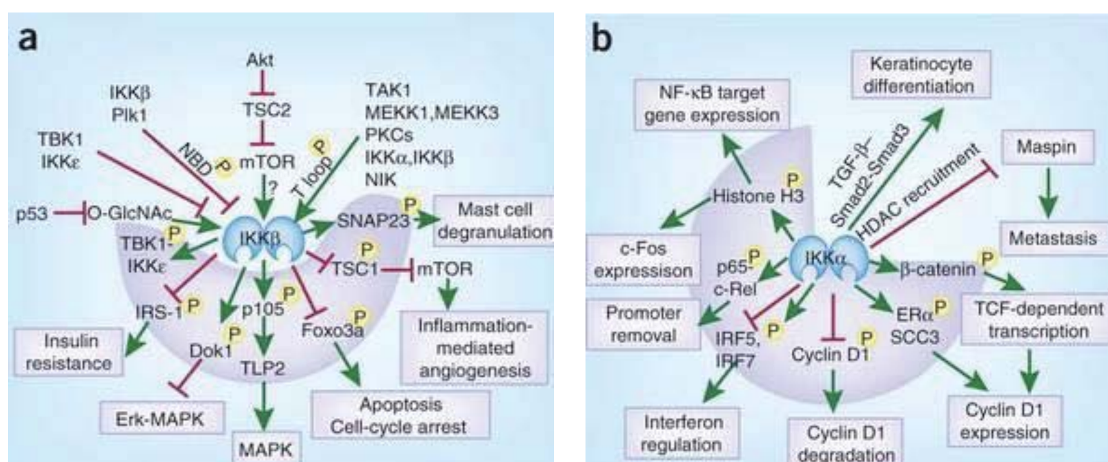
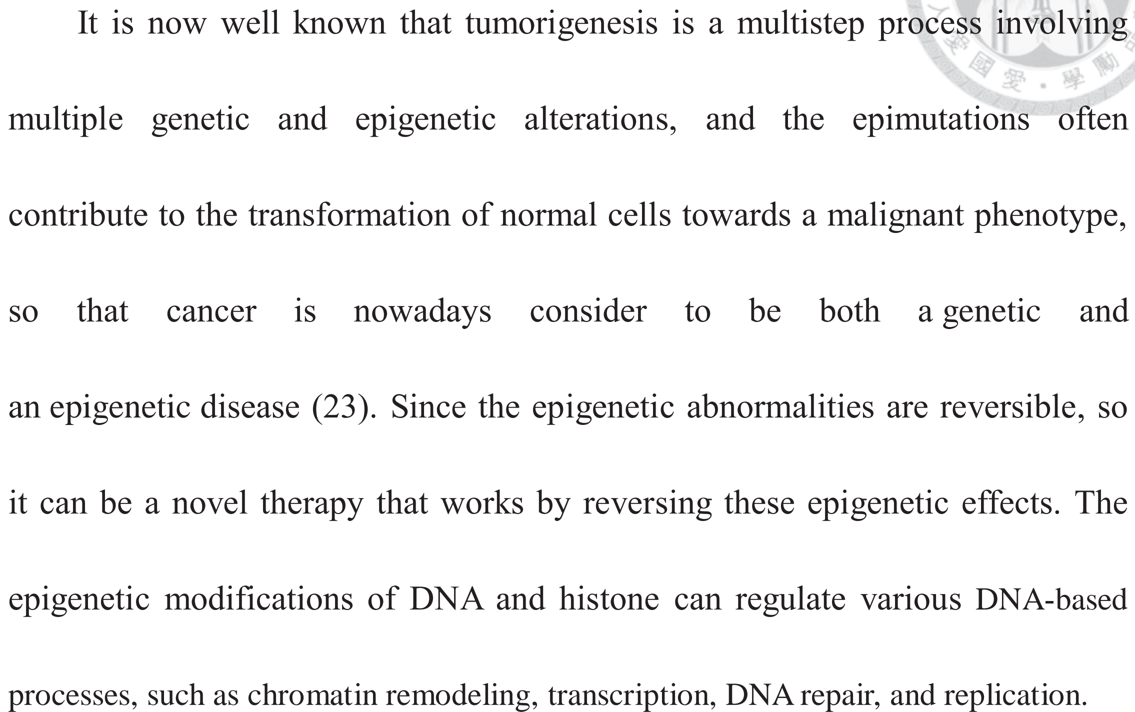


Figure I-3. NF-κB-independent functions of IKK complex subunits (13).

1.3 Histone Methyltransferase in Epigenetic and Cancer Progression



It is now well known that tumorigenesis is a multistep process involving multiple genetic and epigenetic alterations, and the epimutations often contribute to the transformation of normal cells towards a malignant phenotype, so that cancer is nowadays consider to be both a genetic and an epigenetic disease (23). Since the epigenetic abnormalities are reversible, so it can be a novel therapy that works by reversing these epigenetic effects. The epigenetic modifications of DNA and histone can regulate various DNA-based processes, such as chromatin remodeling, transcription, DNA repair, and replication.

Histone methylation different form acetylation and phosphorylation, does not change the overall charge of the molecule. Lysine may be mono-, di-, or tri-methylated, and arginine residues may be symmetrically or asymmetrically methylated by different histone methyltransferases (HMTs) (24). The different levels of lysine methylation may be associated with either transcription activation or repression. H3K4me3 promotes transcription, whereas H3K27me3, H3K9me3 are associated with gene silencing (25). Dysregulation of HMTs has been associated with cancer progression. For example, H3K4 methyltransferase MLL is a mutated in acute leukemias due to translocations and it has been linked with leukaemic transformation (26). Overexpression of H3K9 methyltransferase G9a is linked to cell proliferation,

metastasis and invasion, carcinogenesis in prostate cancer, gastric cancer, lung cancer and breast cancer (27-30).

1.4 G9a Function in Cancer Progression

G9a belongs to the SET domain containing Su(var) 3–9 family of proteins. G9a is histone methyltransferase that catalyze the methylation of Histone 3 at lysine 9, resulting in mono-, di-methylation of K9 (H3K9me1 and H3K9me2) and a less extent to H3K27 (31). G9a contains an evolutionarily conserved SET domain which recognizes histone tails and modify specific lysine residue, and a domain containing ankyrin repeats which is involved in protein-protein interactions (31, 32). In human and mouse cells the G9a and NG36 gene are expressed within a single NG36/G9a transcript (33). Two isoforms of G9a, respectively are 140 and 160 kDa, have been identified. Long form G9a protein is a product of both NG36 and G9a transcripts. In addition, a short isoform has been characterized which lacks exon 10. Functional characterization of these two isoform has shown the distinct subcellular distribution. While short form was observed in both the cytoplasm and nucleus, long form G9a was extensively concentrate within the nucleus (33). A closely related paralog GLP/EHMT15 has been identified which exhibits 45% identity with human G9a and differs primarily in the N-terminus and endogenouslythey function exclusively as a heteromeric complex with G9a (34).

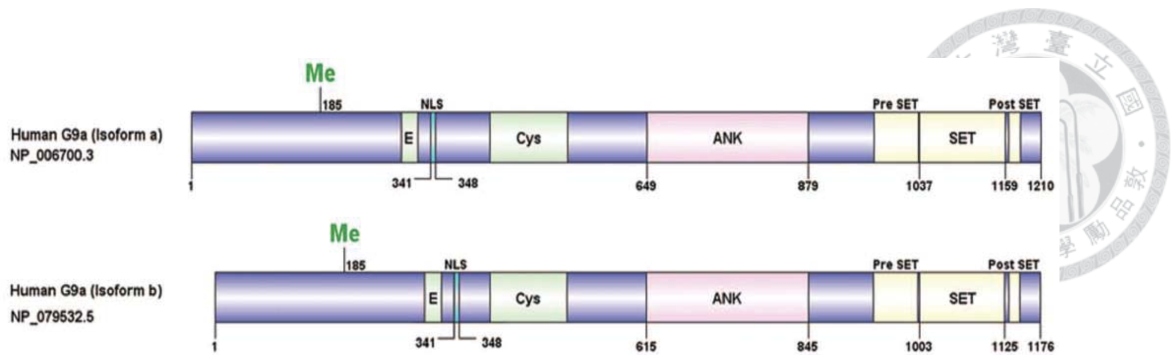
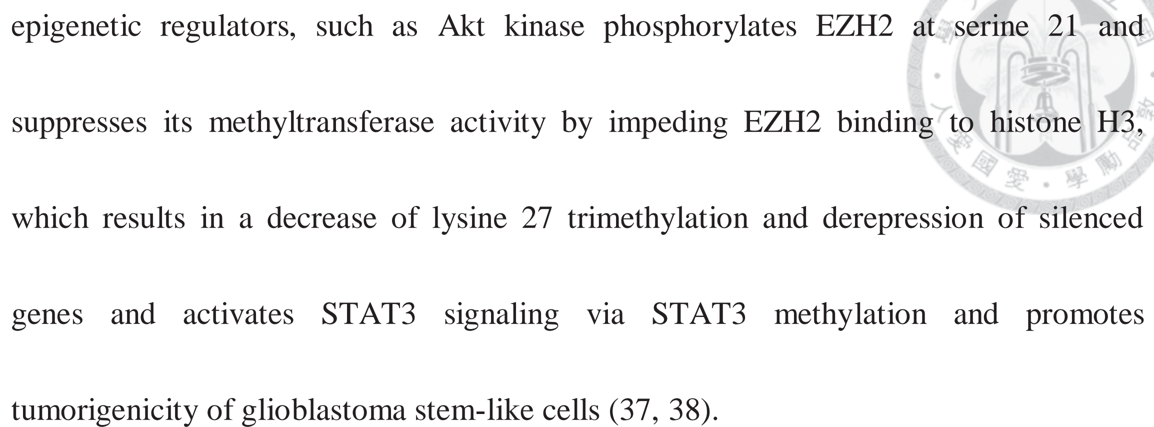


Figure I-4 Schematic representation of G9a domain structure (35).

Studies on its role in carcinogenesis, where downregulation of G9a causes chromosomal instability, inhibition of cell growth, increased cellular senescence and cell death triggered by autophagy in cancer cells (27, 36). In Runx3-expressing gastric cell lines, hypoxia increases the accumulation of G9a and H3K9me2 marks which leads to Runx3 gene silencing. Deletion of the SET domain in G9a results in a failure to repress Runx3 under both normoxic and hypoxic conditions (28). In aggressive lung cancer, high levels of G9a correlate with poor prognosis with increased cell migration and invasion in vitro and metastasis in vivo. The metastatic function of G9a in lung cancer is due to repression of Ep-CAM. (29). Similar studies in CLBC have revealed the role of G9a in promoting EMT by repression of E-cadherin expression. G9a interacts with Snail mediated the repression of E-cadherin. HP1 and DNMTs are recruited to the E-cadherin promoter resulting in DNA methylation and H3K9me2 (30). .

Because of posttranslational modification is important in modulating functions of



epigenetic regulators, such as Akt kinase phosphorylates EZH2 at serine 21 and suppresses its methyltransferase activity by impeding EZH2 binding to histone H3, which results in a decrease of lysine 27 trimethylation and derepression of silenced genes and activates STAT3 signaling via STAT3 methylation and promotes tumorigenicity of glioblastoma stem-like cells (37, 38).

In this study, we found out that IKKα/β can interact and phosphorylate G9a to increase its protein stability and regulate its functions in cancer progression. To clarify the mechanism and regulation of G9a function may help us to enhance the cancer therapeutic efficacy in the future.

Chapter 2.
Materials and Methods

Cell Culture

Human embryonic kidney 293T cells and Breast cancer cells MCF-7 were grown in DMEM medium, BT549 was grown in RPMI-1640 medium. All media (Gibco/Invitrogen, Carlsbad, California, USA) were supplemented with 10% fetal bovine serum (Biological Industries, Ltd. Israel) and 2 mmol/L L-glutamine, 100 μg/ml streptomycin, and 100 untis/ml penicillin (Gibco/Invitrogen, Carlsbad, California, USA). Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 ℃ and were passaged with trypsin/EDTA (Sigma-Aldrich, St Louis, MO, USA) twice to thrice each week and grown to 80% to 90% confluency before any treatment.

Antibodies and Reagents

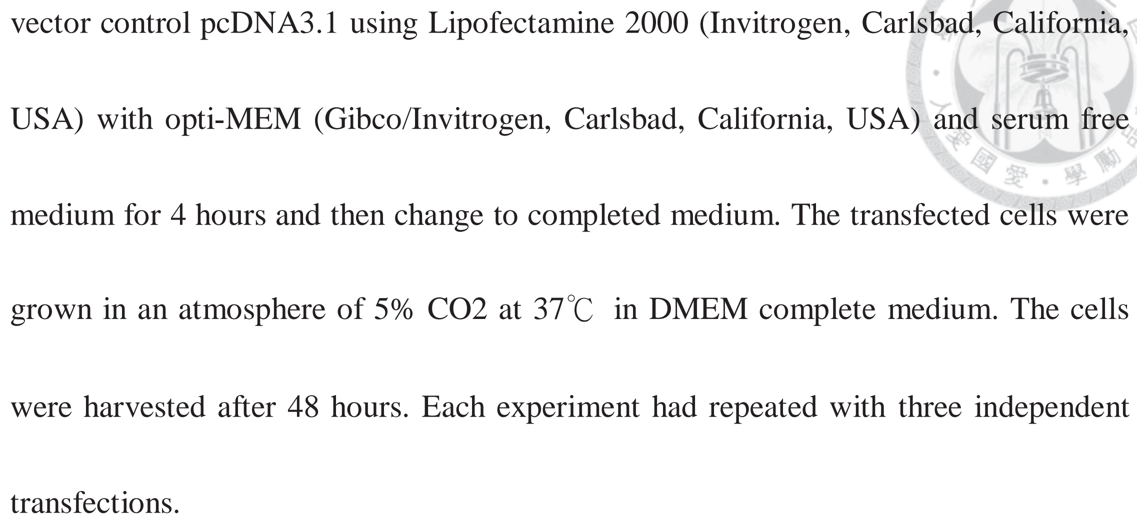
The primary antibodies used in this study were anti-Flag, anti-α-tubulin (Sigma-Aldrich, St Louis, MO, USA), anti-G9a, anti-GLP, anti-pIKKα/β (S176/S177), anti-IKKα, anti-IKKβ, anti-pIκBα (S32/S36), anti-IκB, anti-H3K9me2 (Cell Signaling Technology, Beverly, MA, USA), anti-HA (GeneTex Inc, San Antonio, Texas, USA). Anti-G9a antibody (Abcam, Cambridge, MA, USA) was using in Immunoprecipitation was purchased. IKK inhibitors: IKK16 were purchased from Selleckchem (Houston, Texas, USA) and recombinant cytokine protein: IL-1β were purchased from PeproTech (Rocky Hill, NJ, USA).

Western Blot Analysis

Cells were washed with PBS twice and lysed in RIPA buffer (50 mM Tris-HCl, pH7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 5 mM Na_3VO_4, 100 mM NaF) supplemented with proteinase inhibitor cocktail (G-Bioscience, St. Louis, MO, USA) and stored with occasional vortex for 30 minutes on ice. The lysates were centrifuged at 13,000 rpm for 20 minutes at 4℃, and then collected the supernatant. Protein concentrations were determined by BCA protein assay kit (Thermo Scientific Pierce, Rockford, IL, USA). For each sample, 30 μg of total protein was separated on 8% or 12% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk in PBST for 1 hour and then incubate with primary antibody overnight. After washing with PBST 10 minutes thrice, the membranes were incubated with HRP-conjugated secondary Antibody for 1 hour. After washing thrice again, the immunoreactive protein bands were detected by chemiluminescent detection (ECL, Perkin-Elmer, Wellesley, MA, USA).

Transient Transfection and Lentivirus Infection

293T cells were transiently transfected with Flag-G9a (Full length), HA-IKKα, HA-IKKβ, Flag-IKKβ CA (constitutively active), Flag-IKKβ KD (kinase dead) and



vector control pcDNA3.1 using Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA) with opti-MEM (Gibco/Invitrogen, Carlsbad, California, USA) and serum free medium for 4 hours and then change to completed medium. The transfected cells were grown in an atmosphere of 5% CO2 at 37℃ in DMEM complete medium. The cells were harvested after 48 hours. Each experiment had repeated with three independent transfections.

The G9a, IKKα, IKKβ shRNAs and shRNA control lentivector (pLKO_TRC005) were purchased from National RNAi core Facility in Academic Sinica (Taipei, Taiwan).

The target sequences of

G9a shRNA is 5'-GCTCCAGGAATTTAACAAGA-3',

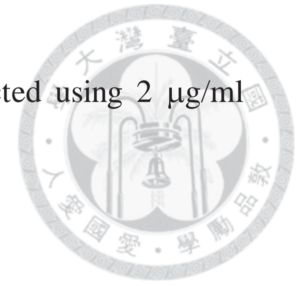
IKKα shRNA #1 is 5'-GCAGATGACGTATGGGATATC-3',

IKKα shRNA #2 is 5'- TAGGGTCTGGGATTCGATATT-3'

IKKβ shRNA #1 is 5'-CATGAATGCCTCTCGACTTAG-3'

IKKβ shRNA #2 is 5'- GCTGGTTCATATCTTGAACAT-3'

Lenitiviruses were produced by co-transfecting 10 μg shRNA-expressing vector, 10 μg pCMVΔR8.91 and 1 μg pMD.G constructs into 1×10^6 293T cells by calcium phosphate. After 16 hours incubation, replace the medium to fresh complete medium. After 48 hours, viral supernatant were harvested and were filtered with 0.45 μm filter



and used to infect cells with 8 μg/ml polybrene. Cells were selected using 2 μg/ml puromycin for 2 days.

***In vitro* Cell Proliferation Assay**

MCF-7 cells infected shRNA-control vector, IKKα and IKKβ shRNA and G9a shRNA were plated to 24-well dish initially, containainging 1×10^4 cells per well. The growth rate of knockdown cells was determined by MTT assay. The amount of MTT formazam product was analyzed at a wavelength of 570 nm.

***In vitro* Colony Formation Assay**

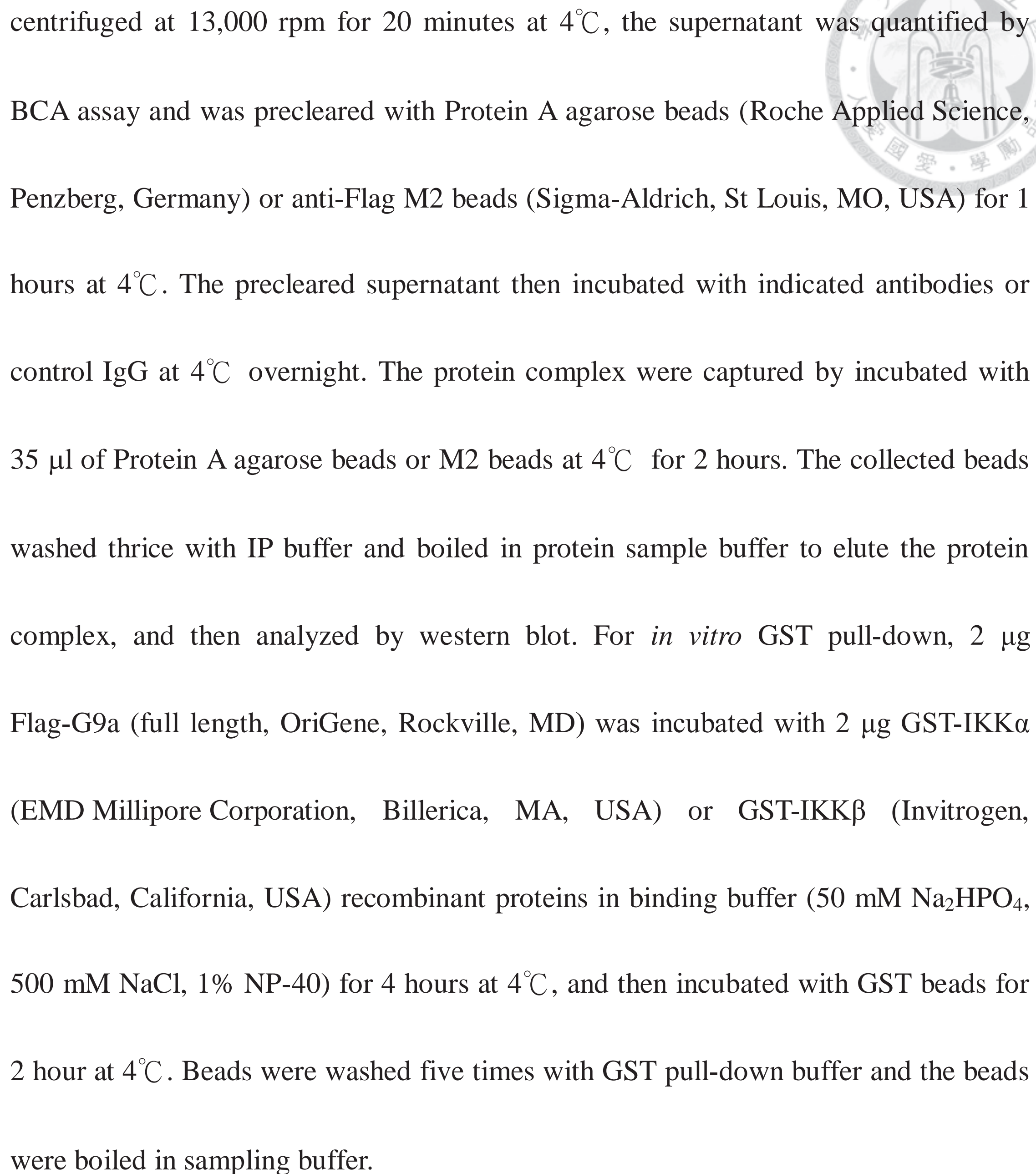
MCF-7 cells infected shRNA-control vector, IKKα and IKKβ shRNA and G9a shRNA were plated to 6-well dish initially, containainging 1×10^2 cells per well. Incubate the cells in a CO_2 incubator at 37°C for 1-3 weeks until cells in control plates have formed colonies with substantially good size. Then cells were fixed in methanol for 10 minutes and then stained with 0.05% crystal violet overnight. Wash excess crystal violet with PBS and allow dishes to dry. The colonies were counted under the light microscope

In vitro Invasion and Migration Assay

Invasion assays were determined using the modified Boyden chambers with filter inserts for 24 well plates. (8 mm pore size, Millipore, Billerica, MA, USA). Matrigel (35 μg, BD Biosciences, San Jose, CA) coated filters were used for invasion assay. For MCF-7 cell, 2×10^4 cells in 100 μL of DMEM serum free medium were seeded into the upper chamber and allowed to invade into the lower chamber with 1 mL completed medium for 24 hours. After 24 hours, cells were fixed in methanol for 10 minutes and then stained with 0.05% crystal violet overnight. The chambers were then washed by PBS once and cells in the upper chamber were carefully removed using cotton-tipped swabs. Quantification was performed by counting the stained cells. The cells on the underside of the filters were counted under the light microscope. The number of cells that migrated was normalized to the growth rate for each cell. Each experiment has repeated thrice. Boyden chambers with filter insets only were used for migration assay. 1×10^4 MCF-7 cells were fixed after 24 hours and followed the same procedure with invasion assay.

Co-Immunoprecipitation and GST Pull Down Assay

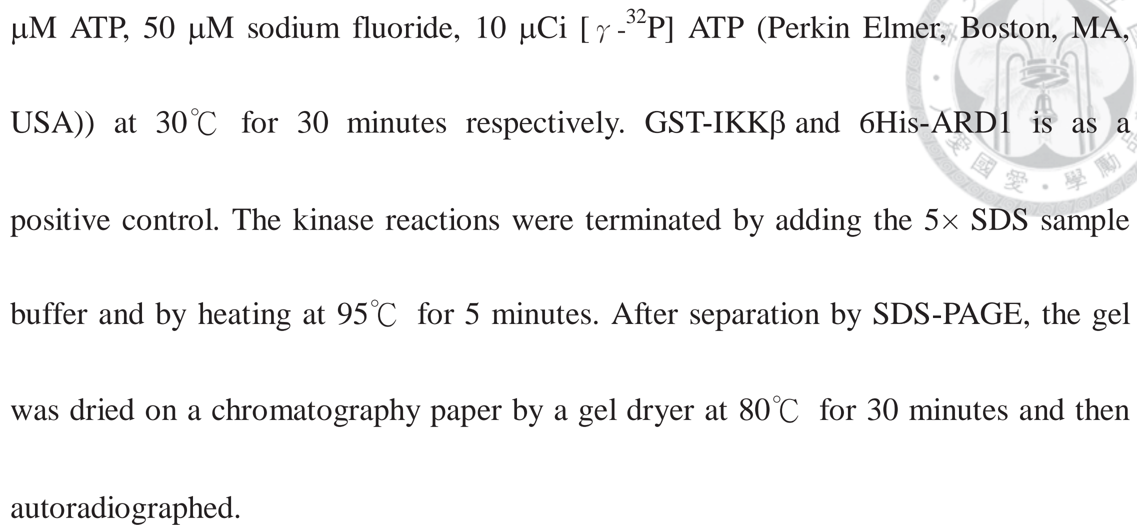
Cells were lysed by sonication in IP lysis buffer (20 mM Tris, pH 8.0, 150 mM, 1 mM EDTA, 0.5% NP-40) supplemented with protease inhibitor cocktail. Cell lysates were



centrifuged at 13,000 rpm for 20 minutes at 4℃, the supernatant was quantified by BCA assay and was precleared with Protein A agarose beads (Roche Applied Science, Penzberg, Germany) or anti-Flag M2 beads (Sigma-Aldrich, St Louis, MO, USA) for 1 hours at 4℃. The precleared supernatant then incubated with indicated antibodies or control IgG at 4℃ overnight. The protein complex were captured by incubated with 35 μl of Protein A agarose beads or M2 beads at 4℃ for 2 hours. The collected beads washed thrice with IP buffer and boiled in protein sample buffer to elute the protein complex, and then analyzed by western blot. For *in vitro* GST pull-down, 2 μg Flag-G9a (full length, OriGene, Rockville, MD) was incubated with 2 μg GST-IKKα (EMD Millipore Corporation, Billerica, MA, USA) or GST-IKKβ (Invitrogen, Carlsbad, California, USA) recombinant proteins in binding buffer (50 mM Na_2HPO_4, 500 mM NaCl, 1% NP-40) for 4 hours at 4℃, and then incubated with GST beads for 2 hour at 4℃. Beads were washed five times with GST pull-down buffer and the beads were boiled in sampling buffer.

***In vitro* Kinase Assay**

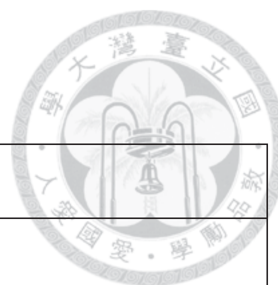
To assay the phosphorylation of G9a, 2 μg Flag-G9a recombinant protein was incubated with 500 ng GST-IKKα, GST-IKKβ, GST in a final volume of 30 μl consisting of kinase buffer (Tris-HCl 25 mM (pH 7.6), 10 mM $MgCl_2$, 1 mM DTT, 40



μM ATP, 50 μM sodium fluoride, 10 μCi [γ-^{32}P] ATP (Perkin Elmer, Boston, MA, USA)) at 30℃ for 30 minutes respectively. GST-IKKβ and 6His-ARD1 is as a positive control. The kinase reactions were terminated by adding the 5× SDS sample buffer and by heating at 95℃ for 5 minutes. After separation by SDS-PAGE, the gel was dried on a chromatography paper by a gel dryer at 80℃ for 30 minutes and then autoradiographed.

RNA Isolation and Reverse Transcription Polymerase Chain Reaction.

Total RNAs from cells were isolated using TRizol reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instruction. 5 μg total RNA of each sample was reverse transcribed into cDNA using the SuperScript® III first strand synthesis kit (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instruction. The cDNAs were then performed the SYBR Green PCR amplification using KAPA SYBR® FAST qPCR kit (Kapa Biosystems, Woburn, MA, USA) with forward and reverse primers and real-time fluorescence detection were performed using the agilent technologies Mx3000p qPCR system. PCR cycling conditions were as follows, 95 °C for 5 minutes followed by 35 cycles at 95 °C for 20 seconds, 60 °C for 20 s seconds, and 72 °C for 20 s seconds.



The sequence of primer using for RT-PCR were listed below:

Gene		Sequence (5'- 3')
G9a	Forward Primer	TCCAATGACACATCTTCGCTG
	Reverse Primer	CTGATGCGGTCAATCTTGGG
b-actin	Forward Primer	CATGTACGTTGCTATCCAGGC
	Reverse Primer	CTCCTTAATGTCACGCACGAT
E-Cadherin	Forward Primer	ATTTTTCCCTCGACACCCGAT
	Reverse Primer	TCCCAGGCGTAGACCAAGA
EpCAM	Forward Primer	TGATCCTGACTGCGATGAGAG
	Reverse Primer	CTTGTCTGTTCTTCTGACCCC
RUNX3	Forward Primer	AGGCAATGACGAGAACTACTCC
	Reverse Primer	CGAAGGTCGTTGAACCTGG

Statistical Analysis.

The data are presented as the mean ± Standard deviation (S.D.) The 2-tailed Student's *t* test was used to compare data between 2 groups. All statistical tests included two-way analysis of variance. P values of less than 0.05 were considered to be statistically significant.

Chapter 3.

Results

3.1 IKKα/β Directly Interacts and Phosphorylates G9a

To explore possible posttranslational modifications in G9a by bioinformatics analysis, we used different bioinformatics prediction system (PhosphoNET, kinasephos 2.0, GPS 2.1) to predict the possible phosphorylation site. Interestingly, from the results, we predicted G9a may contain a putative IKKα/β phosphorylation motif (Fig. 1A). To further investigate whether G9a is a substrate of IKKα/β, first we examined the interaction between G9a and IKKα/β by exogenous co-immunoprecipitation (co-IP) in 293T cells. The co-immunoprecipitation results demonstrated that G9a physically associated with IKKα and IKKβ (Fig. 1B). Importantly, we also observed the interaction of G9a and IKKα/β by using endogenous co-IP in MCF-7 and BT549 breast cancer cells (Fig. 1C). In addition, GST pull-down assay further supported that GST-IKKβ can bind to G9a, but GST-IKKa had not been observed the interaction. These data indicated that G9a directly interacts with IKKβ (Fig. 1D). Given the physical interaction between IKKα or IKKβ and G9a, we examined whether G9a is a physiological substrate of IKKα or IKKβ. *In vitro* kinase assay demonstrated that GST-IKKα and GST-IKKβ can phosphorylate Flag-G9a efficiently, but not GST protein. Previous report had showed 6His-ARD1 phosphorylated by GST-IKKβ, so we use it as the positive control (39) (Fig. 1E). This result demonstrated that both IKKα and IKKβ phosphorylated G9a *in vitro*.

3.2 IKKα/β Increases the Protein Stability of G9a

According to the data of interaction of G9a and IKKα/β in 293T cells, we surprisingly found that G9a expression level was higher in 293T cells co-expressing IKKα or IKKβ than G9a alone (Fig. 2A). The mRNA level of G9a was not changed under this condition (Fig. 2B). In addition, GLP (G9a paralog protein) expression level was not changed. This observation suggested that IKKα/β may upregulate the expression level of G9a through the translation level or protein stability. To further verify this obeservation, we co-expressed the equal amount of Flag-G9a and a series amount of HA-IKKα or HA-IKKβ in 293T cells. As the results shown, G9a expression level increased progressively when exogenous IKKα/β expression level increased. (Fig. 2C). To clarify whehter IKKα/β affect the translation or stability of G9a. We treated cycloheximide to inhibit the translation process in the 293T cells with overexpression of Flag-G9a and HA-IKKα/β . The Flag-G9a expression level was decreased after 12 hours when expressed Flag-G9a only, but co-expression of HA-IKKα/β can prolong the half-life of G9a. (Fig. 2D). It suggested that IKKα/β upregulate G9a through increasing the protein stability. Next, we established IKKα/β-knockdown MCF-7 cells by using 2 different clones of shRNA. Knockdown of either IKKα or IKK can decrease the protein expression level of G9a but not GLP (Fig. 2E). In addition, the mRNA levle of G9a was not changed (Fig. 2F). This data suggested that IKKα/β are

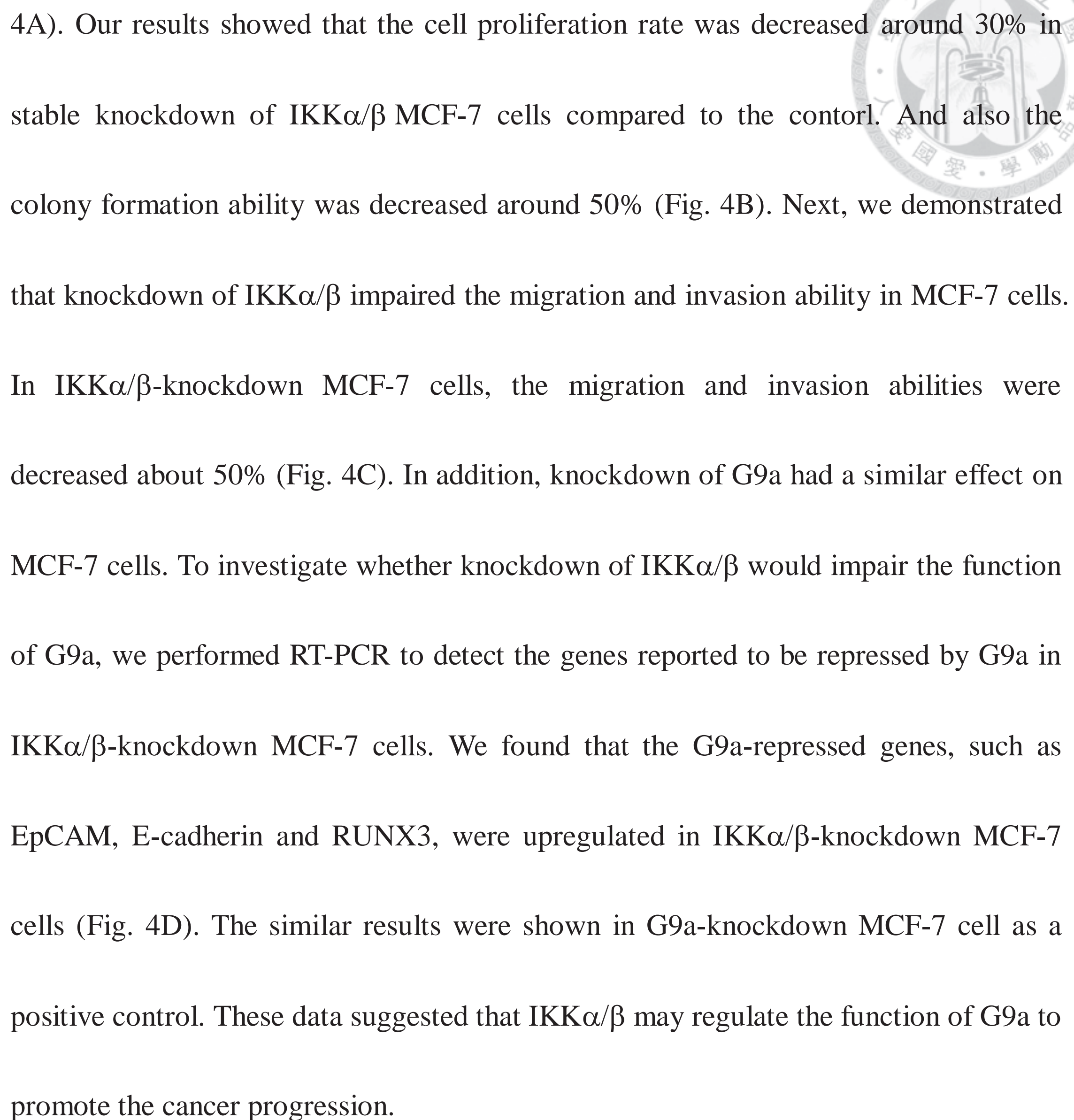
essential for maintaining the protein stability of G9a.

3.3 IKKα/β Kinase Activity Enhances the Protein Stability of G9a

When we co-expressed Flag-G9a and constitutively active Flag-IKKβ (CA), the expression level of G9a was further increased; on the contrary, when co-expressed Flag-G9a and kinase dead Flag-IKKβ (KD), the protein expression of G9a was limited (Fig. 3A). These results showed that the kinase activity of IKKα/β may further enhance the stability of G9a. To clarify whether the kinase activity is important to the stability of G9a, we use cytokine IL-1β to activate IKKα/β. In BT549 cells, we showed that G9a was accumulated by 20 ng/ml IL-1β stimulation after 10 minutes (Fig. 3B). We also showed the accumulation of G9a was increased in dose-dependent manner by IL-1β treatment in MCF-7 (Fig. 3C). Furthermore, we used IKK16 which can inhibit IKKα/β kinase activity to block the accumulation of G9a after IL-1β stimulation in BT549, MCF-7 cells. (Fig. 3D,E).

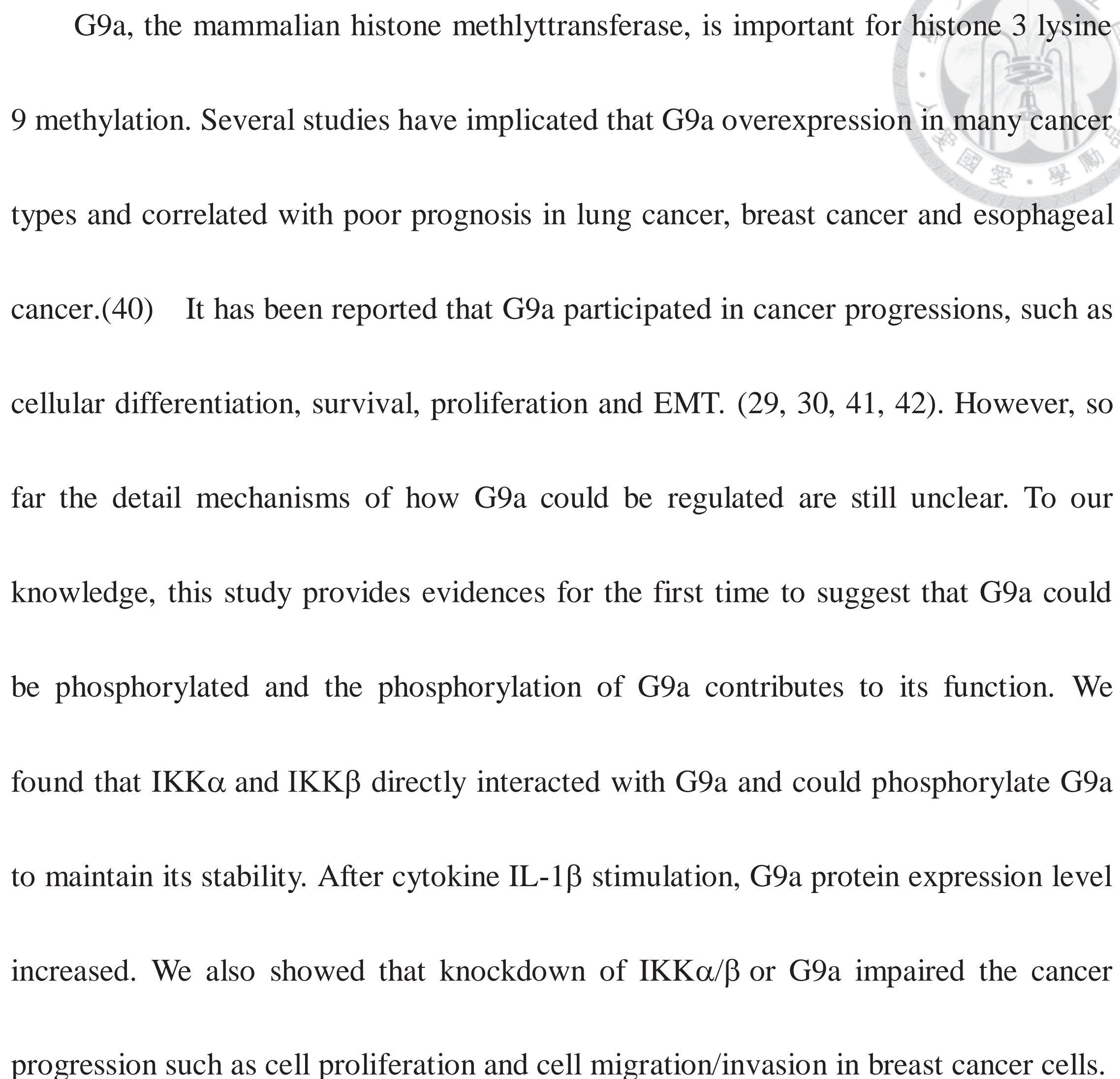
3.4 Knockdown of IKKα/β impairs G9a in Cancer Biological Functions

To determine whether IKKα/β regulated G9a to mediate cancer progression, we establish IKKα-or IKKβ-knockdown MCF-7 stable cell lines by using IKK -shRNA#1 and IKKβ-shRNA#1. We examined cell proliferation by MTT assay (Fig.



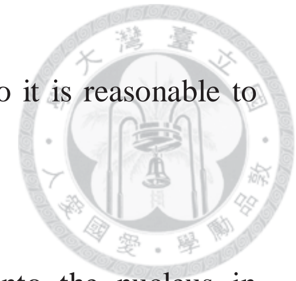
4A). Our results showed that the cell proliferation rate was decreased around 30% in stable knockdown of IKKα/β MCF-7 cells compared to the contorl. And also the colony formation ability was decreased around 50% (Fig. 4B). Next, we demonstrated that knockdown of IKKα/β impaired the migration and invasion ability in MCF-7 cells. In IKKα/β-knockdown MCF-7 cells, the migration and invasion abilities were decreased about 50% (Fig. 4C). In addition, knockdown of G9a had a similar effect on MCF-7 cells. To investigate whether knockdown of IKKα/β would impair the function of G9a, we performed RT-PCR to detect the genes reported to be repressed by G9a in IKKα/β-knockdown MCF-7 cells. We found that the G9a-repressed genes, such as EpCAM, E-cadherin and RUNX3, were upregulated in IKKα/β-knockdown MCF-7 cells (Fig. 4D). The similar results were shown in G9a-knockdown MCF-7 cell as a positive control. These data suggested that IKKα/β may regulate the function of G9a to promote the cancer progression.

Chapter 4.
Discussion



G9a, the mammalian histone methlyttransferase, is important for histone 3 lysine 9 methylation. Several studies have implicated that G9a overexpression in many cancer types and correlated with poor prognosis in lung cancer, breast cancer and esophageal cancer.(40) It has been reported that G9a participated in cancer progressions, such as cellular differentiation, survival, proliferation and EMT. (29, 30, 41, 42). However, so far the detail mechanisms of how G9a could be regulated are still unclear. To our knowledge, this study provides evidences for the first time to suggest that G9a could be phosphorylated and the phosphorylation of G9a contributes to its function. We found that IKKα and IKKβ directly interacted with G9a and could phosphorylate G9a to maintain its stability. After cytokine IL-1β stimulation, G9a protein expression level increased. We also showed that knockdown of IKKα/β or G9a impaired the cancer progression such as cell proliferation and cell migration/invasion in breast cancer cells.

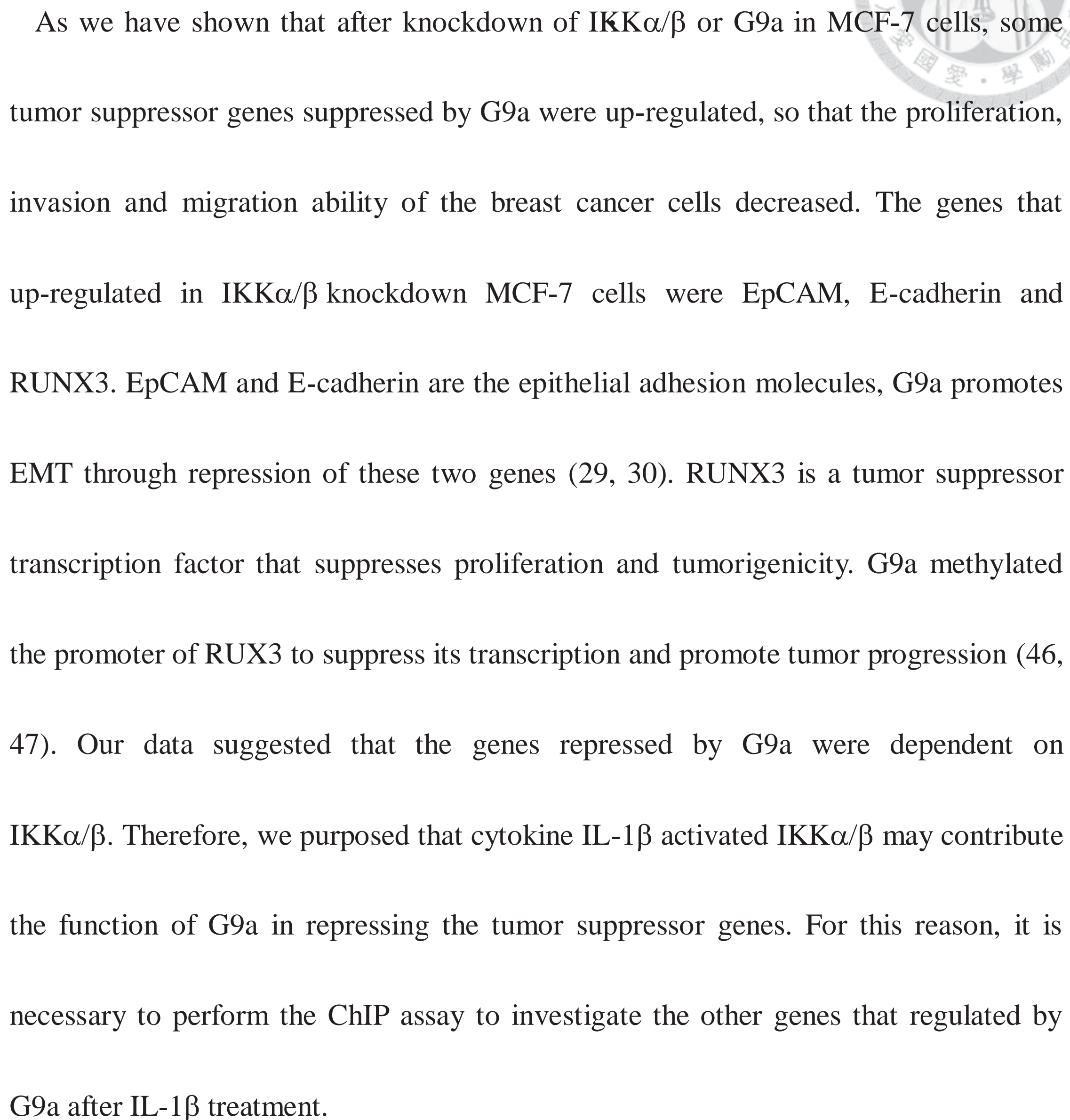
We have demonstrated IKKα and IKKβ can both interact and phosphorylate with G9a. So far the phosphorylation site of G9a has not been reported yet. Therefore, we would perform the mass spectrometry to identify the phosphorylation site. Moreover, we can construct the G9a mutant form to investigate the specific function of G9a in cancer progression. Although IKKα and IKKβ may contribute distinct effect to G9a, our co-immunoprecipitation and *in vitro* kinase assay results suggested that IKKβ might play a major role in phosphorylation of G9a. It has been reported that IKKβ is an



oncoprotein to promote different cancer progression (20-22, 43), so it is reasonable to hypothesize that IKKβ regulates G9a to promote cancer.

Previous reports have shown that IKKβ could translocate into the nucleus in response of DNA damage, but the major of IKKβ localizes in cytosol. IKKα and G9a could localize in nucleus and cytosol. Therefore, we hypothesized that IKKβ interacts G9a in cytosol and IKKα interacts G9a in cytosol or nucleus. We would perform the immunofluorescence experiments to further investigate the localization of IKKα/β and G9a.

It has been reported that hypoxia can induce the expression of G9a (28, 44). In addition, IKKβ can be activated under hypoxic stress (15). It was possible that G9a was phospohorylated by IKKβ under hypoxia and enhanced G9a protein stability. In our model, we showed that after IL-1β stimulation G9a protein expression was increased and IKK inhibitor can block the accumulation of G9a. These data suggested that the activation of IKKα/β is necessay to enhance the stability of G9a. Since DNA damage induced senescence-associated secretory phenotype cell can trigger proteasomal degradation of G9a and GLP through Cdc14B- and $p21^{Waf1/Cip1}$-dependent activation of APC/C^{Cdh1} ubiquitin ligase and decline H3K9 di-methylation on IL-6, IL8 promoter to produce IL-6, IL8 (45). IKKα/β might also play a role in preventing the ubiquitination of G9a. Furthermore, IL-1β activated IKKα/β may sequester G9a in



cytosol and decrease the H3K9 di-methylation to produce IL-6 and IL-8.

As we have shown that after knockdown of IKKα/β or G9a in MCF-7 cells, some tumor suppressor genes suppressed by G9a were up-regulated, so that the proliferation, invasion and migration ability of the breast cancer cells decreased. The genes that up-regulated in IKKα/β knockdown MCF-7 cells were EpCAM, E-cadherin and RUNX3. EpCAM and E-cadherin are the epithelial adhesion molecules, G9a promotes EMT through repression of these two genes (29, 30). RUNX3 is a tumor suppressor transcription factor that suppresses proliferation and tumorigenicity. G9a methylated the promoter of RUX3 to suppress its transcription and promote tumor progression (46, 47). Our data suggested that the genes repressed by G9a were dependent on IKKα/β. Therefore, we purposed that cytokine IL-1β activated IKKα/β may contribute the function of G9a in repressing the tumor suppressor genes. For this reason, it is necessary to perform the ChIP assay to investigate the other genes that regulated by G9a after IL-1β treatment.

Overall, we proposed that IKKα/β can interact and phosphorylate G9a to increase its protein stability and regulate its functions in cancer progression. To clarify the mechanisms and regulation of G9a function could provide alternative therapeutic strategy to benefit more cancer patients.

Chapter 5.
Figures and legends

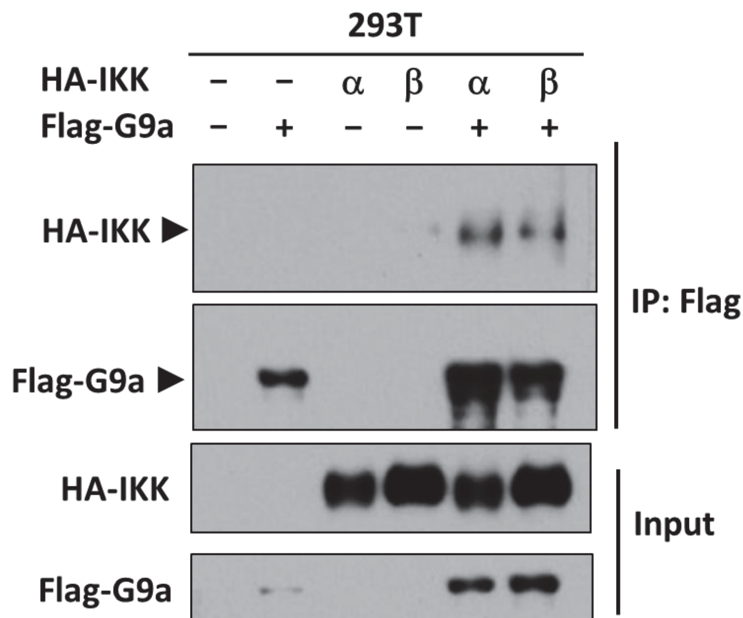
Figure 1

A

IκBα		D	R	H	D	S	G	L	D	S	M	K	K	39
IκBβ		E	W	C	D	S	G	L	G	S	L	G	P	26
NF-κB p105		R	D	S	D	S	V	C	D	T	G	V	E	930
FOXO3a		F	N	F	D	S	L	I	S	T	Q	N	V	651
TSC1		F	Y	R	D	S	L	P	G	S	Q	R	K	518
β-CATENIN		S	Y	L	D	S	G	I	H	S	G	A	T	40
G9a	779	N	A	Q	D	S	G	G	W	T	P	I	I	790
Consensus					D	S	Ψ	X	X	S/T				

Ψ, hydrophobic amino acid

B



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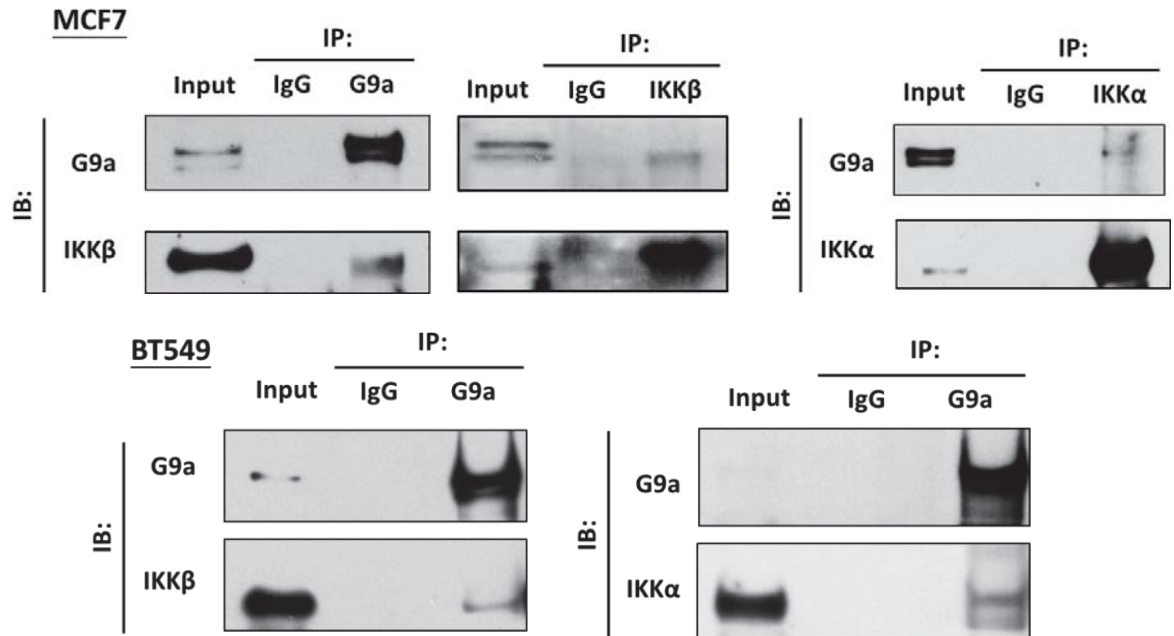

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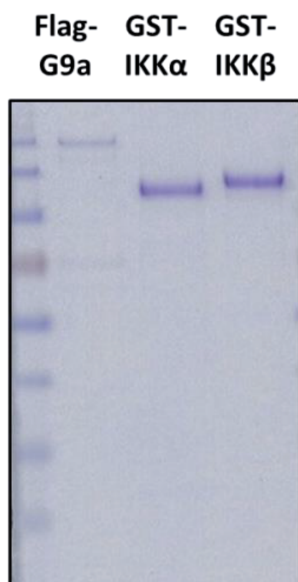

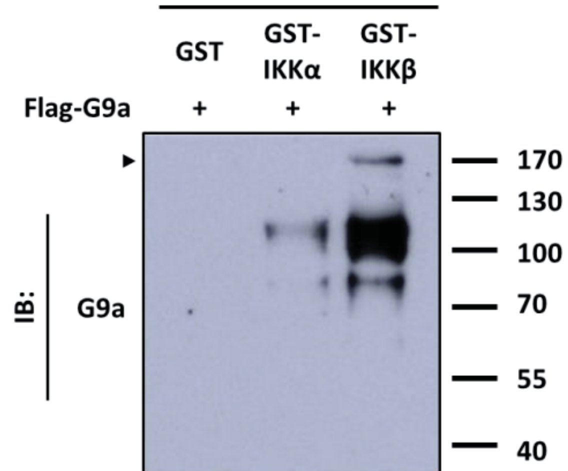

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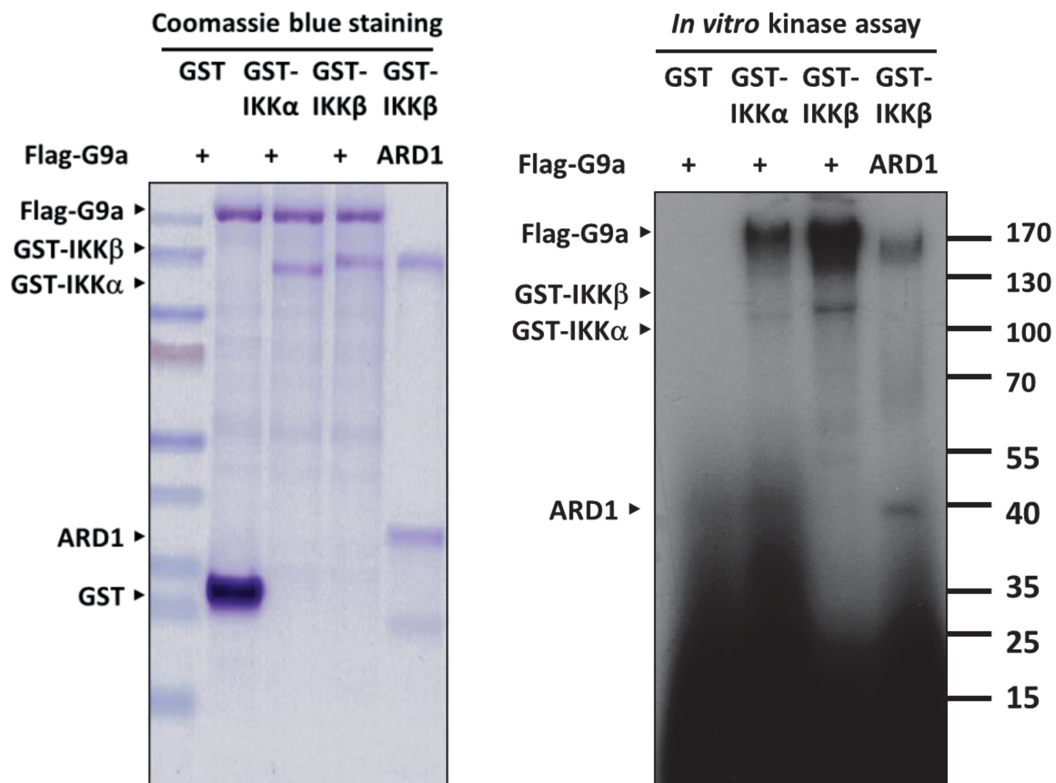
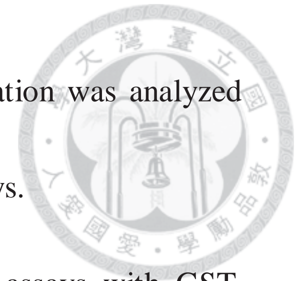


Figure. 1 IKKα and IKKβ physically interacts and phospohorlates G9a

(A) One of the putative IKKα/β phosphorylation motif (DSψXXS/T) that G9a may contain. D, aspartic acid; S, serine; T, threonine; ψ, hydrophobic amino acid; X, any amino acid.

(B) Exogenous interaction of G9a and IKKα or IKKβ. 293T cells were co-transfected with HA-IKKα/β and FLAG-G9a, and cell lysates were immunoprecipitated with anti-FLAG M2 Beads.

(C) Endogenous interaction of G9a and IKKα or IKKβ in MCF-7 and BT549 cells. Cell lysates were immunoprecipitated with specific antibodies to G9a and IKKα/β



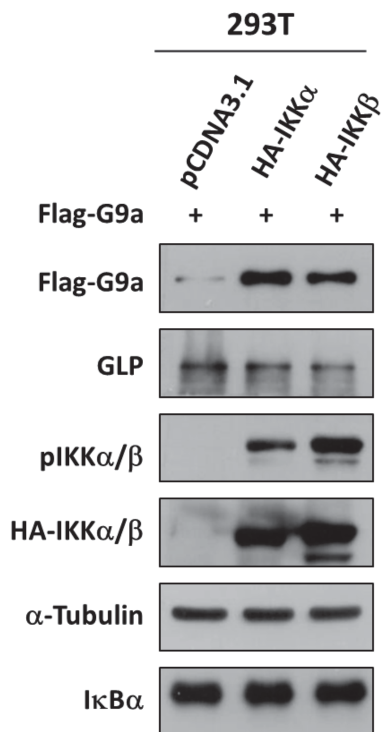
to identify the association of endogenous proteins. The association was analyzed by reciprocal co-immunoprecipitation and western blotting assays.

(D) *In vitro* binding between IKKα/β and G9a. GST pull down assays with GST, GST-IKKα, GST-IKKβ and Flag-G9a recombinant protein. Bound proteins were detected by western blotting using specific anti-G9a antibody. Recombinant proteins used in this assay were shown by Coomassie blue stain.

(E) G9a phosphorylated by IKKα/β. GST-IKKα and GST-IKKβ were used for *in vitro* kinase assay and Flag-G9a was as the substrate. GST beads and 6His-ARD1 proteins were used as negative and positive controls, respectively.

Figure 2

A



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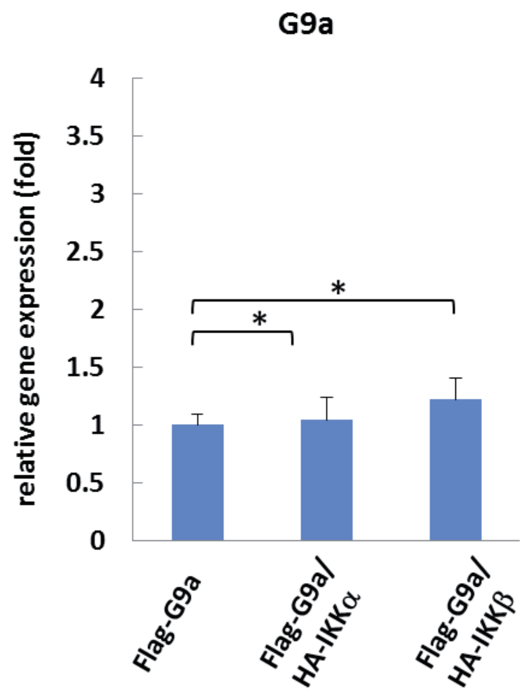

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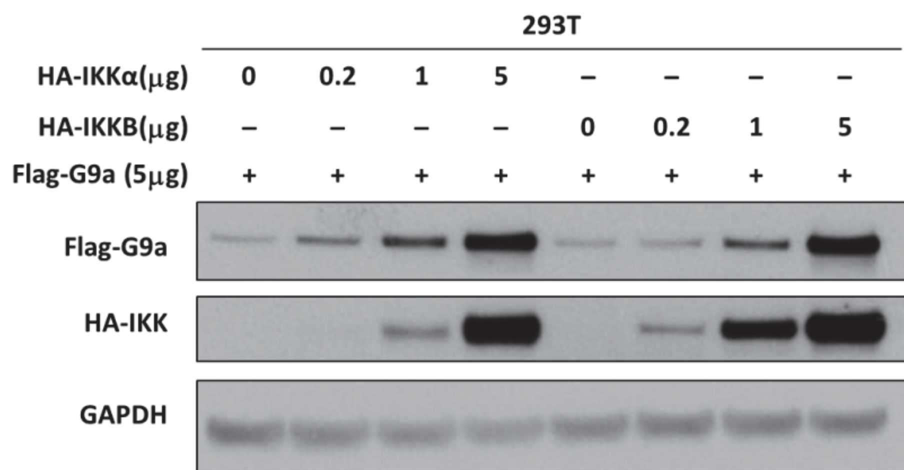

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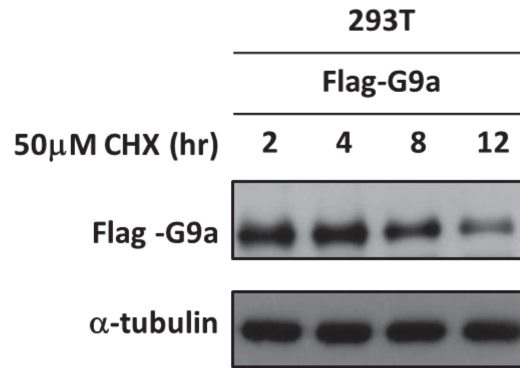

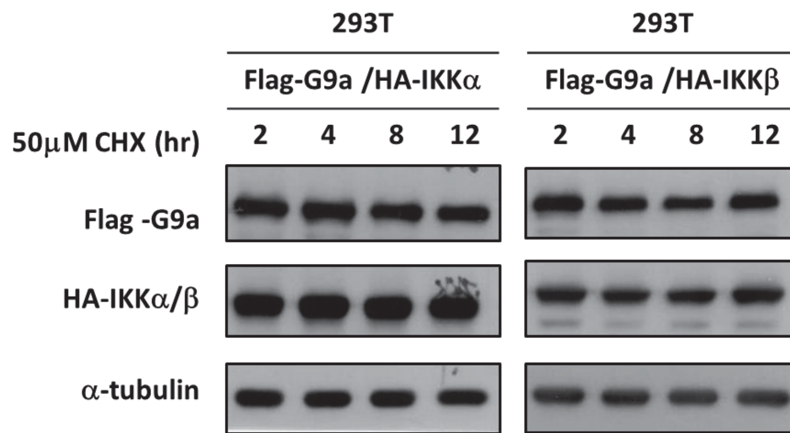

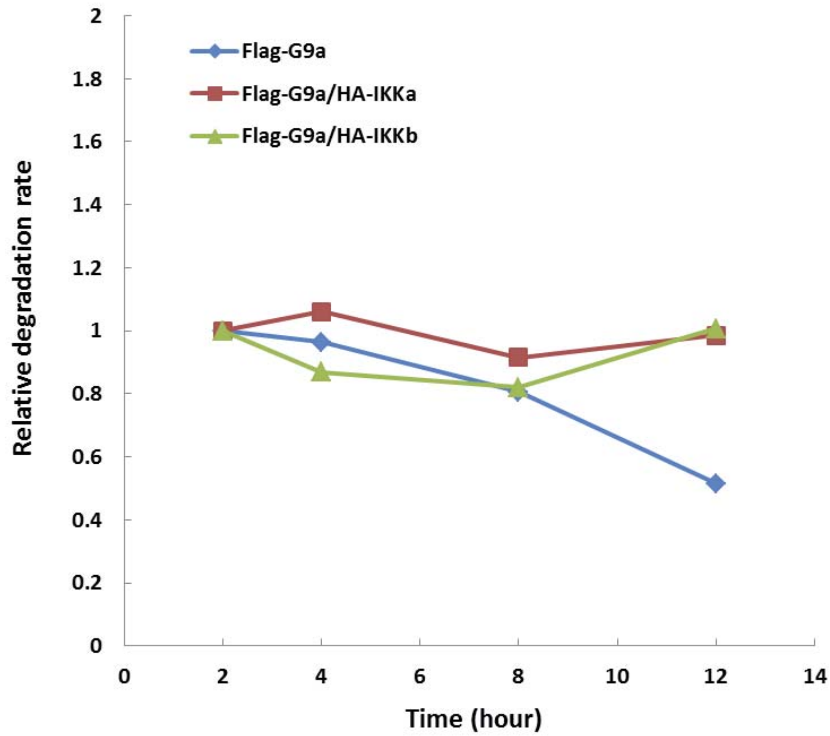

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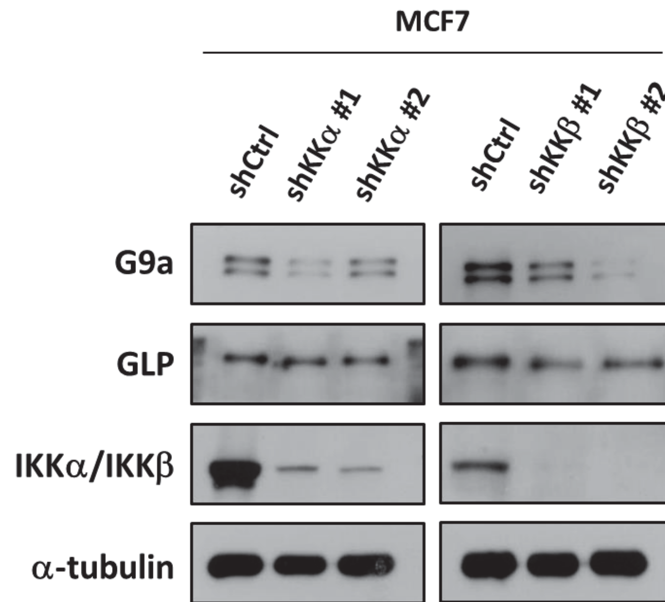

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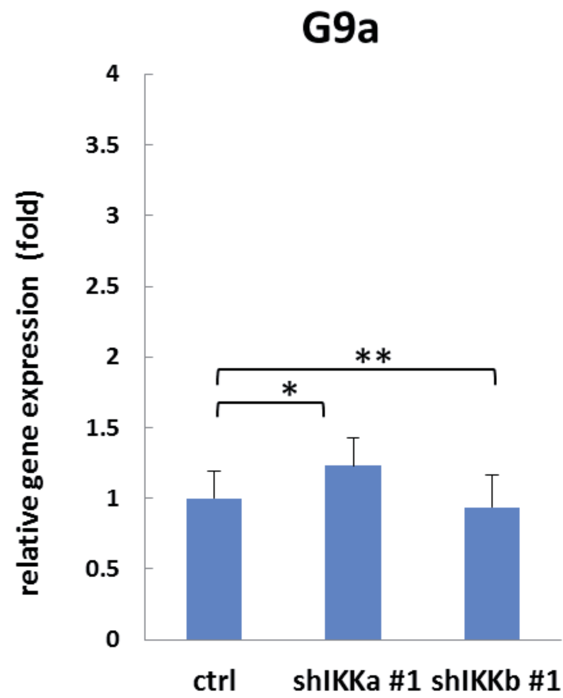


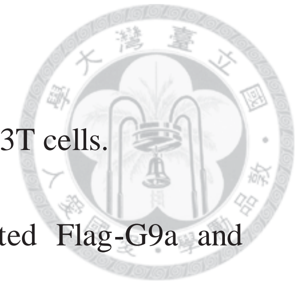


Figure 2. IKKα/β increases the protein stability of G9a

(A) Overexpression of IKKα/β increased protein level of G9a in 293T cells.

(B) The mRNA level of G9a did not change while cotransfected Flag-G9a and HA-IKKα/β.

(C) Protein expression level of G9a progressively increased with exogenous IKKα/β expression level in 293T cells.

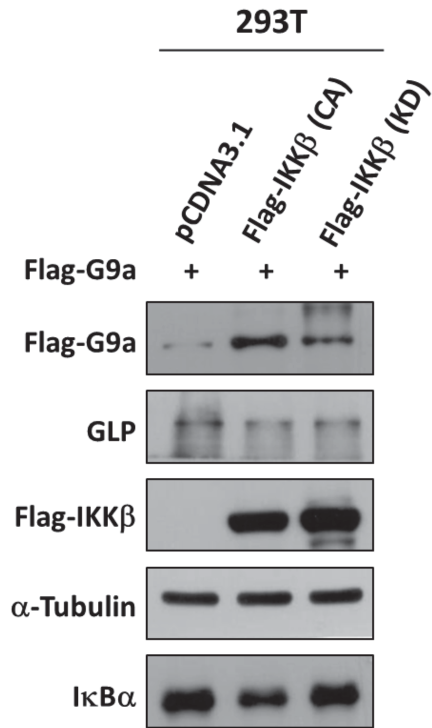
(D) Overexpression of IKKα/β enhanced protein stability of G9a. Time course analysis of G9a stability after 50μM cycloheximide (CHX) treatment in 293T cells. Flag-G9a protein levels at 0, 4, 8 and 12 hours after cycloheximide treatment were analysed by western blotting.

(E) Knockdown of IKKα/β by shRNA decreased the expression of G9a.

(F) The mRNA level of G9a did not change after knockdown of IKKα/β in MCF-7.

Figure 3.

A



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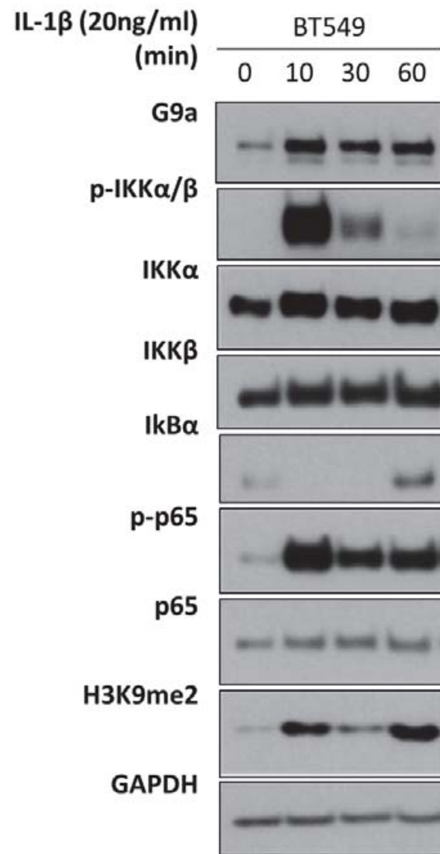

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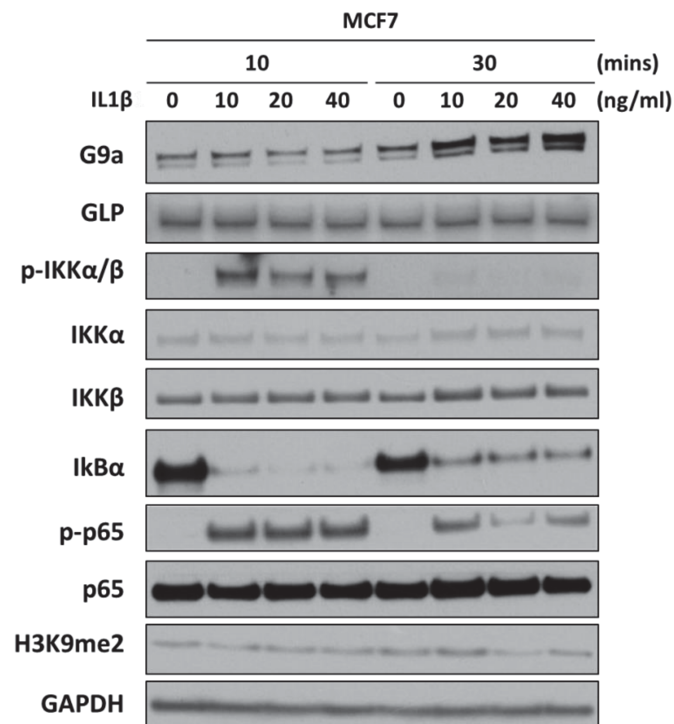

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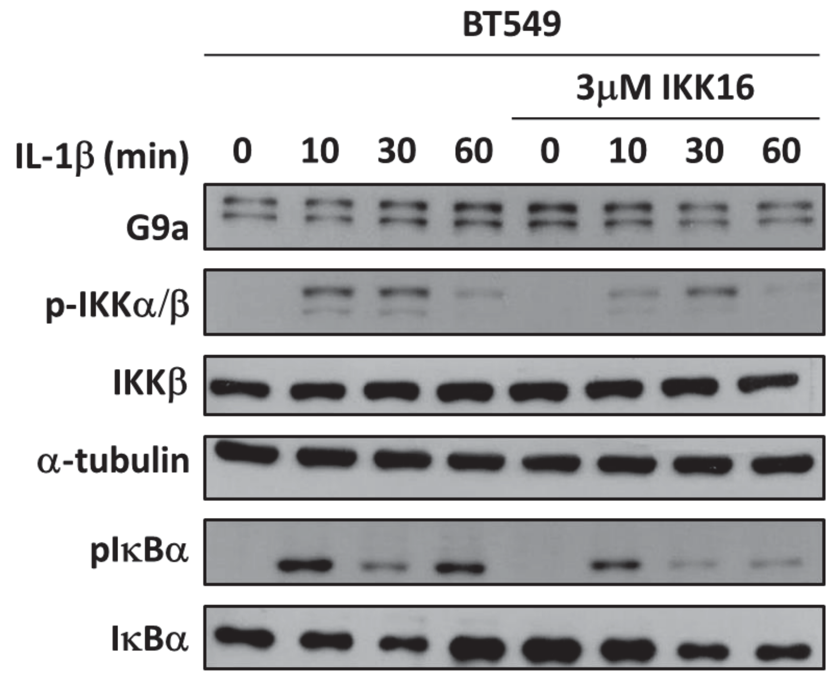

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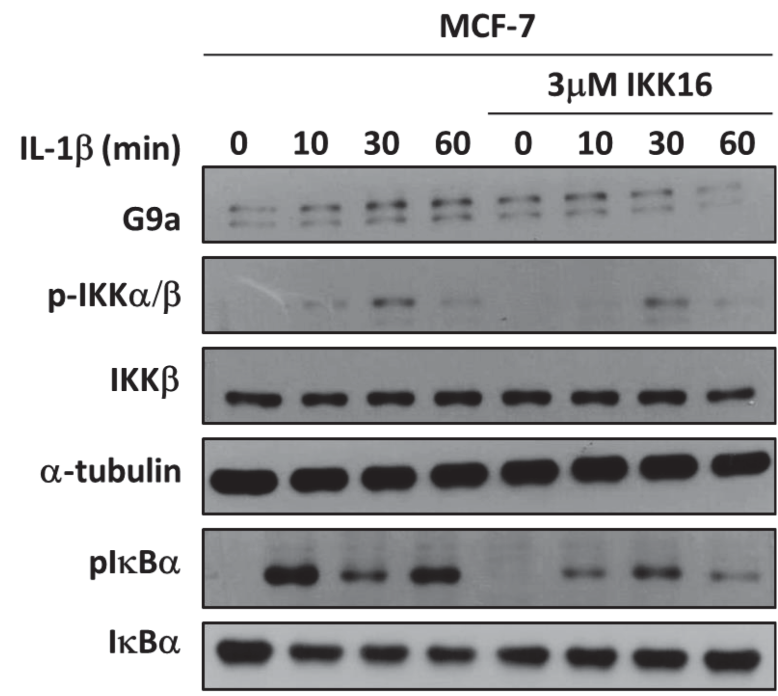


Figure 3. Increase of G9a protein is dependent on IKK kinase activity.

(A) The expression level of G9a was further increased when co-transfected with constiutively acitve Flag-IKKβ (CA) but was limited with kinase dead Flag-IKKβ (KD).

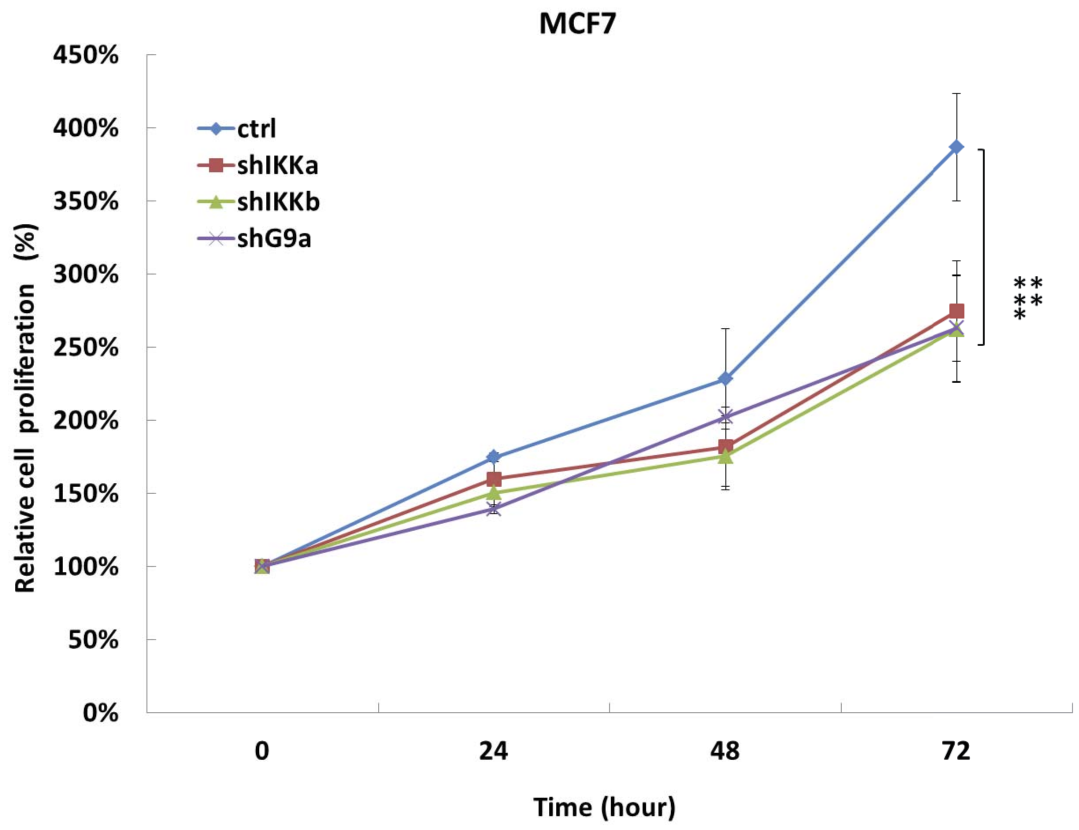
(B) Activation of IKKα/β increased G9a protein level. Time course analysis of G9a protein level after 20ng/ml IL-1β to activate IKKα/β in BT549 cells. Cells were serum starvation overnight and G9a protein levels at 0, 10, 30 and 60 minutes after IL-1β treatment were analyzed by western blotting.

(C) Different doses of IL-1β can enhance the protein expression of G9a progressively in MCF-7 cells. Cells were serum starvation overnight and G9a protein levels at 10, 30 minutes after four different doses of IL-1β treatment (0, 10, 20, 40 ng/ml) were analyzed by western blotting.

(D, E) IKK16 (IKKα/β inhibitor) can inhibit the accumulation of G9a that was increased by IL-1β stimulation in BT549, MCF-7 cells. Cells were serum starvation overnight and pretreated with 3μM IKK16 (IKKα/β inhibitor) for 2 hours before IL-1β treatment. G9a protein levels at 0, 10, 30 and 60 minutes after IL-1β stimulation were analyzed by western blotting.

Figure 4

A



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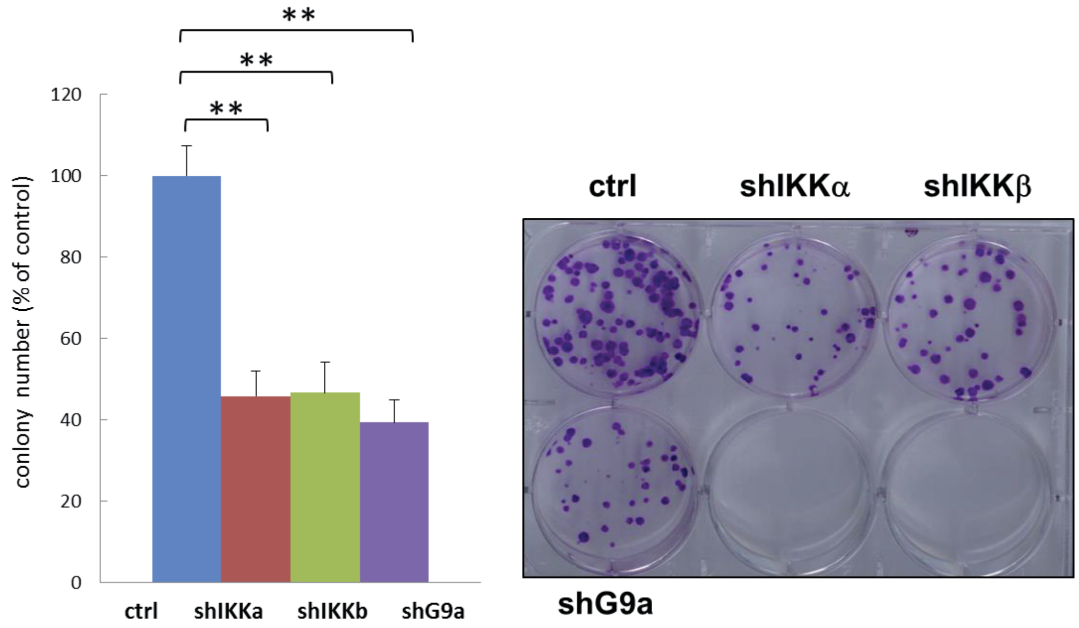

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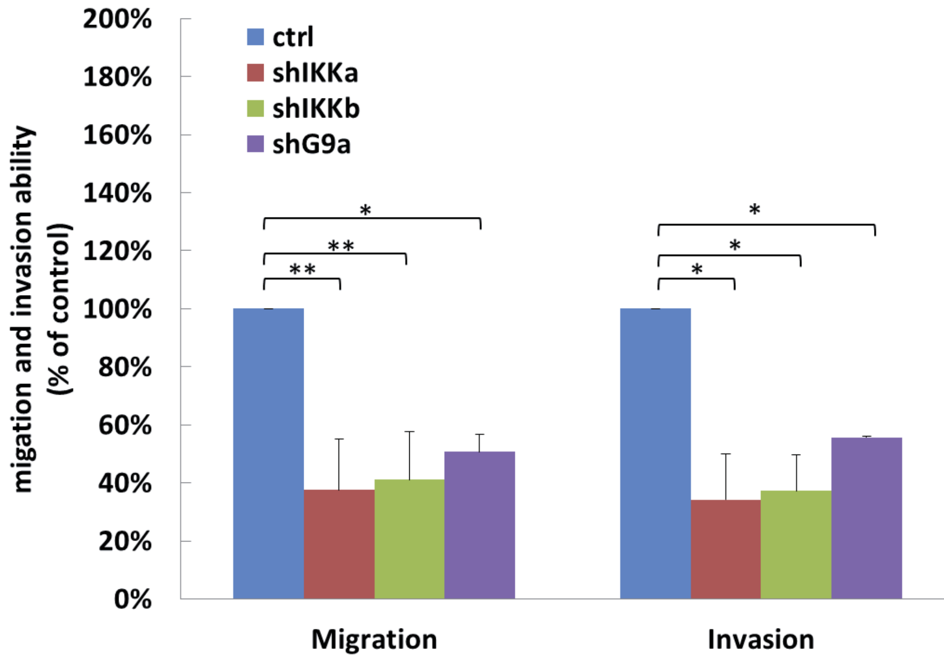
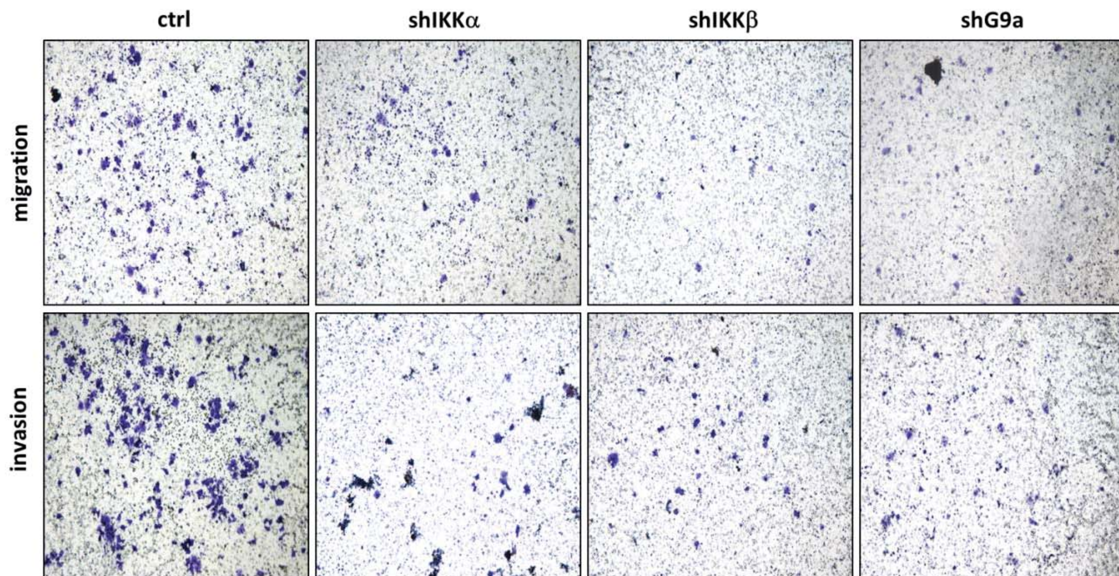




D

G9a

relative gene expression (fold)

2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0

**

ctrl
shG9a

E-cadherin

relative gene expression (fold)

5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0

**
**
*

ctrl
shIKKa
shIKKb
shG9a

EpCAM

relative gene expression (fold)

5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0

**
**
**

ctrl
shIKKa
shIKKb
shG9a

RUNX3

relative gene expression (fold)

9
8
7
6
5
4
3
2
1
0

**
**
**

ctrl
shIKKa
shIKKb
shG9a

Figure 4. The effect of knockdown IKKα/β in cancer function.

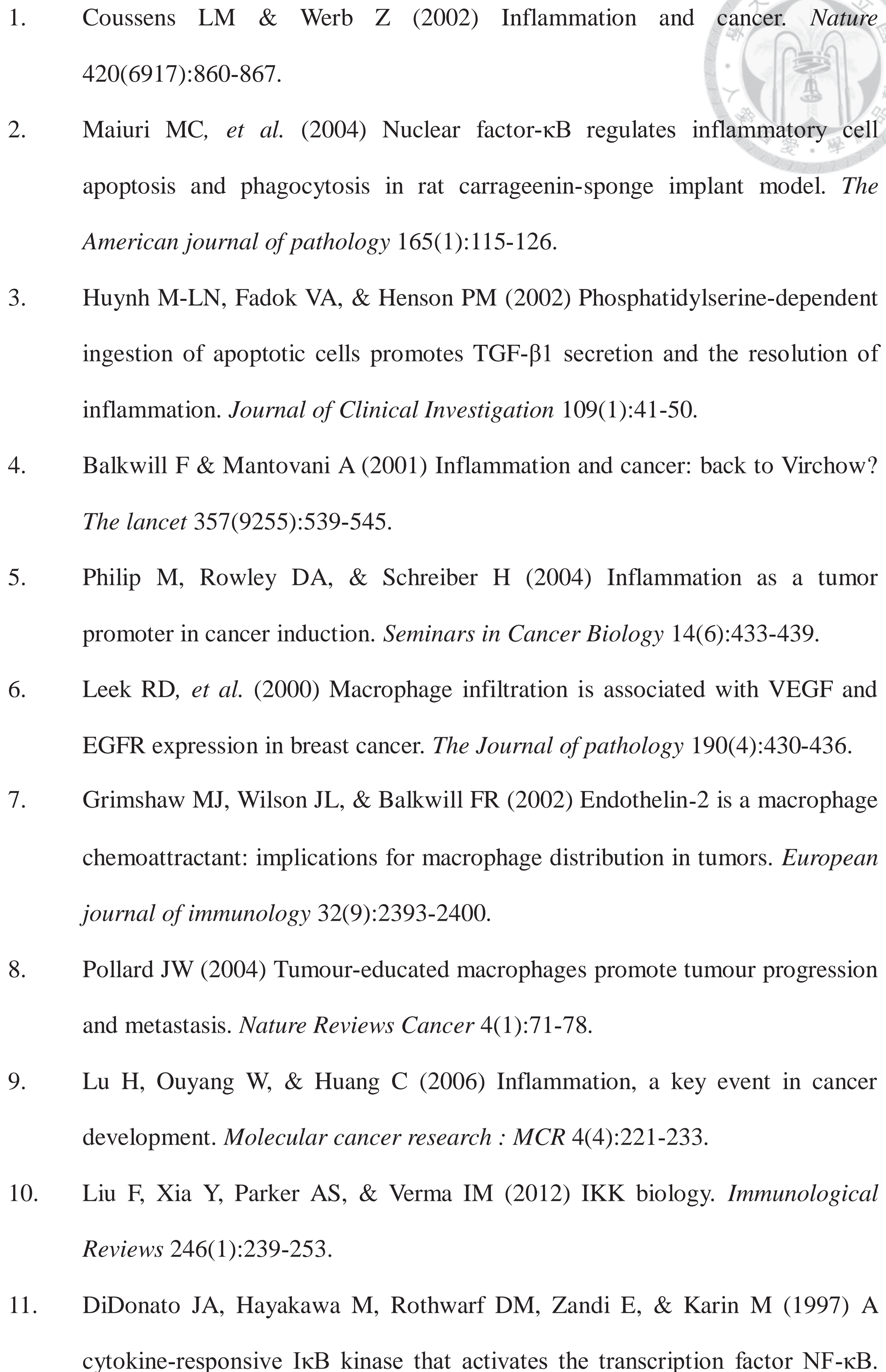
(A) The cell proliferation of MCF-7 cells was analysed by MTT assay. MCF-7 cells infected with IKKα-shRNA #1, IKKβ-shRNA #1 and G9a-shRNA decreased the cell proliferation ability compared to MCF-7 cells infected with shRNA-vector. Data are shown as mean ± SD. *$P<0.05$, **$P<0.01$.(two-tailed Student’s t-test).

(B) Measurement of the colony formation ability of MCF-7 cells. MCF-7 cells infected with IKKα-shRNA #1, IKKβ-shRNA #1 and G9a-shRNA decreased the migration and invasion ability compared to MCF-7 cells infected shRNA-vector. Data are shown as mean ± SD. *$P<0.05$, **$P<0.01$.(two-tailed Student’s t-test).

(C) Measurement of the migration and invasion ability of MCF-7 cells. MCF-7 cells infected with IKKα-shRNA #1, IKKβ-shRNA #1 and G9a-shRNA decreased the migration and invasion ability compared to MCF-7 cells infected shRNA-vector. Data are shown as mean ± SD. *$P<0.05$, **$P<0.01$.(two-tailed Student’s t-test).

(D) qRT-PCR analysis of gene exprssion in MCF-7 cells infected with IKKα-shRNA #1, IKKβ-shRNA #1 and G9a-shRNA, shRNA-vector. Data are shown as mean ± SD. *$P<0.05$, **$P<0.01$.(two-tailed Student’s t-test).

Chapter 6.
References



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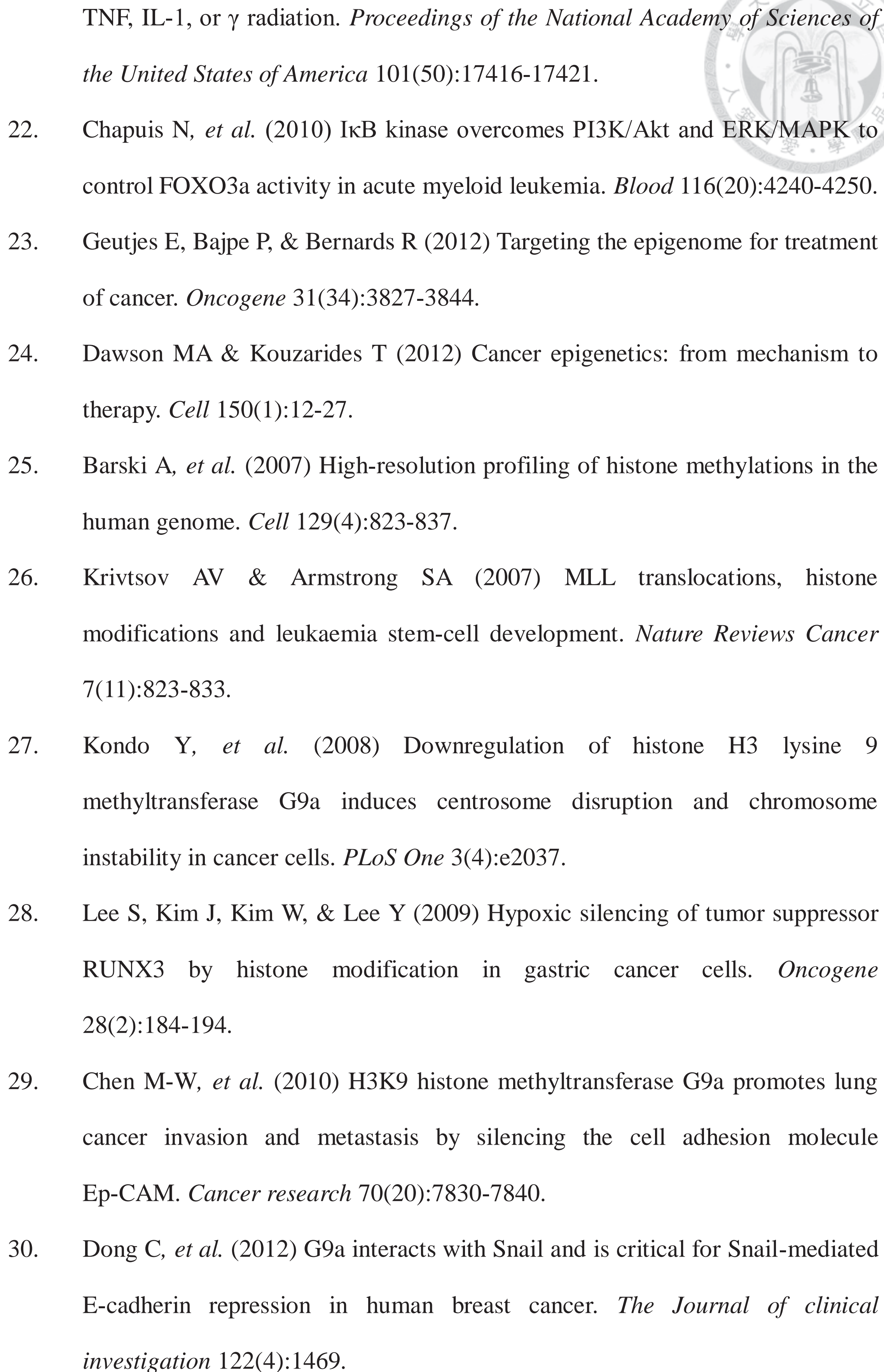

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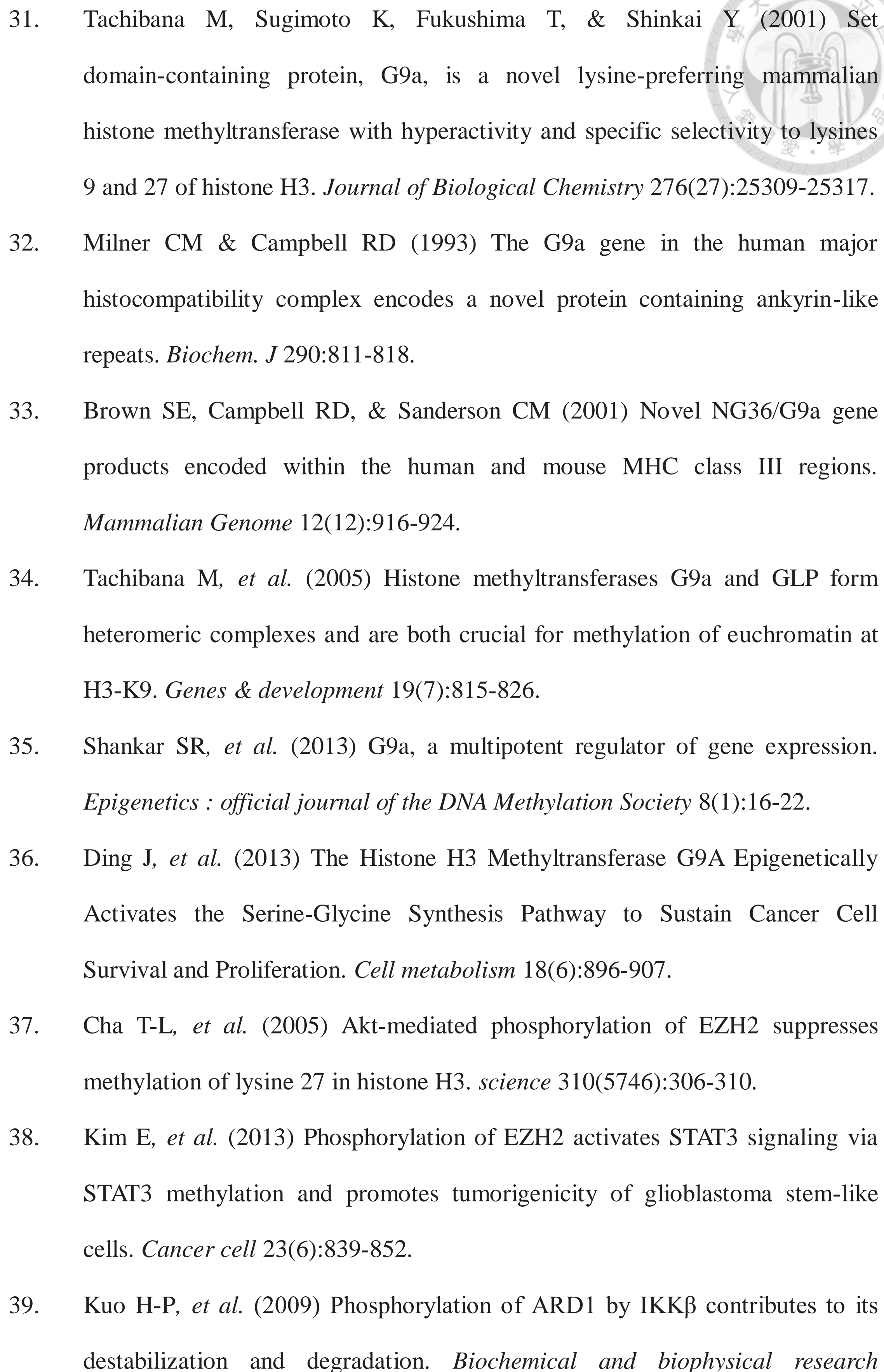

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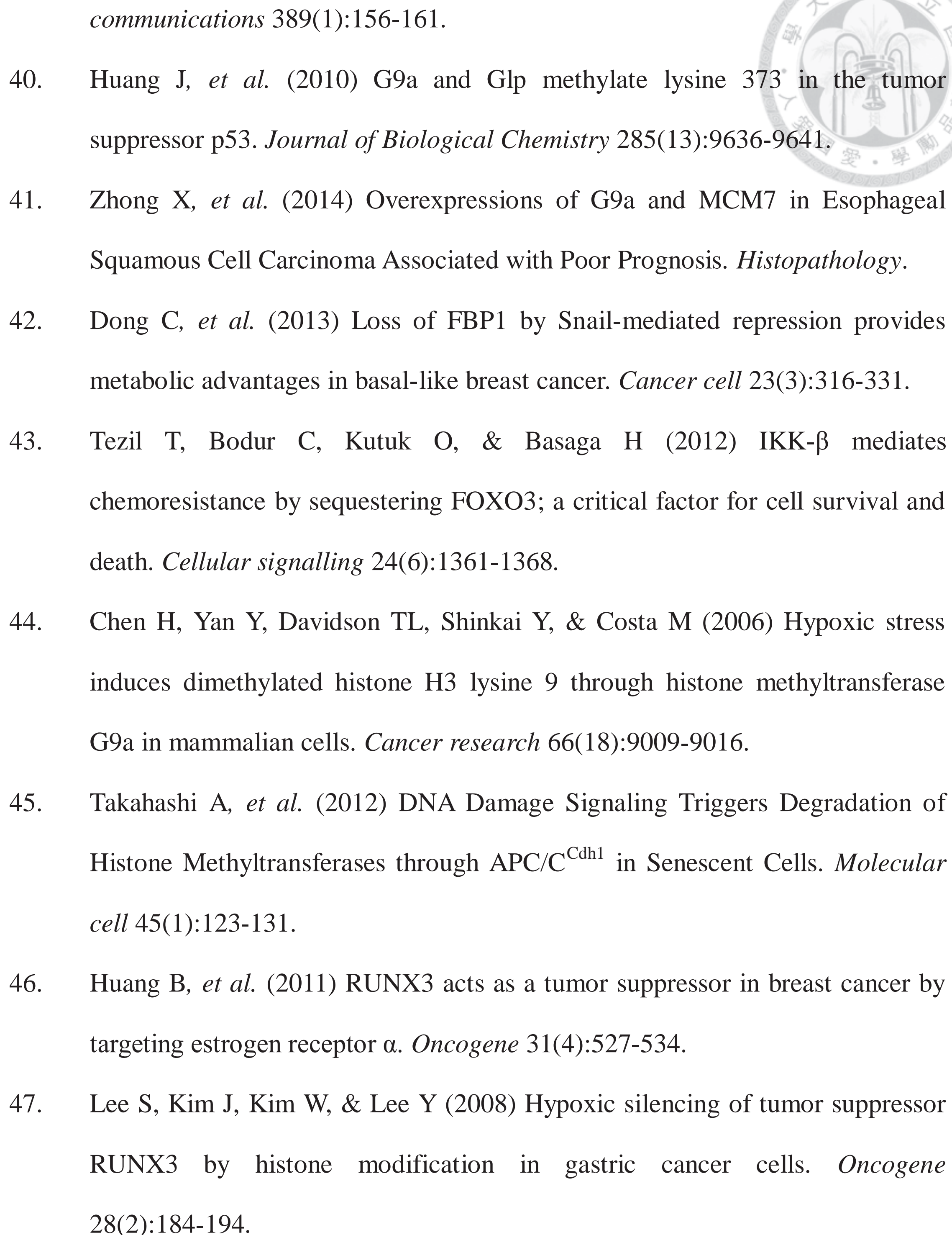

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