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NRIP 穩定雄性激素受體蛋白以及增進雄性激素受體轉錄活性之機制探討

Investigation of the mechanism of NRIP – stabilizing AR protein and enhanced AR transcription activity

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本論文係 林冠良 君（學號 R96445130 ）在國立臺灣大學微生物學所完成之碩（博）士學位論文，於民國 98 年 7 月 24 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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中文摘要

核受體交互作用蛋白 NRIP (nuclear receptor interaction protein)為本實驗室利用雄性激素受體(Androgen receptor, AR)的 C 端作餌，以 yeast-two hybrid 選殖系統所鑑定而得；實驗室先前的研究發現：NRIP 不但可以直接與 AR 交互作用，並且也是一個 AR 共同活化子，在雄性激素存在之下能增強 AR 下游的 Prostate-specific antigen(PSA)轉錄活性。在前列腺細胞株 LNCaP 抑制 NRIP 表現，會使得細胞進行細胞凋亡，這個現象顯示 NRIP 在前列腺癌細胞存活上扮演重要的角色。

最近，本實驗室發現 NRIP 是一個 AR 所調控的基因，不但在有雄性激素存下可以增加 NRIP 的轉錄，且 NRIP 的 promotor 具有雄性激素反應區域 (androgen response element)。此外，本實驗室也發現 NRIP 可以穩定 AR 蛋白。在 NRIP 表現被抑制時會導致 AR 經由 26 S proteasome 降解。於是，本論文主要目標是要更進一步探討 NRIP 穩定 AR 蛋白的機制。

據現階段的研究知道 DDB1-Cullin4 複合體具有 E3 接合酶的作用，能夠促使各種蛋白的降解，而 DDB1 和 NRIP 兩者皆具有 WD40 domain。由 DDB1 的 pull down 的實驗中發現 NRIP 會和 DDB1 交互作用；因此，DDB1-Cullin4 路徑是否牽涉 NRIP 穩定 AR 蛋白的機制值得深入探討。本實驗首先利用 co-IP 實驗證實 NRIP 和 DDB1 的交互作用；利用 site-directed mutation 製備 NRIP 突變蛋白後，標定出上負責和 DDB1 作用的 motif。此外，也發現一個參與 DNA 損傷修補作用的 DDB2 蛋白可與 AR 進行交互作用，並在細胞中表現 DDB2 時，AR 蛋白隨即減少。而在探討 NRIP 如何穩定 AR 蛋白的機制,我們利用 in vivo competition assay 證明 NRIP 有可能透過與 DDB2 競爭對 AR 的結合，使得 AR 蛋白被穩定下來。另外，我們利用失去和 DDB1 結合能力的突變型 NRIP 送到 LNCaP 細胞中，發現突變型的 NRIP 仍保有增加 AR 下游基因調控的轉錄活性，證實 NRIP 調控 AR 轉錄活性不是透過 DDB1-CUL4 這條路徑。

而最後，為了在動物模式中研究 NRIP 的功能，我們建構了 NRIP 基因剔除小鼠，並且在 DNA, RNA 以及蛋白質的層次上皆證明小鼠已缺失 NRIP。此外，我們分離出 NRIP 基因剔除胚胎纖維母細胞，表現 NRIP 可以增加 AR 蛋白的表現量，並且我們發現在 MEF 細胞中發現 NRIP 與細胞生長有密切相關。未來，我們將觀察 NRIP 基因剔除所引起的表徵，並更進一步研究 NRIP 在生物體中扮演的角色。

關鍵字: 雄性激素受體、核受體交互作用蛋白、DDB1、DDB2、基因剔除小鼠。

ABSTRACT

By using yeast-two hybrid system, Nuclear Receptor Interaction Protein (NRIP) is found in our lab. NRIP is an AR coactivator which can directly interact with AR. It can activate prostate-specific antigen (PSA) transcription in an androgen-dependent manner. When knockdown of NRIP in LNCaP cell causes cell apoptosis that reveals a vital role of NRIP in cell survival in prostate cancer cell.

Recently, we found that NRIP is also a novel AR target gene. Transcription of NRIP can be increased in the presence of androgen, and then we identified the androgen response element in the promoter of NRIP. Furthermore, we found the role of NRIP in AR protein stability .Where the knockdown of NRIP contributes to AR degradation through proteasome.

DDB1-Cullin4 complex contains E3 ligase function for various proteins' degradation. From DDB1 pull down assay, it was reported that NRIP is one of DDB1 interaction proteins, therefore we are interested to examine the fact whether NRIP can bind to DDB1. The interaction between NRIP and DDB1 was confirmed by reciprocal co-immunoprecipitation .We found that WDxR motif on NRIP responsible for DDB1 binding was identified using site-direct point mutation. When knockdown DDB1 or CUL4A, we observed an enhancement of AR protein level in LNCaP cells. When ectopic expression of NRIP can reduce AR protein ubiquitin level. Hence, we hypothesize

NRIP-stabilizing AR protein involved in DDB1-Cul4A complex.

DDB2 is a well-known WD40 protein that associates with DDB1. DDB1-DDB2 heterodimer was involved in NER pathway which UV-induced DNA damage repairs. We found that DDB2 can associated with AR and introduced DDB2 can reduce AR protein level in LNCaP cell. This degradation of AR protein is dependent on presence of DHT. To further demonstrate the mechanism of NRIP-stabilizing AR protein; we used *in vivo* competition assay to indicate the mechanism of NRIP-stabilizing AR. The mechanism of NRIP-stabilizing AR may be through competition with DDB2 for binding to AR. Furthermore, we introduced mutant NRIP (can not interact with DDB1) into LNCaP cell to observe the PSA gene expression, the result excluded the DDB1-CUL4 pathway in the mechanism of NRIP by which contributes to enhance AR mediated transcription activity.

To approach investigating the function of NRIP *in vivo,* we had generated NRIP knockout mice and confirmed the genotype of NRIP KO mice in DNA, RNA and protein level. Based on the role of NRIP in AR stability in cell culture, we observed an enhancement of AR protein when introducing NRIP in NRIP KO MEF cell. Moreover, the proliferation of KO MEF cell was slower than wild-type cell. It suggests that NRIP may play a role in cell growth. In the future, we would investigate more functions of NRIP *in vivo* using knockout mice model.

Key word: AR、NRIP、DDB1、DDB2、knockout mice。

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Chapter1

INTRODUCTION

1.1 Androgen, Androgen receptor and its coregulator

Androgen, an AR ligand, acting via the androgen receptor plays critical roles in prostate development, growth, and pathogenesis. There are two potent androgens-testosterone and dihydrotestosterone (DHT) in humans and mammals. Testosterone is converted to DHT by 5α-reductase isozymes.

Androgen receptor is a classical type I steroid nuclear receptor, which function as ligand-inducible transcription factor. In the presence of ligand , AR can translocate into nucleus and form homodimer binding to inverted repeat DNA response elements and recruit kinds of coregulators to regulate gene expression (Hsieh, Small et al. 2007) (Chmelar, Buchanan et al. 2007).

The structure of AR is composed of N-terminus regulatory domain including activation function 1 (AF-1) , a DNA binding domain, hinge region containing ligand-dependent nuclear localization signal , and a C-terminal ligand-binding domain (LBD) with an additional transactivation function 2 (AF-2) (Lee and Chang 2003). Analysis of the amino acid sequence of the AR , which reveals the presence of a putative PEST (Pro-Glu-Ser-Thr) sequence in the hinge region (between amino acids 638 and 658) that is conserved between different species, suggest that AR is a short

half-life protein and its turnover may be through proteasome-mediated degradation (Sheflin, Keegan et al. 2000).

The multiple functions of AR was regulated by its coregulator which can enhance (coactivator) or suppress (corepressor) AR transcription activity. One AR interaction protein ARIP4 (androgen receptor interaction protein 4) can interact with AR DNA binding domain and enhance AR transcription activity by chromatin remodeling (Rouleau, Domans'kyi et al. 2002). Gelsolin, which is an actin binding protein, can enhance AR transcription activity by facilitating AR translocation (Fliss, Rao et al. 1999). ARA267 (AR-associated protein 267) is a methyltransferase which can interact with AR DBD-LBD and stimulate androgen-induced AR transcription activity by histone methylation on H3-K36 and H4-K20 (Wang, Yeh et al. 2001).

1.2 Mechanisms of androgen receptor degradation

The turnover of androgen receptor plays an important role in regulation of androgen receptor. There are three pathways reportedly to be involved in AR degradation：(A) AR can be phosphrylated by PI3K/AKT and subsequently undergoes ubiquitination by MDM2 E3 ligase. After ubiquitination, AR was degraded through 26S proteasome (Lin, Wang et al. 2002) (Gaughan, Logan et al. 2005). (B) Androgen-induced AR translocation can be interfered by PTEN

(phosphatidylinositol-3, 4, 5-trisphosphate 3-phosphatase). The interaction between AR and PTEN may expose the active site of the AR for the recognition of caspase-3, leading to AR degradation (Lin, Hu et al. 2004). (C) In DDB1-CUL4B complex, AhR (dioxin receptor) can be activated in the presence of ligand (3-methylcholanthrene) and then interact with Arnt (aryl hydrocarbon receptor nuclear translocator) to form heterodimer and translocate into nucleus. The heterodimer can associate with DDB1-CUL4B complex to assemble a functional E3 ligase. Sex steroid hormone receptor AR or ER can be a target substrate and ubiqutinated by this E3 ligase (Ohtake, Baba et al. 2007).

1.3 The characteristic and discovery of Nuclear Receptor Interaction Protein

NRIP (nuclear receptor interaction protein) was isolated by yeast two-hybrid screening assay in which HBD of androgen receptor (amino acids 595-918) was used as the bait and human HeLa cDNA library was used as preys (Tsai, Lee et al. 2005). NRIP gene locus locates on chromosome 1q24.2. Full length NRIP cDNA contains 2583 bp that encodes 860 amino acids and with a molecule weight of 160 kda. Structure of NRIP contains seven WD40 domains (five in the N-terminus and two in the C- terminus), and one IQ motif. WD40 domain is derived from the conserved tryptophan (W) and aspartic (D) acid residues in length of approximately 40 amino acids residues. WD40 domain

form β-propeller structures that acts as a platform for the stable or reversible association of binding partners or small ligands (Orlicky, Tang et al. 2003). The IQ motif is a basic amphiphilic helix, and usually presents in 1-7 copies serving as a binding site for calmodulin which indicates NRIP function may be regulated by Ca^{2+} (Bahler and Rhoads 2002) .

NRIP mRNA was expressed in brain, heart, prostate, liver and especially highly expressed in skeletal muscle and testis (Tsai, Lee et al. 2005) . Based on amino acids sequence analysis, there is 86% identity between human and mice NRIP. Besides, the mouse NRIP also contained seven WD40 domains, the highly conserved protein sequence, which implies NRIP might have evolutionary significance.

NRIP is a transcriptional cofactor which can not only interact with androgen and glucocorticoid receptor but also increase AR- and GR-mediated transcriptional activity. Moreover, NRIP can protect androgen receptor given that knockdown of NRIP instabilizes AR protein. The transcription of NRIP is also regulated by androgen though ARE (androgen response elements) on the promoter of NRIP (Chen, Tsao et al. 2008).

In recent study, DDB1-pull down assay shows that NRIP (also called DCAF6 or IQWD1 in these study) is one of DDB1 interaction proteins (He, McCall et al. 2006; Jin, Arias et al. 2006), which implies that some functions of NRIP may be associated with DDB1-CUL4 complex. The role of NRIP in protein degradation needs further

investigation.

1.4 DNA damage binding protein and Cullin4

Ubiquitin E3 ligase contains two important functions: catalysis of isopeptide bond formation and recruitment of substrates to this catalytic activity. The cullin (CUL) family is evolutionarily conserved proteins that assemble a large family of cullin-dependent E3 ligase. The human cullin family includes CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5 and CUL7. All cullins contain a conserved carboxy-terminal domain which binds to small RING finger protein: ROC1 (Regulator of Cullins-1, also called Rbx1) or ROC2 (Regulator of Cullins-2) (Petroski and Deshaies 2005). The small RING finger protein can recruit E2 ubiquitin-conjugating enzyme to proceed to ubiquitination. Cullin-dependent E3 ligase require to interact with an adaptor protein to target specific substrate, rather than binding to substrate directly as other E3 ligase. For example, CUL1-dependent ligase rely on interaction with an adaptor protein SKP1 (S-phase kinase-associated protein 1) to bridge an F-box protein to target specific substrate (Petroski and Deshaies 2005).

CUL4 (cullin4) belongs to Cullins (CULs) family which fuction as ubquitin E3 ligase by recruiting ring finger protein (ROC1) and various substrate receptors. To target specific substrate, CUL4 utilizes the C-terminus to bind with ROC1 and the N-terminus to interact with linker protein (DDB1) which recruits various substrate receptors to

target specific substrate. A well-known model is DDB1-DDB2-CUL4 complex which involves in NER (nucleotide excision repair) pathway after UV-irradiation (Nouspikel 2009). DDB1-CUL4A is recruited to the DNA damage foci through the interaction of DDB2 at the damaged DNA. The E3 ligase subsequently ubiquitylates histones around the damaged DNA foci. After ubiquitination, the histones may dissolve from the damaged nucleosome that makes the damaged DNA exposed. Later, the NER pathway factor XPC (Xeroderma pigmentosum group C-complementing protein) is recruited to the damaged site and the NER pathway proceeds (Chu and Yang 2008; Scrima, Konickova et al. 2008; Nouspikel 2009).

Recent studies on DDB1 function, more than DNA repair, DDB1 was involved in transcription (Higa, Wu et al. 2006), cell cycle (Sansam, Shepard et al. 2006), embryonic development and genomic stability (Cang, Zhang et al. 2007). The new roles of DDB1 rely on assembling with CUL4 to form ubiquitin ligase. Moreover, the CUL4-DDB1 ubiquitin ligase may recruit DCAFs, which belong to WD40 repeat-containing protein and function as substrate receptors to target specific substrate. DCAFs means a family of **D**DB1 and **C**UL4-**a**ssociated **f**actor**s**, and the function of CUL4-DDB1 E3 ligase was determined by association of the specific DCAFs (Lee and Zhou 2007).

DDB2 (DNA damaged binding protein 2, also named p48), a member of DCAFs,

contains three WD40 domain and was originally found to involve in nucleotide excision repair along with DDB1 (Takao, Abramic et al. 1993). In additon to DNA repair, DDB2 may function as transcription factor to regulate gene expression. It had reported that DDB2 acts as a co-factor of E2F1 (Hayes, Shiyanov et al. 1998) and that associated with chromatin-acetylating transcription coactivator STAGA complex **(** SPT3-TAFII31-GCN5L acetylase complex) (Martinez, Palhan et al. 2001). In addition, DDB2-deficient mice not only were hypersensitive to UV-induced skin carcinogenesis but also developed a high rate of malignant tumor in internal organ which indicate DDB2 function as a tumor suppressor (Itoh, Iwashita et al. 2007). Even if DDB2 is considerd as a tumor suppressor, recent study has reported that DDB2 is a candidate for oncogene in breast cancer which may contribute to breast tumor progression (Kattan, Marchal et al. 2008). Besides, the other DCAF protein COP1 (constitutive photomorphogenic-1) recruits c-Jun to the CUL4-DDB1 E3 complex to proceed ubiquitination and degradation (Wertz, O'Rourke et al. 2004). A DCAF protein-Cdt2 can regulate ubiquitination and degradation of p21 (Abbas, Sivaprasad et al. 2008). Except to protein ubiquitination, DCAF protein-WDR5 was also reported to regulate gene transcription activity through histone methylation (Higa, Wu et al. 2006) . Variable function of DCAFs suggest that additional role of DDB1-CUL4 complex still be investigated.

1.5 Aim of the Thesis

NRIP is an AR coactivator, which can enhance AR-mediated transcripton activity. Moreove, the feedback regulation and feed-forward loop between AR and NRIP was observed that androgen –AR signaling can upregulate NRIP gene expression and NRIP can stablilize AR protein. NRIP is also known as a member of DCAFs which can interact with DDB1-CUL4 complex. Hence, we raise a question whether DDB1-Cul4 E3 ligase contributes to AR degradation. Therefore, in this study, we were interested to investigate the mechanism of NRIP-stabilizing AR via DDB1-CUL4 pathway. In addition, to further characterize the function of NRIP, we would generate and construct NRIP knockout mice.

The major goals of this thesis are:

(a) To confirm NRIP-DDB1 interaction and find the interaction domain.

(b) To investigate effect of NRIP and DDB1 on AR protein stability.

(c) To find out DDB2 involved in AR degradation.

(d) To verify whether the mechanism by which NRIP enhance AR-mediated transcription activity is through DDB1-CUL4 pathway.

(e) To construct and generate NRIP knockout mice, and to delineate the mechanism of NRIP-stabilizing AR protein.

Chapter2

MATERIALS AND METHODS

2.1 Cell culture and Drug Treatment

HEK 293T (Human embryonic kidney epithelial) cells and MEF (mouse embryonic fibroblast) cells was maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine,100 U/ml of streptomycin and 100 U/ml of penicillin at 37℃ 5%CO_2 condition . LNCaP and 22RV1 cell were grown in RPMI 1640 containing 10% FBS, L-glutamine and antibiotics at 37℃ 5%CO_2 condition. For dihydroxytesterone (DHT) treatment, cells were maintained in the medium containing 5% charcoal–dextran-stripped (CDS) FBS (HyClone) for at least 2 days and cells were treated with DHT in 1~10nM. For ubiquitination analysis .cell were treated with 10 mM of MG132 (Cabiochem, Darmstadt, Germany) at the indicated times.

2.2 Mice strain and Generation of the NRIP knockout mouse

Mouse NRIP genomic DNA (bMQ134h07) was obtained by screening a BAC library (Geneservice, http://www.geneservice.co.uk/) derived from the 129/sv mouse strain. NRIP knockout Chimeric male mice were generated by core lab and breeded with wild-type C57BL/6J females. Germline transmission was obtained from their agouti progeny. To type the genotype, mouse tail genomic DNA was extracted and the genotype was confirmed by PCR (described later). After genotyping, the heterozygous

NRIP knockout (**+** / **-**) was generated (F1). The heterozygous NRIP knockout mice (F1) were mating each other to produce the homozygous NRIP knockout mice (**-** / **-**)(F2).

2.3 Isolation of mouse embryonic fibroblast

Wild-type NRIP mice or NRIP knockout mice was crossed each other. After 14 days, the pregnant mouse was sacrificed and the embryos (E14) were isolated from the uterus, and then embryos were transferred to a petri dish with sterile PBS. Subsequently, use sterile forceps and scissors to remove the embryo heads and limbs and scoop out the liver, intestines and heart. And then the embryo carcass was transferred into a sterile 15mL tube with a sufficient volume of Trypsin/EDTA to cover the carcasses with incubation for 15 minutes at 37℃. Then pipette the tissue a few times to dissociate the tissue Allow the large pieces of cellular debris to settle. And then transfer the supernatant into a fresh tube and add moderate DMEM medium containing 10% FBS (about 1 embryo 10 ml medium). The fibroblast was seeding on 10 cm^2dish (about 1 embryo a 10 cm dish) with incubation at 37℃ with 5% CO2. After 2 days change the medium which should be acidic. When the dishes are confluent, usually in 3-4 days, the cultures are ready for freezing (Freeze cells in 10% DMSO at 2 x 10^6 cells/vial).

2.4 Plasmid constructions and Site-directed mutagenesis

NRIP- and AR-expression plasmids were constructed by Pei-Hong Chen and Sei-Way Chang, respectively. Plasmids expressing DDB1, DDB2, CUL4A and CUL4B were kindly provided by Dr. Yue Xiong (University of North Carolina Chapel Hill, NC).

To construct the site-directed mutants of NRIP, First, wild type NRIP plasmid (pCMV-3xflag-wtNRIP) was used as template. A series of mutagenic primers (DTR172→DTA, DRR222→ DRA) with mutation in nucleotide sequence of NRIP were used to proceed with PCR. The mutagenic primers containing desired mutations are listed below and the mutant NRIP map was described in appendix. The PCR conditions are listed below.

	Volume	Final concentration
Plasmids (50ng/ul)	1	2ng/μl
2.5 mM dNTP	2.5	0.25mM
10X reaction buffer	2.5	1X
10 uM primer (sense)	1	1μM
10 uM primer (antisense)	1	1μM
Pfu ultra polymerase	1	2.5U
Double distilled water	16	
Total volume	25	

95℃	5：00	
98℃	0：40	5 cycle
42℃	0：15	
72℃	5：00	
98℃	0：40	25 cycle
55℃	0：15	
72℃	5：00	
72℃	10：00	

Primer: NRIP (DTA)-sense

5'-CTGTTAGGTGGTTTGATACAGCCATCAAAACTAGCTGCACAAAAG-3'

Primer: NRIP (DTA2)-antisense

5'-CTTTTGTGCAGCTAGTTTTGATGCGTGTATCAA ACCACCTAACAG-3'

Primer: NRIP (DRA)-sense

5'-GCTCAGTACGAATATATGATCGGGCAATGCTGGGCACAAGAGCTAC-3'

Primer: NRIP (DRA2)-antisense

5'-GTAGCTCTTGTGCCCAGCATTCGCCGATCATACATTCGTACTGAGC-3'

2.5 RNA interference

The pSUPER vector-mediated RNAi system was used in this study. The vector uses H1 promotor which was a promoter for the polymerase Ⅲ drived. Based on the protocol, 19 nucleotide stretches within the coding region of these genes-NRIP, DDB1, DDB2, Cul4A, Cul4B was constructed. The following are specific targeted sequence of these genes ：

SiNRIP：5’-GATGATACAGCACGAGAAC-3’

SiDDB1：5’-AGCATTGACTTACCAGGCA-3’

SiCul4A：5’-GGAAGAGACTAATTGCTTA-3’

SiCul4B：5’-CGGAAAGAGTGCATCTGTA-3’

2.6 Transient Transfection

(a) Calcium phosphate method

293 or 293T cells were seeded on 10-cm dishes and grown overnight. When the cells reached about 60% confluency, replaced fresh medium (DMEM) before transfection. For each sample, expression plasmids were diluted in 450 μl sterilized water, and 50 μl 2.5 mM $CaCl_2$ was gently added into the diluted DNA. And the solution was mixed by vortex at level 4 for 10 secconds. Next, 500 μl Hepes (280mM Nacl, 50 mM HEPES, 1.5ml Na_2HPO_4 titrate to pH=7.0 with NaOH)was dropped into the Ca^{2+}-DNA mixture while vortexing at the same time and then mixed by vortex for another 30 secconds. The DNA solution was dropped into dish and then swirl to mix.

After incubating with 5 hours in 37℃ under 5% CO_2 conditions and then the medium containing DNA precipitates were replaced by fresh DMEM medium containing 10 % FBS. Cell lysates were harvested after 48 hours and used to perform Western blot or co-immunoprecipitation assay.

(b) Electroporation method

Before electroporation, LNCaP, 22RV1, and MCF7 cells were washed with PBS and then trypsinized. The cells were washed bu antibiotics-free and FBS-free RPMI medium, and then counted cells to achieve the cell density $1x10^7$ cell/ml. About 700ul of the suspended cells were mixed with expressive plasmids in eppendorff tube and incubated 10 minutes at room temperature. The cells were transferred to prechilled BTX disposable cuvette (4 mm gap) .The ElectroSquarePorator™ ECM830 apparatus (HARVARD BIOSCIENCE COMPANY) was used to perform electroporation procedure as described by manufacturer. The working ondition for LNCaP, 22RV1, MCF7 were 170V and 70msec .after electroporation, the transfected cells were plated into 10cm plate. Twenty-four hours later ,the medium was changed with fresh RPMI containing 10%FBS. The cell lysate was harvested at 48 or 72 hours.

2.7 Co-immunoprecipitation

For the interaction between NRIP-DDB1 or AR-DDB2, the plasmids were co-transfected by Calcium phosphate method. Cell lysates were collected by triton-100 lysis buffer (150 mM NaCl, 1% TritonX-100, 50 mM Tris, pH7.4) containing protease inhibitor. For each immunoprecipitation, 1 mg of cell lysate was diluted in 200 μl of Triton-100 buffer containing protease inhibitor and immunoprecipitated with 1 μg of anti-FLAG (Sigma) or anti-HA (GeneMark) antibody at 4℃ for 1 hour . Immunoprecipitation products were pulled down by 30 μl of Protein G sepharose beads (Amersham Biosciences) and washed 3 times with triton-100 buffer. After centrifugation at 10,000 x g for 1 minute, supernatant was carefully removed and 20 μl of 2X protein sample buffer was added to elute the proteins. The sample was boiled at 95℃ for 10 minutes and then centrifugated at 13000 x g. After centrifugation, the supernatant was subjected to Western blot.

2.8 Western blot

Protein samples were quantified and separated on 10% SDS-PAGE, and transferred to PVDF membrane using Semi-Dry Transfer Unit (GE Healthcare Life Sciences). Membrane was blocked with 5% skimmed milk-TBST and then blotted with anti-FLAG, anti-HA or anti- β -actin primary antibodies. Anti-mouse Ig-HRP (Amersham

Biosciences) was added after 3 washes by TBS-T and signals were detected by ECL Western blotting detection system (Amersham Biosciences) and captured by X-ray film (Fuji film).

2.9 RNA extraction

To lyse and homogenize cells, the cell was harvested and 1 ml of TRIzol reagent (Invitrogen) was added to each cell sample, and then pipeted to mix. The homogenized samples were transferred to microfuge tubes and incubated at room temperature for 5 minutes. To separate the RNA phase, 0.2 ml of chloroform was added .The mixture was shaken vigorously by hand for 15 seconds and then incubated at room temperature for 2 minutes. The samples were centrifuged at 13,000 x g for 15 minutes at 4℃. Following centrifugation, the upper colorless aqueous phase (about 0.5 ml) was transferred to a fresh tube and mixed with 0.5 ml of isopropanol. The samples were incubated at room temperature for 10 minutes and centrifuged at 13,000 x g for 10 minutes at 4℃. The RNA precipitate then formed pellet on the side and bottom of the tube. To wash the RNA, the supernatant was removed and 1 ml of 75% ethanol was added. The pellet was gently flicked and centrifuge at 7,500 x g for 5 minutes at 4℃, briefly air-dried for 5 t0 10 minutes. Finally, 50μl of DEPC-treated water was added to dissolve the RNA pellet by gently pipetting. The Concentration of the RNA samples was measured by

spectrophotometry. The RNA samples were stored in -70℃ for later use.

2.10 RT-PCR Analysis

Reverse transcription was performed with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). For each sample, 3 μg of total RNA was used to synthesize cDNA. First, RNA was mixed with 1 μl of 50 μM oligo(dT)$_{20}$, 1 μl of 10 mM dNTP mix, and DEPC-treated water to 10 μl. The RNA/primer mixture was incubated at 65℃ for 5 mins and then placed on ice for 1 min. Next, 10 μl of the cDNA synthesis mix (2 μl of 10X RT buffer, 4 μl of 25 mM $MgCl_2$, 2 μl of 0.1 M DTT, 1 μl of RNaseOUT (40 U/ μl), and 1μl of SuperScript III reverse transcriptase (200 U/ μl) was added. The mixture was mixed gently, collected by brief centrifugation and incubated at 50℃ for 50 minutes. The reaction was terminated at 85℃ for 5 min, chilled on ice and then collected by brief centrifugation. 1 μl of RNase H was added and incubated at 37℃ for 20 minutes. cDNA samples were stored at -20℃ or used for PCR analysis.

The following primers were used for PCR：

β-actin

Forward primer：5’-ACCTTCAACACCCCAGCCATG-3’

Reverse primer：5’-CTGGAAGAGTGCCTCAGGGCA-3’

PSA

Forward primer：5’-ATGTGGGTCCCGGTTGTCTTCCTCACC-3’

Reverse primer：5’-TCAGGGGTTGGCCACGATGGTGTCCTT-3’

AR

Forward primer：5’-CAGGAATTCCTGTGCATGAAAGCCATGCTACTC-3’

Reverse primer：5’-TCACTGGGTGTGGAAATAGAT-3’

NRIP

Forward primer：5’-ATGTCTCGGGGTGGCTCCTACCCACAC-3’

Reverse primer：5’-ACTGGTTCACCTGTCCCTGGTTTGG-3’

NRIP-F1(mouse)：5'-GTTGTGGGACGTGAGGAAAA-3'

NRIP-F2(mouse)：5'-AATGTGCATGATGGCTGTGT-3'

NRIP-F3(mouse)：5'-TTTTGACAACCATCCGTTCA-3'

NRIP-R(mouse)：5'-ATGACTCCATCTCCAGAGCAA-3'

NRIP-R2(mouse)：5'-AACTGGTTTTGATGCGTGTG-3'

β actin-F(mouse)：5'-CCATCATGAAGTGTGACGTTG-3'

β actin-R(mouse)：5'-CTGCGCAAGTTAGGTTTTGTC-3'

2.11 Realtime PCR

Complementary DNA was prepared from the total RNA using SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen). Quantitative PCR reactions were performed by Sybr-Green fluorescent dye (ABI). The primers for realtime PCR are listed below:

NRIP-realtime-F(mouse)：5'-ACGCATCAA AACCAGTTGCA-3'

NRIP-realtime-R(mouse)：5'-CAGCACGCCTACAGTTGATTA AAATA-3'

GAPDH-realtime-F(mouse)：5'-AGCCTCGTCCCGTAGACAAAAT-3'

GAPDH-realtime-R(mouse)：5'-CCGTGAGTGGAGTCATACTGGA-3'

2.12 DNA typing PCR

For DNA typing of NRIP KO mice, mouse tail was cutted and the genomic DNA of the tails was extracted by DirectPCR kit (Viagen Biotech, Inc.). 200 ng genomic DNA were used as template and mixed with 2 μl of 10X reaction buffer, 2μl of 2.5 mM dNTP, 1 μl of 10 μM forward primer (AU or KU), 1 μl of 10 μM reverse primer (XD), 1 μl of Taq DNA polymerase (Genemark), and total volume was filled with double distilled H_2O until 20 μl. The PCR products were resolved on 3% agarose gel to proceed DNA typing. The genomic DNA typing primers were listed below：

NRIP30235AU primer: 5'-AGGTAGATTTCTGAGTTTGAGG-3'

NRIP32625KU primer: 5'-GCTTACTTTCATTTATCCCTCTTTG-3'

NRIP33256XD primer: 5'-GACATTCTTATCAGCTACACTAG-3'

2.13 Southern blot

The genomic DNA from mouse tail was extracted and digested by restriction enzyme Sca1. The digested DNA was separated on 0.7% agarose gel , and then denature DNA by incubating the gel in denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 30 mins. Then wash the gel with milli-Q water. Denatured DNA gel was incubated in neutralization solution (1M Tris-Cl pH=8, 1.5 M NaCl) and then wash the gel with milli-Q water. After that, the gel was transferred with Nylon membrane (milliopore) , then DNA was ary-dry and UV cross-linked (1200 x 100 μ J/cm^2)for fixation. The transferred Nylon membrance was incubated with prehyhybridization buffer(50% Formamide , 5X SSC , 5X Denhardts , 0.1% SDS) for prehybridization, and then incubated with hybridization buffer (50% Formamide , 5X SSC , 5X Denhardts , 5X Dextran , 0.1% SDS) and DNA probe for hybridization. DNA probe was prepared by PCR with primers designed on NRIP intron 1 region and labeled by Random Primers DNA labeling system (GIBCOBRL, LIFE TECHNOLOGIES). After hybridization, the membrane was washed with wash solution (2 X SSC , 0.1% SDS or 0.2X SSC , 0.1% SDS) until the signal detected by the GM counter < 50 cpm. Finally, the signal was captured by X-ray film (Fuji film) at -70℃ for 1~3 days.

2.14 Cell proliferation assay

MEF cells were seeded at 1 x 10^5 cells per well in 6-well plate using DMEM containing 10% FBS and grown overnight. The cells were trypsinized and the total cell numbers were counted 24, 48, 72, 96 hours after seeding using hemocytometer by trypan blue staining.

Chapter3

RESULTS

Part one： The mechanism of NRIP –stabilizing AR protein and enhancing AR mediated transcription activity.

3.1 DDB1-CUL4 complex involved in AR degradation

Previously, we have demonstrated that NRIP is an AR-interacting protein, which has also been found with the characteristic of stabilizing AR protein and enhancing AR mediated transcription activity. To further investigate the mechanism by which NRIP can stabilize AR protein and the effect of NRIP on ubiquitylation level of AR, we co-expressed NRIP, AR and flag-tagged ubiquitin in HEK 293T cell. After two days, the cell lysate was harvested and 1 mg of cell lysate was employed to immunoprecipitation assay with anti-AR antibody. The following Western blot analysis was utilized to examine the ubiquitylation level of AR protein by anti-flag antibody. The result showed that NRIP can reduce AR ubiquitin level compared with vector control (Figure 1. right panel lane 5, lane 6 and input was shown on left panel).

Recently, it has been reported that DDB1-Cul4 E3 ligase was involved in the degradation of AR protein and NRIP (also known as DCAF6 or IQWD1) was found to be a member of DCAFs which can interact with DDB1 and functions as a substrate

receptor of DDB1-Cul4 E3 ligase complex. In this regard, we wanted to ask whether AR involves in DDB1-CUL4 degradation pathway, so we expressed DDB1 and NRIP in prostate cancer cell line, LNCaP. We found that introduced DDB1 can reduce AR protein level (Figure 2B), however, NRIP expression increased AR protein amount (Figure 2A). Moreover, we used siRNA to knockdown endogenous NRIP (Figure 2C), DDB1 (Figure 2D) or Cul4A (Figure 3), AR was increased in siDDB1 or siCUL4A expressing cells and knockdown of NRIP reduced AR protein level. These data suggest that DDB1-Cul4A pathway might be involved in AR degradation and confirm reported evidence that NRIP can enhance AR protein stability (Chen, Tsao et al. 2008).

3.2 Interaction between NRIP and DDB1

Based on the previous study, NRIP (DCAF6) was identified as one of the DDB1 interaction proteins (Jin, Arias et al. 2006). To examine the interaction, we expressed flag-tagged NRIP and HA-tagged DDB1 in 293T cell and co-immunoprecipitation was preformed reciprocally. As shown in Figure 4, the interaction signal was observed in 293T cell expressing flag-NRIP and HA-DDB1 compared with beads only control (Figure 4. Lanes 4 and 5). Likewise, the reciprocal co-immunoprecipitation data confirmed the results that NRIP indeed interacts with DDB1 (Figure 4, lanes 9 and 10).

3.3 WDXR motif on NRIP is essential for binding DDB1

Recent report has shown that DCAF proteins contain WD40 repeated domain and the interaction between DCAFs and DDB1 was through WDXR motif in WD40 repeat domain (Jin, Arias et al. 2006). The character of WDXR motif is WD40 repeated domain followed by X-Arg dipeptide or occasionally an X-Lys dipeptide. NRIP is a DDB1 binding protein and contains two predicted WDXR motif on its third and fourth WD40 domain. To further find out the domain of NRIP interacting with DDB1, we tried to mutate the predicted WDXR motif on NRIP. Site direct mutagenesis was performed on pCMV-3xflag-wtNRIP plasmid to change the amino acid arginine (R) to alanine (A) on the two predicted motif (172R→A, 222R→A as shown on Figure 5A). These mutated NRIP- and wild type NRIP-expressing plasmids were introduced in 293T cell. After 48 hours, the 1 mg of cell lysate were co-immunoprecipitated with anti-HA antibody and subjected to Western blot with anti-flag antibody (Figure 5B). The figure showed that single mutation (DTA or DRA) of the corresponding residue in NRIP substantially reduced interaction with DDB1 (Figure 5B, lanes 6 and 7, compare with lane 5) and double mutant (DTA/DRA) lost the entire activity of binding to DDB1 (Figure 5B, lane 8). Here, we find out that NRIP can interact with DDB1 through both WDXR motif.

3.4 DDB2 influence AR protein stability

To further demonstrate the mechanism of NRIP stabilizing AR, hence, we wanted to find out which WD40 domain protein was involved in AR degradation in DDB1-CUL4 pathway. We tried to search WD40 proteins involved in DDB1-CUL4A pathway to find out the candidate.

DDB2 is a well-known DDB1 binding protein which involved in DNA repair and regulating transcription. DDB1-CUL4 E3 ligase containing DDB2 can ubiquitinate histone H2A (Kapetanaki, Guerrero-Santoro et al. 2006), H3, H4 (Wang, Zhai et al. 2006) and XPC (Xeroderma pigmentosum complementation group C protein) (Sugasawa, Okuda et al. 2005). Besides, DDB2-deficient mice show opposite phenotype of AR knockout mice (increased testis) (Matsumoto, Takeyama et al. 2003; Itoh, Iwashita et al. 2007). Hence, we first attempted to know whether DDB2 involved in AR degradation. We introduced DDB2 into LNCaP cell, and then AR protein level was measured. As showed in Figure 6, expression of DDB2 decreased AR protein compared with the vector control (Figure 6B, lanes 1 and 3). Interestingly, DDB2-induced AR degradation was observed only under the 10nM DHT treatment, but not observed under ETOH treatment (Figure 6A, lanes 1 and 3) (Figure 6B, lanes 1 and 3). It may suggest that AR degradation by DDB2 occurred in nucleus. Furthermore, the similar result could be observed in another prostate cancer cell 22RV1 cell when

introducing DDB2 (Figure 7, lanes 3 and 4).

3.5 DDB2 is a novel AR interaction protein

As shown in Figure 6, we found DDB2 can decrease AR protein levels. Next, we raised a question whether DDB2 can associate with AR. To address this question, we overexpressed myc-tagged DDB2 and flag-tagged AR in 293T cell and then cell lysate was harvested after 48 hours to proceed with co-immuoprecipitation. The Western blotting analysis data showed that when myc-DDB2 and flag-AR were coexpressed in 293T cells, there was an interaction signal (Figure 8 right panel lane 4) compared with beads only control (Figure 8, right panel lanes 5). The same result was also observed by reciprocal experiment (Figure 8, right panel lanes 9 and 10). The result indicated that DDB2 can interact with AR in *vivo*. Hence, we found that AR may be a target substrate of DDB2.

The structure of AR is composed of N-terminus regulatory domain including activation function 1 (AF-1) , a DNA binding domain (DBD), hinge region containing ligand-dependent nuclear localization signal , and a C-terminal ligand-binding domain (LBD) with an additional transactivation function 2 (AF-2). We wanted to find out which domain of AR was targeted by DDB2. In the experiment, HA-tagged truncate N-terminus 1-188 amino acids of AR (Δ 188), truncate N-terminus 1-488 amino acids

of AR (Δ 488), deleted DNA binding domain of AR (Δ DBD) and deleted ligand binding domain of AR (Δ LBD) expression plasmids were constructed (shown on Figure 9A). We coexpressed wild-type or these deletion mutant AR expression plasmids into 293T cell. After 48 hours, cell lysate was harvested to proceed to co-immumoprecipitation with anti-HA antibody and Western blotting analysis was used with anti-myc antibody. The data showed that DDB2 can interact with wild-type and all mutated AR (Figure 9B). But the deleted DNA binding domain of AR (Δ DBD) and ligand-binding domain (Δ LBD) seems to decline the interaction with DDB2 compared to the other mutants (Figure 9B lane 6). It suggested that the DBD and LBD of AR may play a role on interaction between DDB2 and AR. Therefore, we will generate the deletion mutant at DBD and LBD, after that will test the interaction.

3.6 NRIP competes with DDB2 for interacting with AR

Next, we think over the mechanism of NRIP by which contributes enhanced AR protein stability. As we know that both NRIP and DDB2 can associate with AR, thus, we proposed the model that NRIP can compete with DDB2 for binding to AR. To investigate this hypothesis, we coexpressed AR, DDB2 and the increased NRIP amount of plasmids (8 μ g ,16 μ g) in 293T cell. The cell lysate was immunoprecipitated with anti-HA antibody and subjected to Western blot with anti-myc antibody to compare

the amount of immnoprecipitated DDB2. In this result, the higher expression level of NRIP reduced the interaction between DDB2 and AR (Figure 10, lane 7) compared with the control which was not transfected with NRIP expression plasmid (Figure 10, lane 5). The *in vivo* competition assay indicated that there is a competition relationship between NRIP and DDB2 for binding to AR. These results suggest that the competition between NRIP and DDB2 to interact with AR may result in AR protein stabilization.

In sum, we propose a model that the mechanism of NRIP-stabilizing AR protein (Figure 11): When NRIP was absent, ligand-bound AR translocated into nucleus, it may be a substrate and targeted by DDB2, and then be degraded through DDB1-CUL4 pathway. There is the competition relationship between NRIP and DDB2 for interacting with AR. When NRIP was present, it may protect AR from DDB2 targeting. The mechanism allowed AR to escape degradation and to be stabilized.

3.7 The mechanism of NRIP-enhancing AR mediated transcription activity may not be dependent on DDB1-CUL4 pathway.

Moreover, Previous study in our lab (Chen, Tsao et al. 2008), expression of NRIP can enhance AR mediated transcription. Recent study on DCAFs, WDR5, a member of DCAF containing WD40 domain, can associate with DDB1 and function as

transcription factor (Higa, Wu et al. 2006). Hence, we wanted to know whether the function by which NRIP contributes to AR mediated transcription activity is through DDB1-CUL4 pathway. In the experiment, we tried to express wild-type or mutated NRIP (single WDXR motif mutant: DTA, DRA or double WDXR motif mutant: DTA/DRA) in LNCaP cell and these NRIP mutants were measured for PSA gene expression. Interestingly, mutated NRIP (can not associate with DDB1) can also enhance RNA level of PSA, which is an AR downstream targeted gene (Figure 12 lane 5). It may suggest that the transcription activity of NRIP may not be dependent on DDB1-CUL4 pathway.

CHAPTER 3

RESULTS

Part two：Construction and generation of NRIP knockout mice and the mechanism of NRIP –stabilizing AR protein.

3.8 Construction of NRIP knockout mice

Mouse NRIP locus located on chromosome 1. It had 19 exons with 3,067 bps transcript length 3,067 bps and encoding 876 amino acids share 86% identity with human NRIP. To investigate the function of NRIP in animal model, we sought to generate a strain of mice lacking expression of NRIP. To generate NRIP knockout mice, we deleted exon 2 through homologous recombination in ES cells (cooperated with National Research Program for Genome Medicine Transgenic Mouse Models Core lab). Mouse NRIP exon 2 contains 62 bps and deletion of exon 2 would be induced frameshfit on the transcript with a premature stop-codon (Figure 13). The mutant mRNA would encode a nonfunction protein product with 33 amino acids (Figure 13). Mouse chimeras were crossed with wild type C57BL/6 mice to obtain stable NRIP heterozygotes (+ / -), and the hetrozygotes were used to generate NRIP homozygous knockout mice (- / -).

3.9 Confirmation of NRIP knockout mice by genotyping

To confirm the recombination, we designed primers in NRIP intron 1-2 and intron 2-3 (Figure 14A), and genomic DNA from three genotypes of mice was used to proceed to PCR (Figure 14B). The data showed that homologous NRIP KO genotype appeared 700 bps with designed AU-XD primers and disappeared the PCR products with KU-XD primers. With the DNA typing result, we further detected RNA expression of NRIP, total RNA from the heart and testis tissues of WT/KO mice were analyzed by RT–PCR assay (Figure 15B). The PCR primers corresponded to sequences present in exon 1 (primer F1), exon 2 (Primer F2) and exon 3 (Primer R) (Figure 15A). No NRIP-mRNA expression was detected in the heart or testis tissue of the NRIP - / - mice with F2-R primers (Figure 15B, middle panel lanes 2 and4). The two PCR products in the wild-type and knockout mice corresponded to two alternatively spliced mRNAs: the shorter product corresponded to an mRNA lacking exon 2 (Figure 15B, top panel, WT: 330 bps, KO: 268 bps). In this result, we further confirm the deletion of exon2 in NRIP knockout mice. As we mentioned earlier, deletion of exon 2 makes a premature NRIP mRNA. Hence, we would detect the protein expression of NRIP. Protein sample from skeletal muscle tissue of wild-type and knockout NRIP mice was subjected to Western blot with anti-NRIP antibody. In the Western blotting data, endogenous NRIP was not observed in knockout tissue (Figure 16, upper penal lane 4) compared with wild-type

tissue (Figure 16, upper penal lane 3). The LNCaP cell lysate was used as a marker for confirmation of NRIP molecular size (Figure 16, upper lane 1: siNRIP and 2: pSUPER vector). Collectively, we had successfully generated NRIP knockout mice and confirmed the genotype of NRIP knockout mice on DNA, RNA and protein level.

3.10 NRIP can regulate the mouse embryonic cell growth

On previous study of our lab, NRIP seems to regulate variable cell lines proliferation. Since knockdown of NRIP inhibits the cell growth of cervical cancer cell (SiHa, HeLa) and induces apoptosis in prostate cancer cell (LNCaP) (unpublished data). The phenomenon indicates that NRIP may regulate cell growth generally and somehow be involved in cell survival. To investigate the function of NRIP, we had generated NRIP wild-type and knockout mouse embryonic fibroblast (MEF) cell from the E14 embryo. Genomic DNA of the MEF cells was typed by PCR with designed primers (Figure 17A, AU-XD primer: knockout genotype appeared 700bps product and KU-XD primer: knockout genotype disappeared the PCR product) and the difference of RNA splicing form from MEF cells was also confirmed by RT-PCR using F1, F2, R primers as described earlier on Figure 15 (Figure 17B, WT: 330 bps, KO: 268 bps). The isolated wild-type and knockout MEF cell was maintained in DMEM medium condition with 10% FBS. After three passage, the WT and KO MEF cell were seeded at 1×10^5 per

well on the 6 well culture plate and the cell numbers were counted by trypan blue exclusion method at 24h, 48h,72h, 96h. The cell proliferation assay showed that WT MEF cell had higher proliferation ability (Figure 17C). It may suggest the role of NRIP in cell growth.

3.11 Expression of NRIP can enhance AR protein level in MEF cell

In the part one of result, NRIP can stabilize AR protein in LNCaP cell. To further investigate the mechanism in primary cell, the NRIP knockout mouse embryonic fibroblast (MEF) cell was electroporated with pCMV-NRIP-3xflag plasmid. After 48 hours, the cell lysates were harvested and then used to compare these AR protein expression level by Western blotting analysis. The result showed on Figure 18 (middle penal lanes 1 and 2), enhancement of AR protein level was observed when introducing NRIP expression plasmid. It may suggest that NRIP can enhance AR protein level in the primary MEF cell.

Chapter 4

DISCUSSION

Here, we first showed that NRIP can reduce ubiquitylation level of AR protein. To further investigate the mechanism, we had found that DDB1-CUL4A pathway was involved in AR protein degradation and DDB2 may be a substrate receptor to target AR protein. In this thesis, the mechanism by which NRIP contribute to enhanced AR protein stability may be competiting with DDB2 for binding to AR (Figure 11).

AR degradation in DDB1-CUL4 pathway

When silencing DDB1 or CUL4A by siRNA, it can enhance AR protein level in LNCaP cell (Figure 2D and 3) and ectopic expression of DDB2 can induce AR degradation (Figure 6, 7). It suggested that AR degradation may depend on the function of DDB1-CUL4A^{DDB2} E3 ligase. However, some questions remained to be investigated. First, although the ubiquitylation function of DDB2 is well-known through DDB1-CUL4 pathway, no evidence in the thesis can support that DDB2-induced AR degradation relies on DDB1-CUL4 pathway. Second, DDB2 also functions as a transcription activator (Hayes, Shiyanov et al. 1998) or repressor (Minig, Kattan et al. 2009). It remained unclear whether the influence of DDB2 on AR protein level is

through protein degradation or silencing the transcription of AR. Third, the detailed interaction between AR and DDB2 was not clear, the DNA binding domain (DBD) and ligand binding domain (LBD) of AR seems to play a role for this interaction. But, the deletion of DBD or LBD did not fully abolish this interaction; it may suggest that DDB2 target AR through multiple region. Fourth, according to the previous study on AR degradation by DDB1-CUL4 pathway, it has been reported that AhR (dioxin receptor), which is a DDB1-interacting protein, can associate with DDB1-CUL4B to assemble E3 ubiquitin ligase in the presence of its ligand (3-methylcholanthrene) (Ohtake, Baba et al. 2007). AR can be ubiquitinated by this E3 ligase. In the thesis, we found that DDB2 may also influence AR protein degradation in a DDB1-CUL4-dependent manner. This finding may indicate that both of the DDB1-binding protein (AhR and DDB2) can target AR protein and further promote the degradation of AR. Among the DCAF members, both DDB2 and CSA can ubiquitinate the histone on DNA damage region. Nevertheless, histone ubiquitination by DDB2 may occur in global genomic nucleotide excision repair, whereas histone ubiquitination by CSA occur in transcription coupled nucleotide excision repair (Lee and Zhou 2007) (Hakem 2008) (Nouspikel 2009). Based on these reports, it is possible that AhR-induced and DDB2-induced AR degradation pathways occurred in different conditions, which may involve various protein factors, such as post-translational

modifiers and other unknown regulators.

In addition, previous reports also showed that the expression of DDB2 can be triggered by p53 (Tan and Chu 2002) and AR activity can be repressed by p53 (Eastham, Grafton et al. 2000) (Mantoni, Reid et al. 2006). It is probably that DDB2-induced AR protein degradation may be arisen from p53 effect. The dependence of p53 on the stability of AR protein will therefore be further investigated.

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The new tumor suppressor role of DDB2 in nuclear receptor degradation

Based on previous data, we found a new role of DDB2 may involve in AR protein degradation. DDB2 is an essential factor for DNA repair in mammalian cell. Moreover, DDB2 is mutated in the xeroderma pigmentosum (group E). In additon to DNA repair, DDB2 may function as transcription factor to regulate gene expression. Several lines of evidence have suggested the active involvement of DDB2 in tumor progression. It had reported that DDB2 acts as a co-factor of E2F1 (Hayes, Shiyanov et al. 1998) and is associated with chromatin-acetylating transcription coactivator, STAGA complex (Martinez, Palhan et al. 2001). Moreover, expression of DDB2 is induced by p53, BRCA1 and by ionizing radiation in human cells (Tan and Chu 2002) (Hartman and Ford 2002). Recent study on DDB2 knockout mice, DDB2-deficient mice not only were

hypersensitive to UV-induced skin carcinogenesis but also developed a high rate of malignant tumor in internal organ which indicate DDB2 function as a tumor suppressor (Itoh, Iwashita et al. 2007) (Yoon, Chakrabortty et al. 2005) (Itoh, O'Shea et al. 2003).

Here, we showed that DDB2 was involved in AR protein degradation. It may reveal a novel function of DDB2 to suppress tumor development. In further study, it still requires more demonstration on the mechanism of AR degradation induced by DDB2 and the negative regulation of DDB2 on the AR-downstream gene expression.

Putative mechanisms by which NRIP contributes to stability of AR

Our study showed that NRIP compete with DDB2 for binding to AR, and result in enhanced AR protein stability. On previous study, Akt-MDM2 pathway was reported to be involved in AR degradation. The active Akt induced phosphorylation of AR as well as MDM2. Phosphorylated AR subsequently undergoes polyubiquitination by the active MDM2, followed by AR protein degradation (Lin, Wang et al. 2002). It was reported that the interaction is through AR-DBD domain and MDM2-RING finger domain (Lin, Wang et al. 2002). As we know, NRIP utilizes C-terminus sixth, seventh WD40 domain interact with AR-LBD (unpublished data). There is close between DBD (559- 631 amino acids) and LBD (631-915 amino acids). It is unclear whether the interaction between NRIP and AR block the MDM2 binding to AR. Thus, we supposed other

putative mechanism by which NRIP contribute to AR protein stability, MDM2 may be another competitor for binding AR.

Transcription activity of NRIP not rely on DDB1-CUL4 pathway

Previous study in our lab (Chen, Tsao et al. 2008), expression of NRIP can enhance AR mediated transcription. Thus, NRIP was identified as an AR coactivator. WDR5, a WD40 domain protein, and can associate with DDB1 and function as transcription factor (Higa, Wu et al. 2006) . WDR5 was reported to modulate histone methylation and its function was proposed to be associated with DDB1-CUL4 pathway. In the figure 11, we had excluded the possibility that NRIP function on AR mediated transcription activity rely on DDB1-CUL4 pathway. It may suggest that the transcription activity of NRIP may be involved in other pathway.

The transcription activity of AR was regulated by its coregulator. More than 200 androgen receptor coregulator had been identified since the isolation of the first androgen receptor coactivator- SRC1 in 1995 (Onate, Tsai et al. 1995).These coregulators possess different functions and involve in different pathways to regulate AR transcription activity：(1) Chromatin remodeling complex, such as the ATPases Brahma-related gene 1 (BRG1) required for nucleosome repositioning by the mating type switching/sucrose nonfermenting (SWI/SNF) chromatin remodeling complex to

stimulate AR transcription activity (Marshall, Link et al. 2003). (2) Histone modifiers (acetylation or methylation), for example, two member of the members of the p160 SRC gene family, SRC-1 and SRC-3, which possess histone acetyltransferase activity, can enhace AR-mediated transcription (Alen, Claessens et al. 1999). Lysine specific demethylase 1 (LSD1), which specifically demethylates monomethylated and dimethylated H3K9, can interact with the AR and stimulate AR-dependent transcription (Metzger, Wissmann et al. 2005). (3) Splicing factor, like PSF (polypyrimidine tract-binding protein-associated splicing factor) and interact with AR and the RNA polymerase II carboxy-terminal domain (CTD). These coregulators could serve as a "molecular link" between the transcriptional machinery and the splicing machinery (Auboeuf, Dowhan et al. 2005). (4) Cytoskeletal protein, such as Gelsolin interacts with the AR during nuclear translocation and enhances ligand-dependent AR activity (Nishimura, Ting et al. 2003). (5) Protein involved in endocytosis, for instance, Caveolin is a principal component of caveolae membranes that serve as a scaffold protein of many signal transduction pathways. In the presence of ligand, Caveolin-1 can interact with the AR and increases nuclear localization of the AR and potentiates ligand-dependent AR activation (Cheng, Singh et al. 2006).

All the cellular factors mentioned above are apparently good candidates and directions for further investigation. The mechanism by which NRIP enhance

AR-mediated transcription activity will be examined in detail by searching more NRIP-interacting protein as well as AR-interacting protein involved in AR-mediated transcription.

Finding the roles of NRIP in animal model

To further investigate function of NRIP *in vivo*, we had planned to generate NRIP-deficient mice. In NRIP knockout mice, we deleted exon 2 of NRIP which can make NRIP protein be produced failed by transcript frameshift. In the thesis, the NRIP knockout mice were successfully generated and confirmed the genotype in DNA, RNA and protein level.

With previous study, NRIP mRNA was expressed in brain, heart, prostate, liver and especially highly expressed in skeletal muscle and testis (Tsai, Lee et al. 2005). Limb-girdle muscular dystrophy (LGMD), a kind of progressive muscular dystrophy (PMD), in which the pelvic and shoulder girdle musculatures are predominantly or primarily involved. This kind of disease is also characterized by increased SCK (serum creatine kinase) levels, muscle fiber necrosis, and regeneration (Fardeau, Hillaire et al. 1996). It was reported the gene expression of NRIP as well as gene relative to muscle development and function were downregulated in the dystrophic muscle from LGMD patient. It may suggest that NRIP functions as an important factor to regulate normal

skeletal muscle function. In our lab, preliminary data showed that IGFBP6 (insulin-like growth factor binding protein 6), which is associated with myoblast quiescence and can inhibit Rhabdomyosarcoma progression (Gallicchio, Kneen et al. 2001) (Koike, Ito et al. 2005), was up-regulated in NRIP knockout mice. Furthermore, NRIP contain IQ motif, which was found in diverse families of calmodulin (CaM)-binding proteins. Some of these are highly abundant in neuronal and skeletal muscle tissues (Putkey, Kleerekoper et al. 2003). The interaction between NRIP and calmodulin was confirmed by in vitro binding assay in presence of calcium (unpublished data). Collectively, the role of NRIP in muscle contraction and regeneration would be an interesting topic for future studies.

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FIGURES

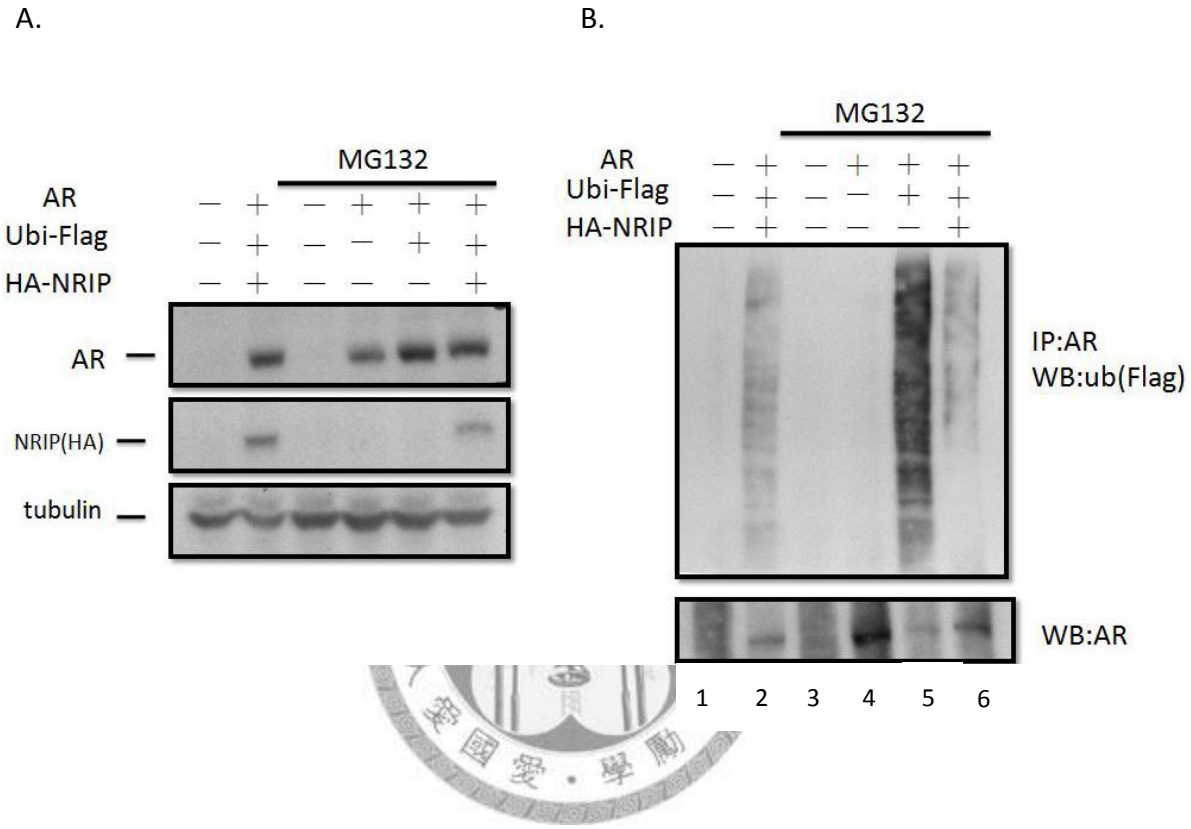


Figure 1. **NRIP reduced the ubiquitin level of AR.** A, Input from NEK293T cells which was transfected with equimolar amounts of AR (pcDNA3.1-AR: 10 μ g), HA-tagged NRIP(pCMV-HA-NRIP: 10μ g), flag-tagged ubiquitin (5μ g). B, After 48 hours ,the cell were treated with 20μ M for 6 hours incubation. The cell lysate was used to proceed with immunoprecipitation with anti-AR antibody and then subjected to Western blotting analysis with anti-flag antibody.

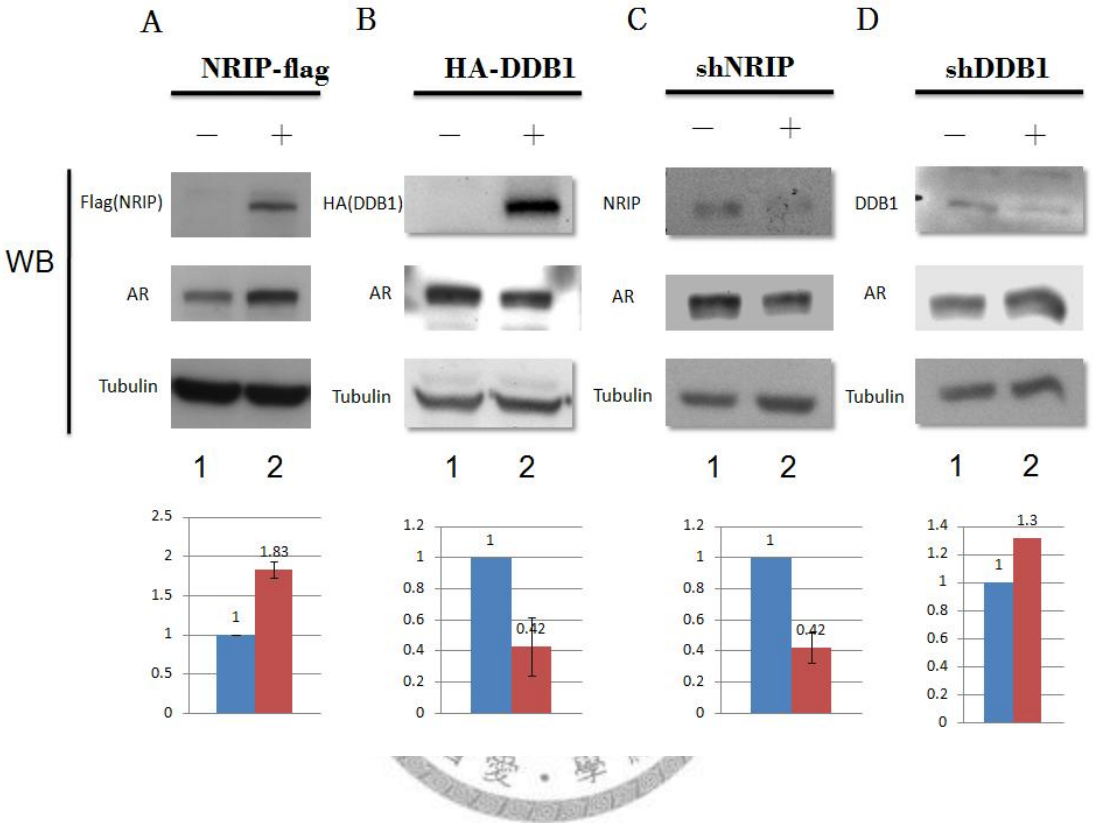


Figure 2. **NRIP enhanced AR protein stability and DDB1 reduced AR protein stability.**

AR protein level was observed in Prostate cancer cell LNCaP, which was electroporated with (A) pCMV14-3xflag-NRIP plasmid (B) pCDNA3.1-HA-DDB1 (C) pSUPER-shNRIP (D) pSUPER-shDDB1 and their corresponding control vector. The quantitative data of AR protein was analyzed by UVP and presented as mean ±SD.

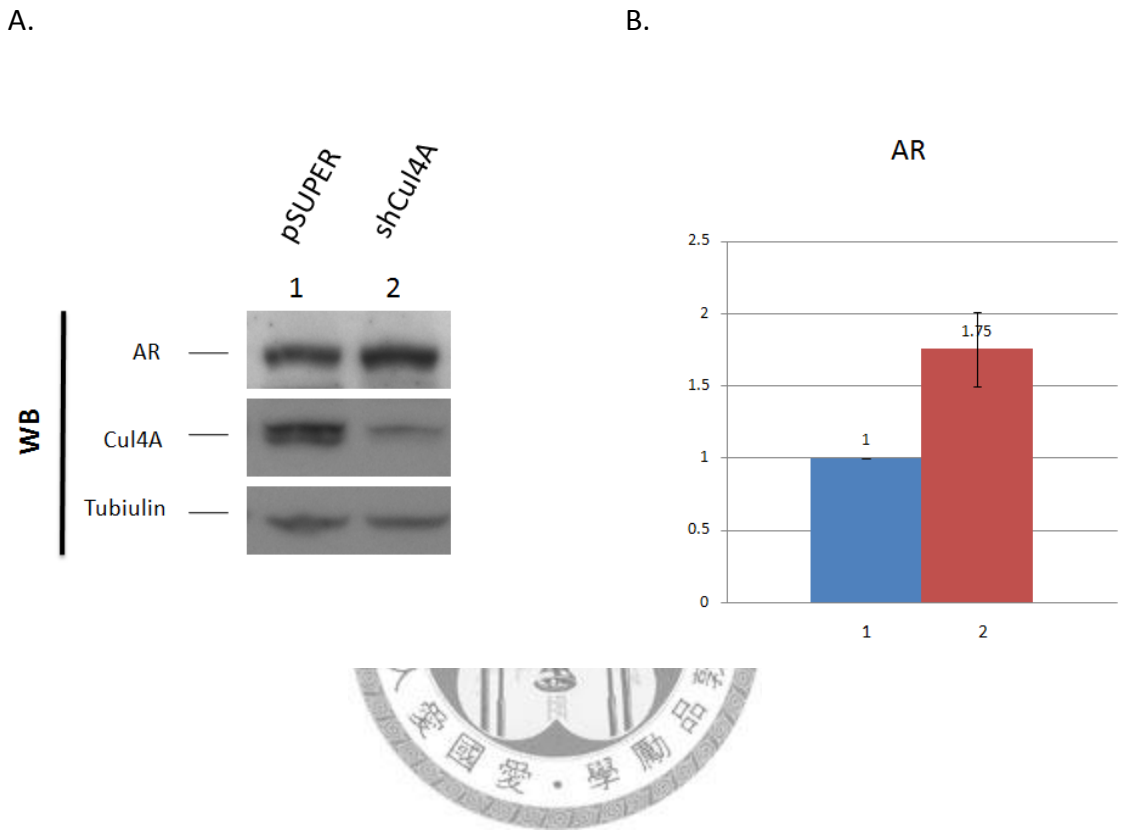


Figure 3. **Knockdown of CUL4A enhanced AR protein level.**

A. LNCaP cell was eletroporated with pSUPER-shCUL4A and vector control (pSUPER). The AR protein level was detected by Western blotting analysis with anti-AR antibody.

B. The shift of AR protein level was compared with control and analyzed by UVP. The quantitative data was presented as mean ±SD.

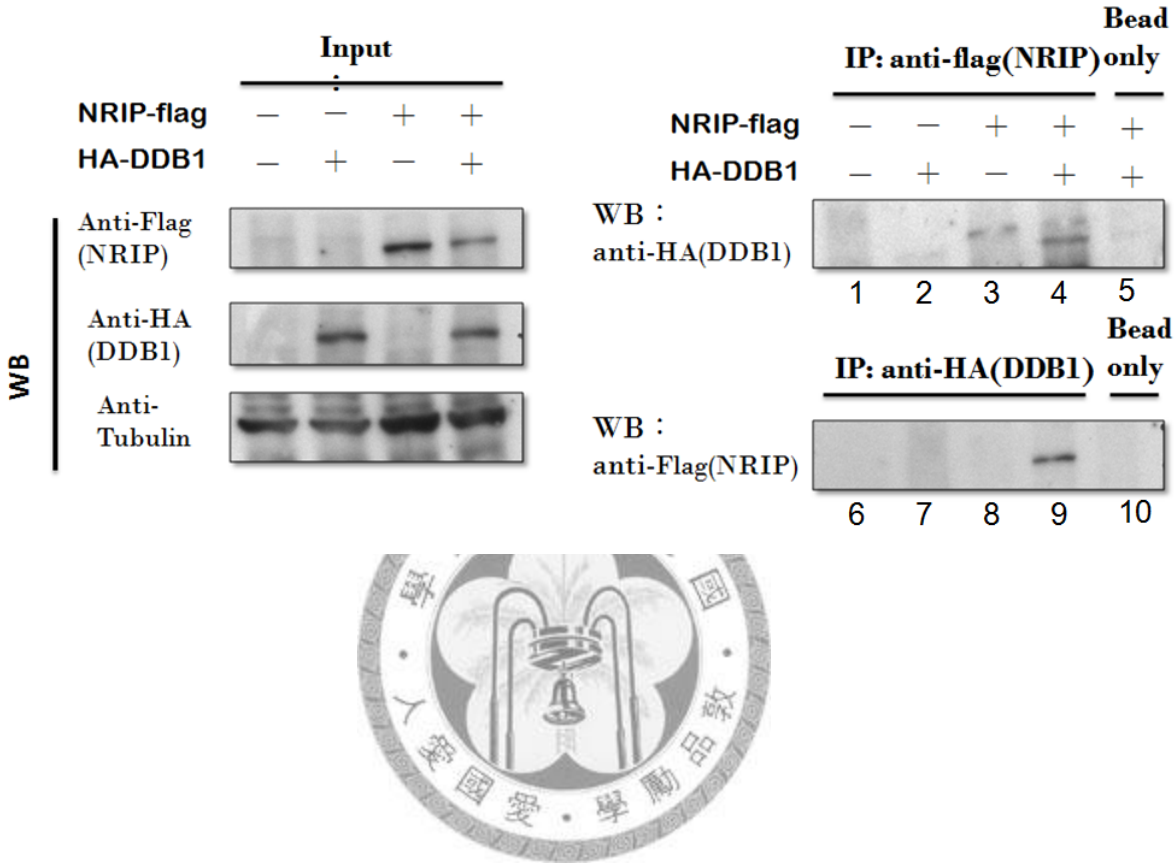


Figure 4. **NRIP can associate with DDB1.** Co-immunoprecipitation indicated the interaction between NRIP and DDB1 *in vivo*. 293T cell lysates were harvested 48h after transfection with pcDNA3.1-HA-DDB1(12μ g) and pCMV14-Flag-NRIP(10μ g). The harvested cell lysate were immunoprecipitated reciprocally with anti-HA antibody or anti-flag antibody.

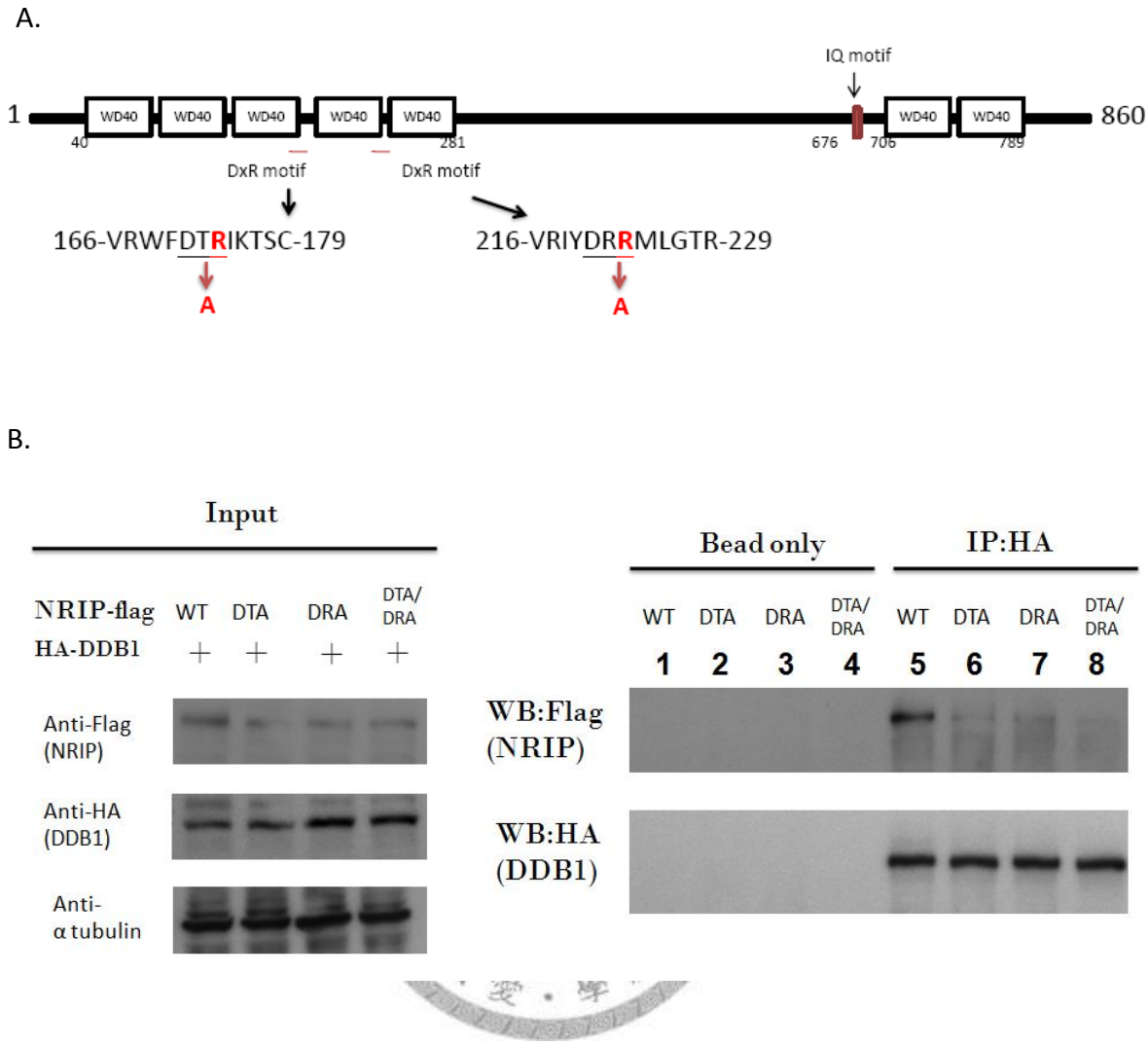


Figure 5. **WDXR motif on NRIP is essential for binding DDB1.** A. Site-direct mutation in predicted WDxR motif on NRIP third and fourth WD40 domain. B. Co-immunoprecipitation indicated the interaction between wild-type /mutant NRIP and DDB1 *in vivo*. The flag-tagged wild-type NRIP or mutant NRIP expression plasmid (pCMV14) (12μ g) was cotransfected with pcDNA3.1-HA-DDB1 (10μ g) in 293T cell, and then immunoprecipitated with anti-HA antibody and was subjected to Western blot with anti-flag antibody.

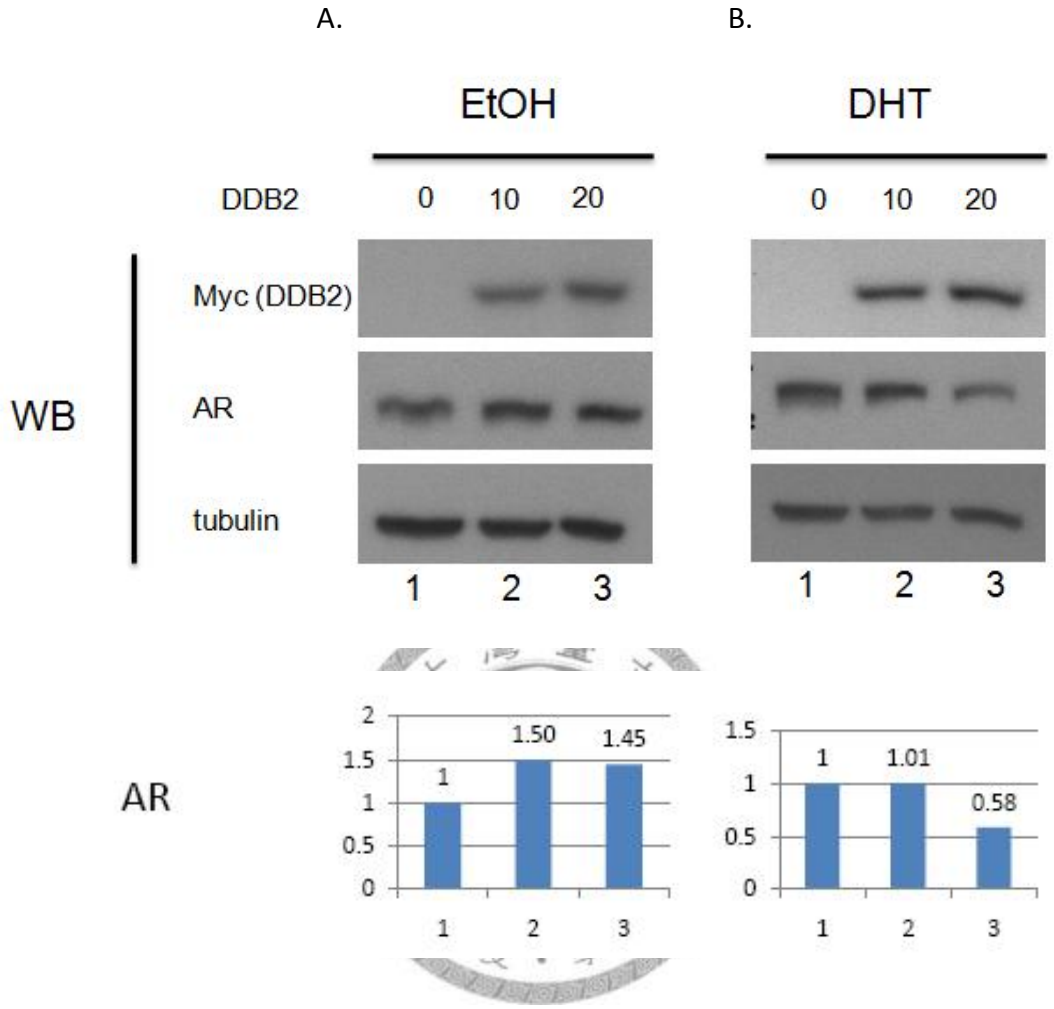


Figure 6. **DDB2 influence on AR protein level in LNCaP cell.** LNCaP cells were eletroporated with pcDNA3.1-myc-DDB2 expression plasmids (10μ g and 20μ g) and control vector. After 24h, cells were then treated with vehicle (A) or 10nM DHT (B) for 24h, the AR protein expression level was analyzed by Western blot. The quantitative data of AR protein was normalized by tubulin signal and analyzed by UVP.

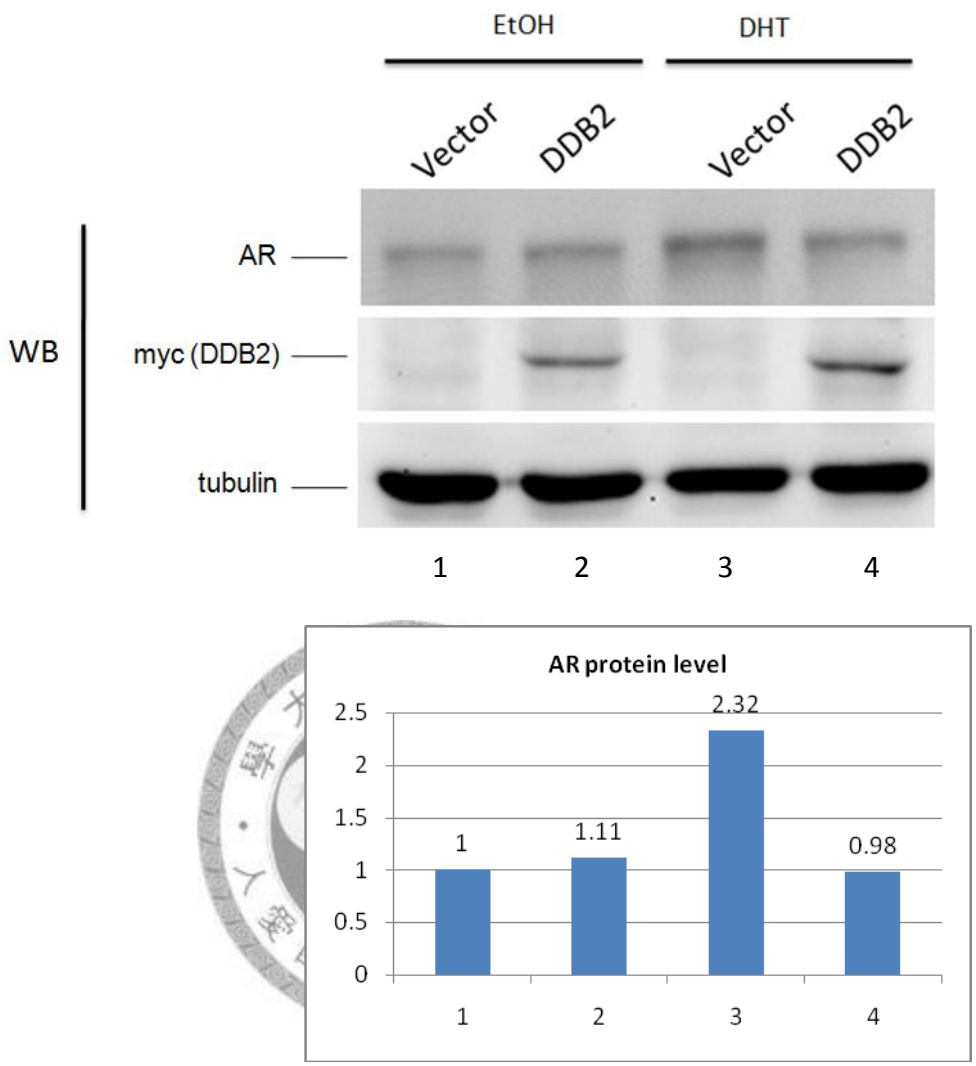


Figure 7. **DDB2 influence on AR protein level in 22RV1 cell.** 22RV1 cells were eletroporated with pcDNA3.1-myc-DDB2 expression plasmids (20μ g) and control vector. After 24h, cells were then treated with vehicle (lanes 1 and 2) or 10nM DHT (lanes 3 and 4) for 24h, the AR protein expression level was analyzed by Western blot. 22RV1 cell with control vector or DDB2 expression plasmids under ethanol vehicle (lane 1and lane 2), 22RV1 cell with control or DDB2 expression plasmids under 10nM DHT treatment (lane 3 and lane 4). The quantitative data of AR protein was analyzed by UVP and normalized by tubulin signal.

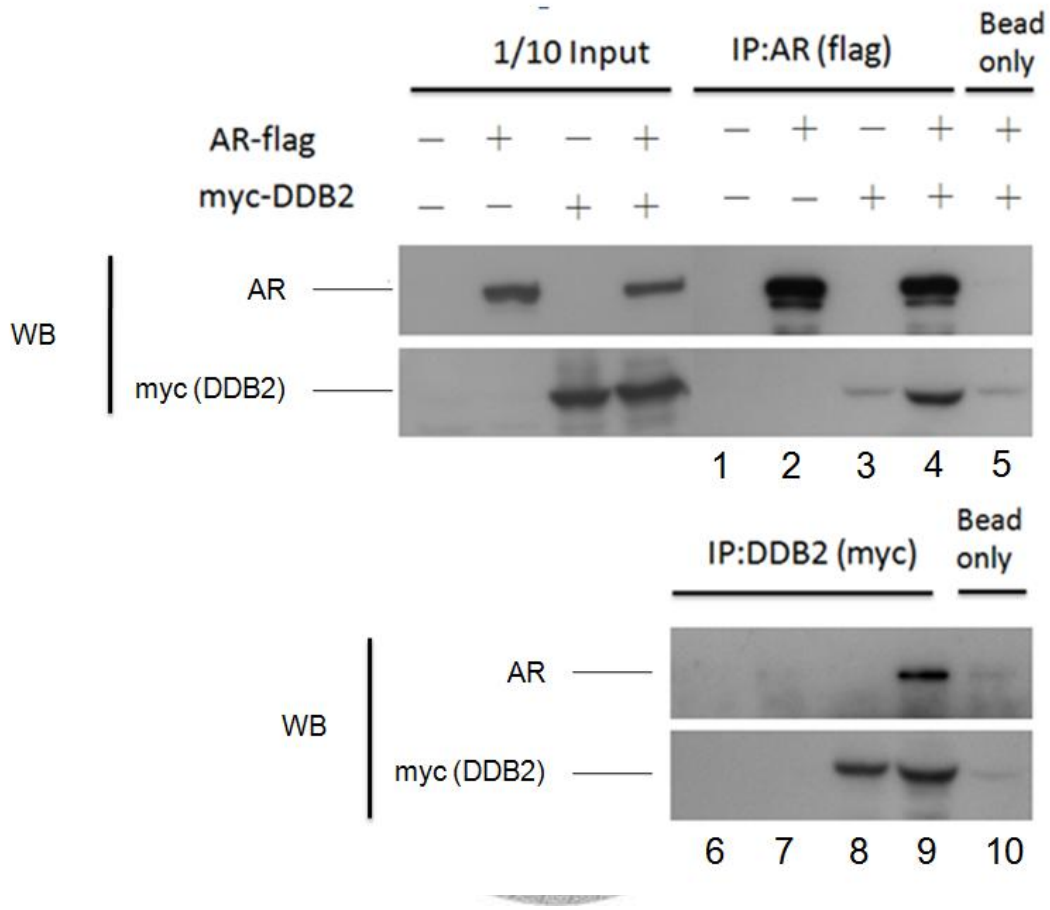


Figure 8. **DDB2can associate with AR.** Co-immunoprecipitation indicated the interaction between DDB2 and AR *in vivo*. 293T cell lysates were harvested 48h after transfection with pcDNA3.1-myc-DDB2 (8μ g) and pSIN-flag-AR-GFP (10μ g), and then immunoprecipitated reciprocally with anti-HA antibody or anti-flag antibody.

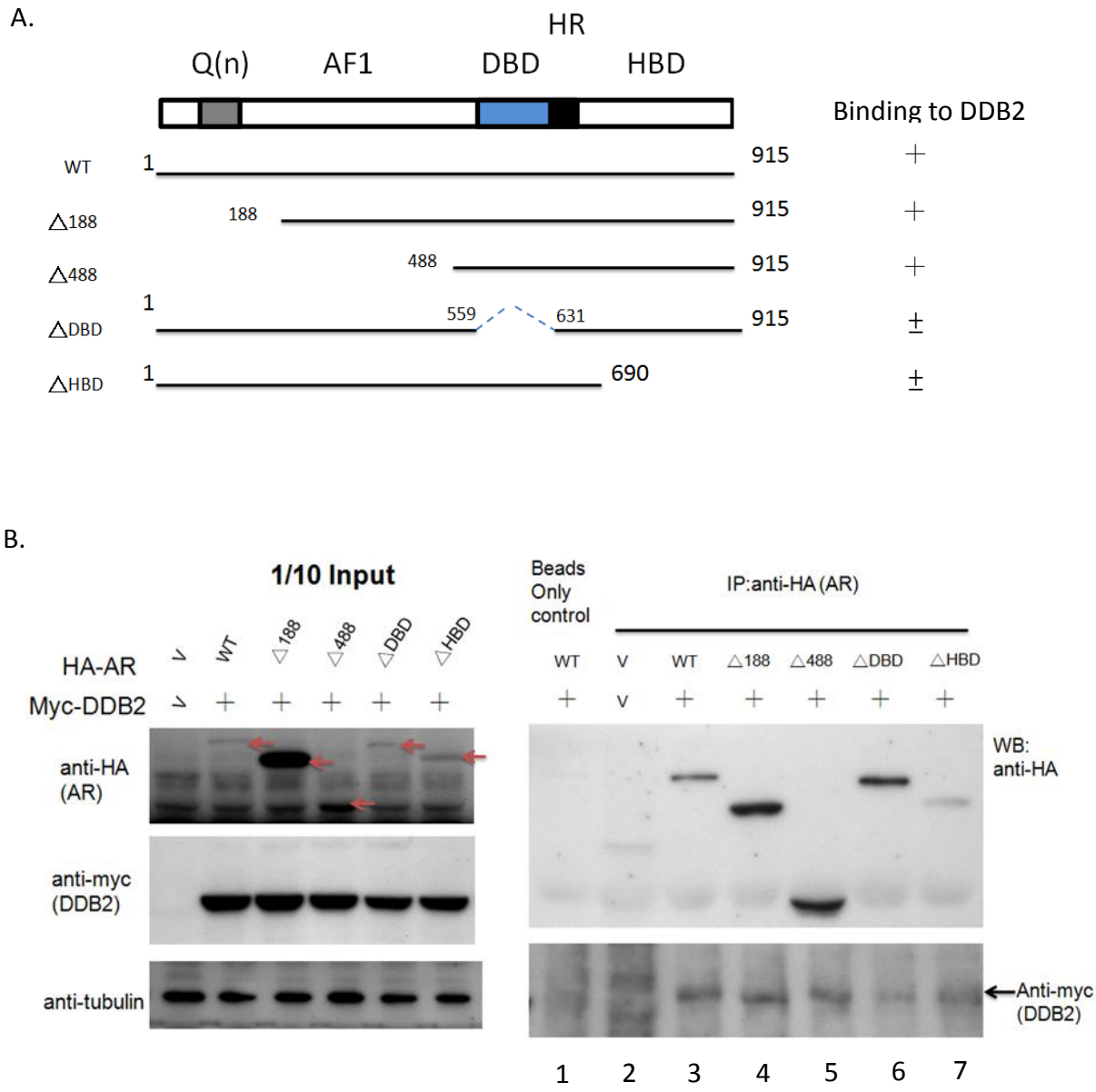


Figure 9.**The interaction map between DDB2 and AR.** A, HA-tagged truncated N-terminus 1-188 amino acids of AR, truncated N-terminus 1-488 amino acids of AR, deleted DNA binding domain of AR and deleted ligand binding domain of AR was shown on upper figure. B, The wild-type or deletion HA-AR (pCMV vector) were cotransfected with pcDNA3.1-myc-DDB2 in 293T cell. After 48 hours, the cell lysate was harvested to proceed to immunoprecipitation with anti-HA antibody and was subjected to Western blot with anti-myc antibody. (v: vector)

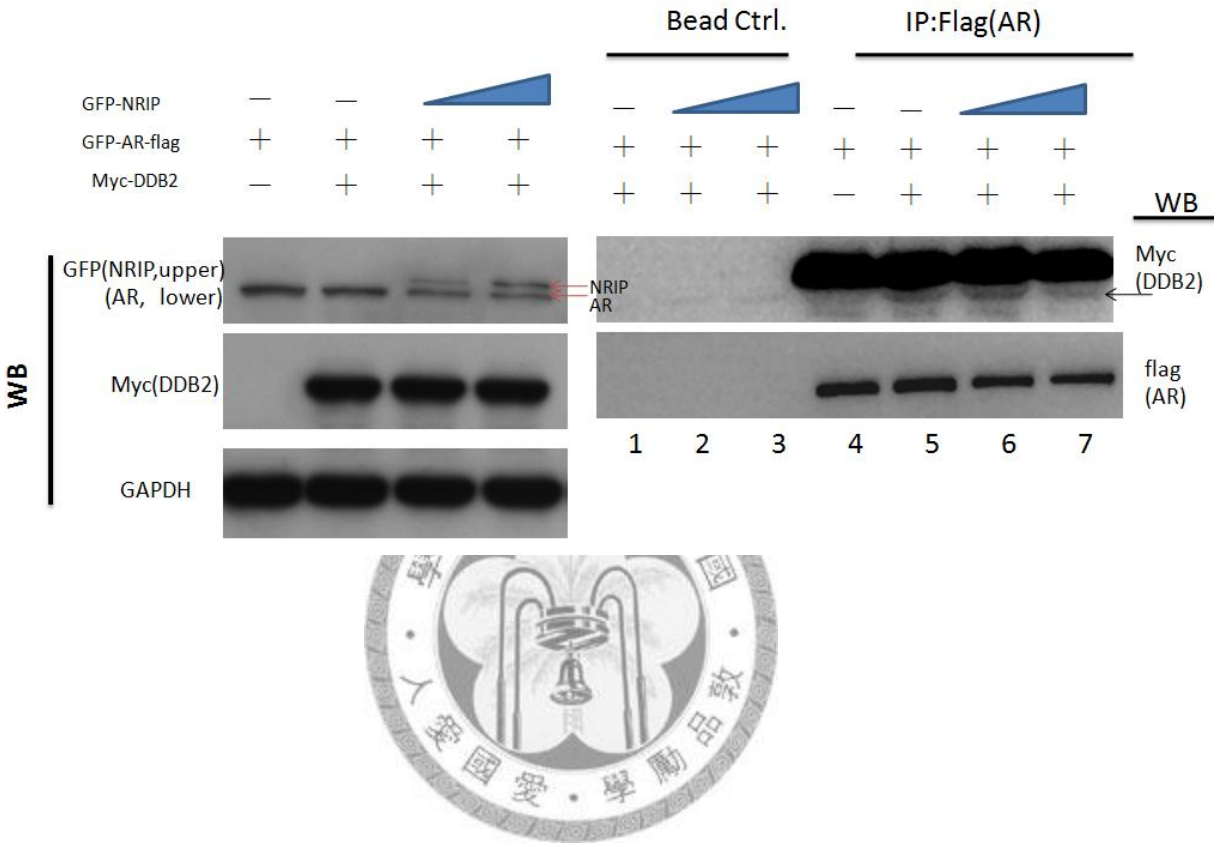


Figure 10. **NRIP and DDB2 compete for binding to AR.** *A*, Co-immunoprecipitation experiments with extracts from transiently transfected 293T cells. Equal amounts of plasmids expressing myc-tagged DDB2 (pcDNA3.1-myc-DDB2: 5μ g) ,flag-tagged AR (pSIN-GFP-AR-flag: 10μ g) ,and dose-expressing amounts of plasmids expressiong GFP-tagged NRIP (pEGFP-NRIP: 8μ g, 16μ g) and were transfected in pairwise combinations or all three together. The cell extracts were prepared after 48 hours and subjected to immunoprecipitation with anti-flag antibody. The immunoprecipitates were separated by SDS-PAGE and analyzed by Western blot assays for the presence of flag-AR with an anti-flag antibody (*IP*). The presence of co-immunoprecipitated myc-DDB2 was detected using anti-myc antibody.

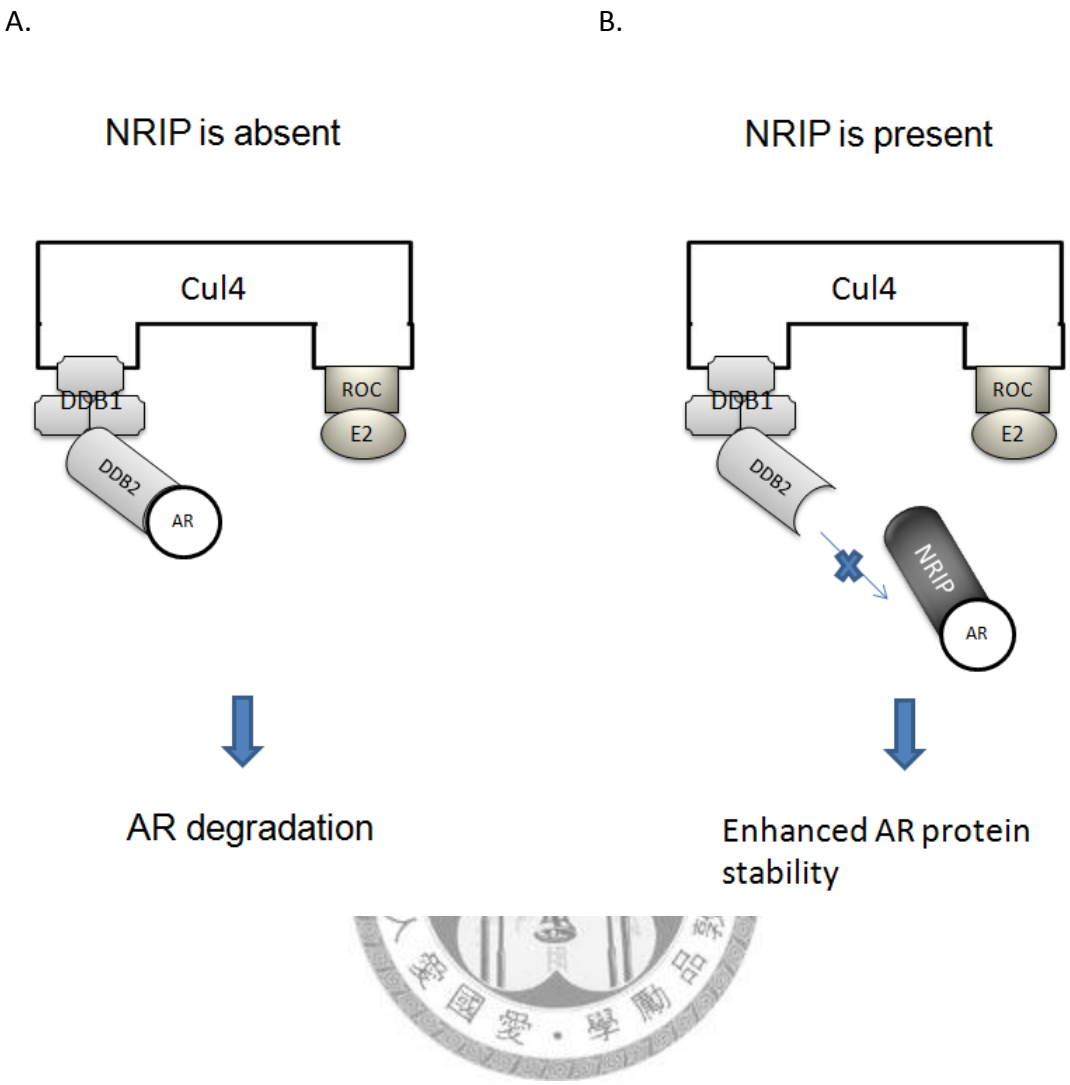


Figure 11. **The proposed models of NRIP-stbilizing AR protein.**
A,When NRIP was absent, ligand-bound AR translocated into nucleus, it may be a substrate and targeted by DDB2, and then be degraded through DDB1-CUL4 pathway. B, When NRIP was present, it may compete with DDB2 for binding to AR. The mechanism allows AR to escape degradation induced DDB1-CUL4 E3 ligase and to be stabilized.

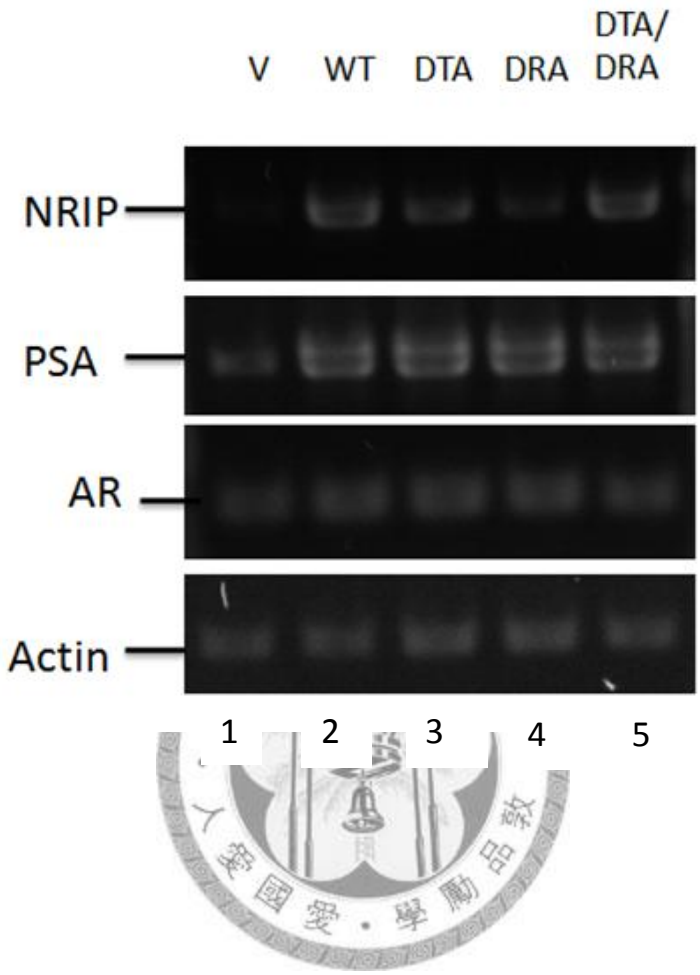
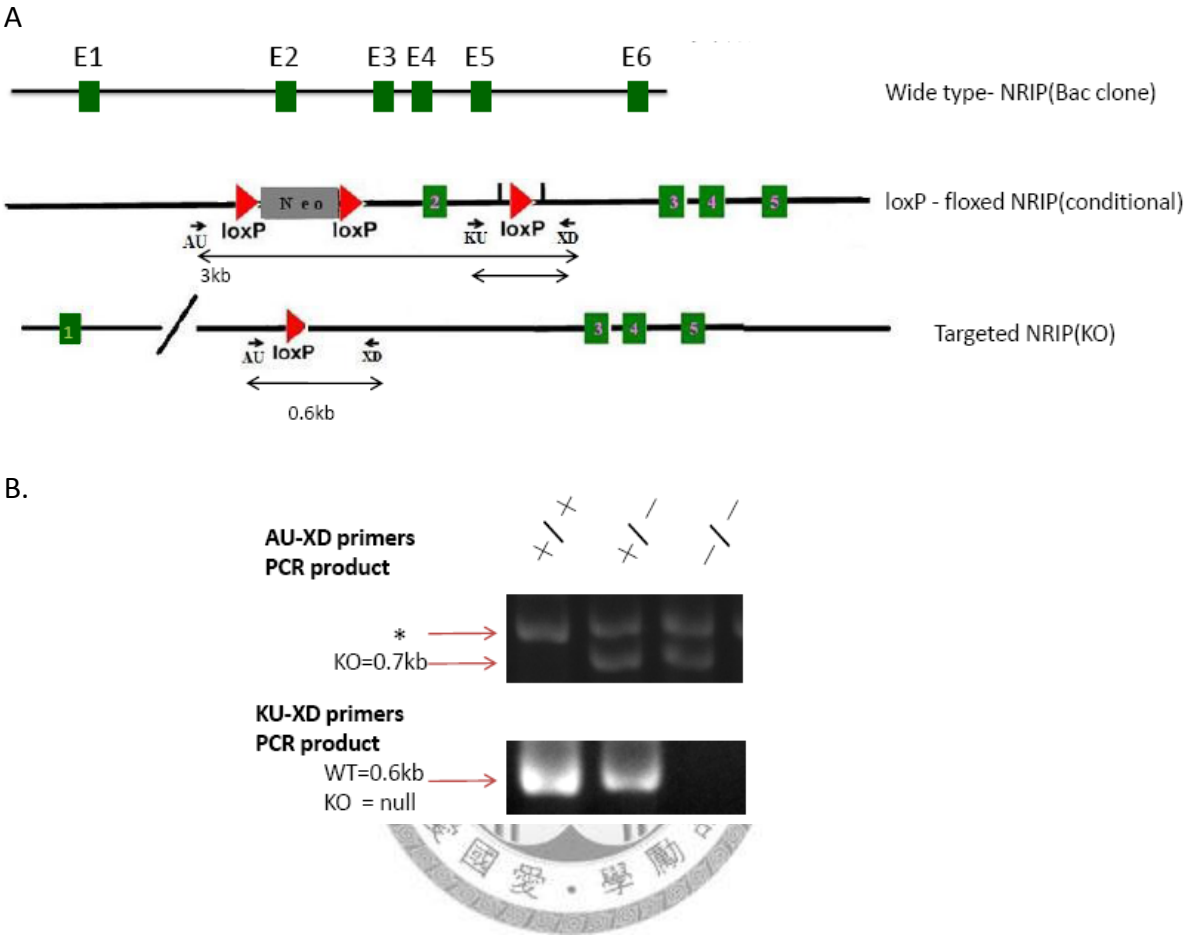


Figure 12. **The effect of wild-type and mutant NRIP on AR mediated transcription activity.** LNCaP cells were eletroporated with wild-type or WDXR motif mutated NRIP expression plasmids. After 48h, Total RNA isolated from transfected cells were reverse-transcribed and amplified by RT-PCR using primers to detect PSA RNA expression level.

Figure 13. **Frameshift of NRIP transcript induced by deletion of exon 2.** The grey labeled region represents exon 2 nucleotide sequence.



Fugure 14. **Production of NRIP knockout mice.** A. Disruption of the NRIP locus. The figure showed the structure of the NRIP wild-type genomic locus, loxP-floxed NRIP genomic locus and targeted locus. B. NRIP KO mice genotyping by PCR, the tail genomic DNA was extracted and was genotyped by PCR with designed primers (showed on A) (∗: nonspecific bond).

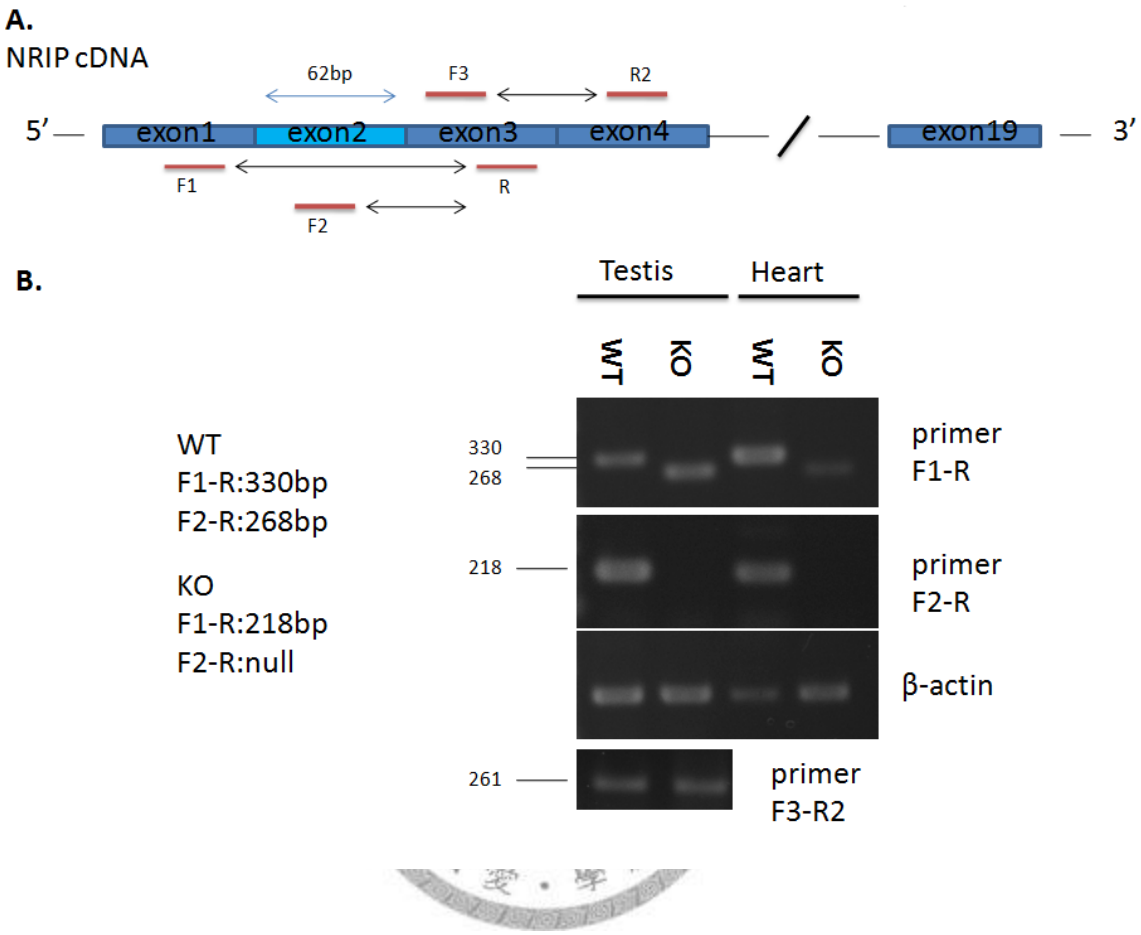


Figure 15. **NRIP knockout mice genotyping by RT-PCR analysis.** A. the designed primers to detect deletion of exon 2, their target site as showed as indicated. B. Total RNA isolated from heart and testis of wild-type and knockout mice were subjected to RT-PCR analysis using the designed primers.

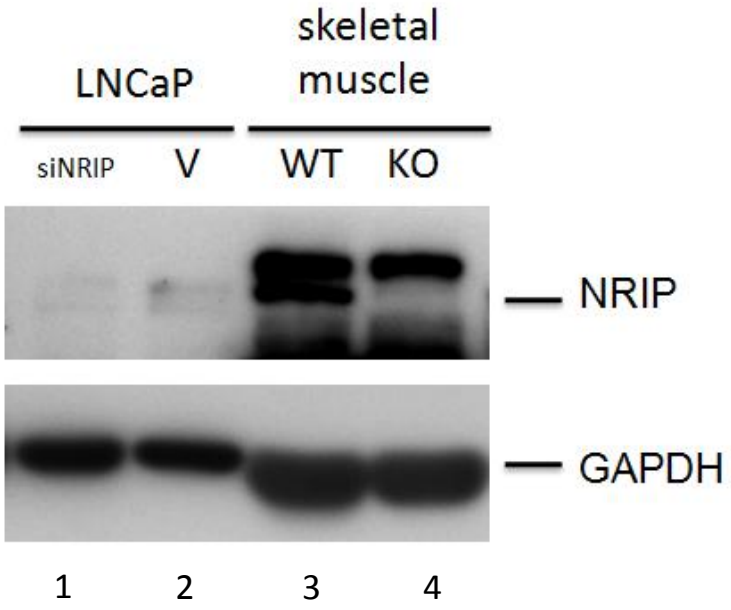


Figure 16. **NRIP protein expression of NRIP wild-type and knockout mice.** Skeletal muscle tissue from NRIP wild-type and knockout mice was subjected to Western blotting analysis with anti-NRIP antibody. The LNCaP cell lysate (knockdown NRIP or not) was used as a mark for the endogenous NRIP molecular size.

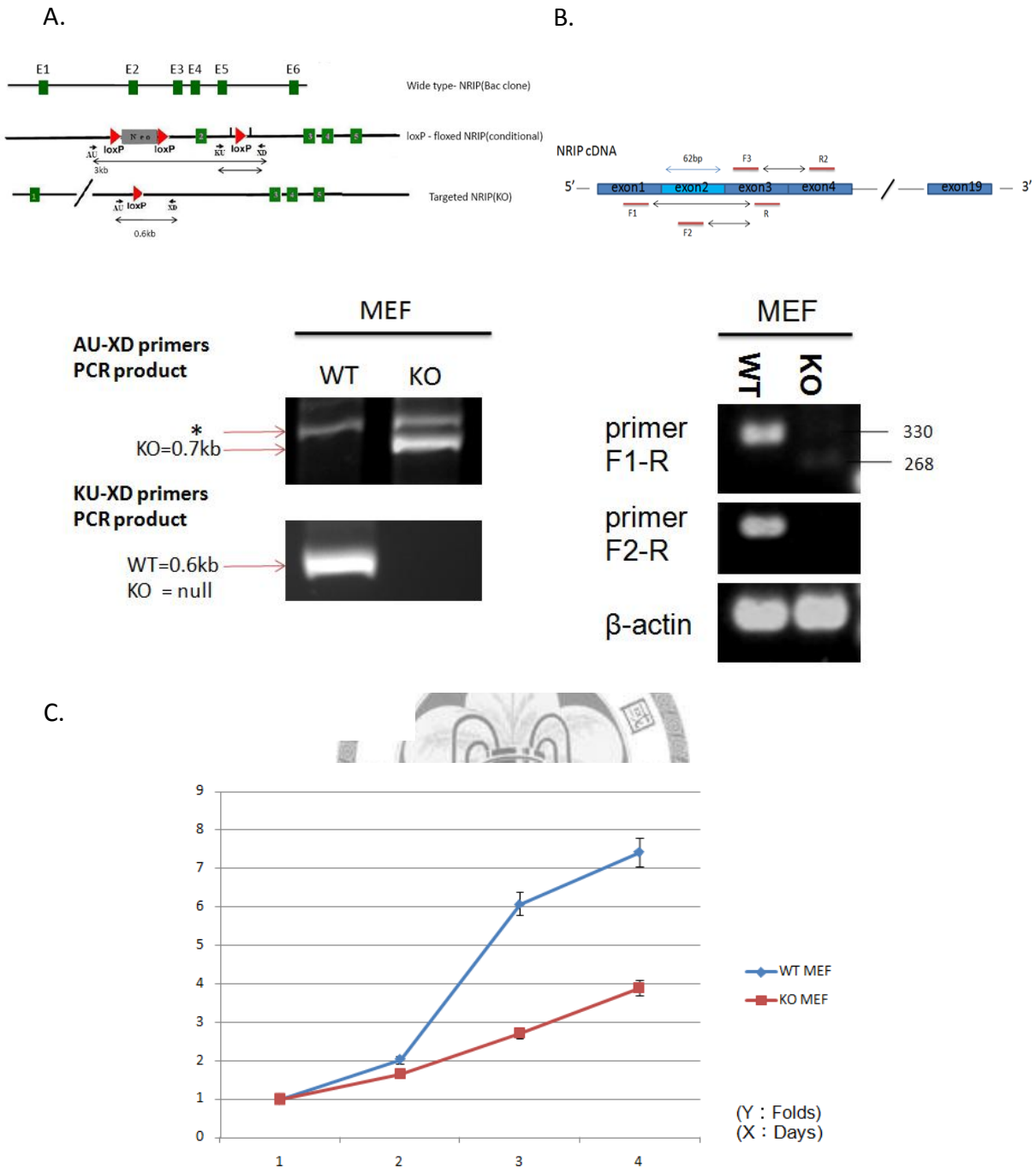


Figure 17. **NRIP can regulate the mouse embryonic cell growth.** The primary mouse embryonic fibroblast (MEF) cell was generated from 14 days old embryo. A, The genomic DNA from MEF cell was extracted and then used to precede with PCR for DNA typing with AU-XD or KU-XD primers. B, Total RNA isolated from MEF cell was reverse-transcribed and amplified by RT-PCR using primers which were described in figure 15A. C, The MEFs cell (passage=3) from NRIP WT or KO mice were seeding equally in 6 well plate and counted after 24, 48, 72, 96 hours. The data were analyzed by three independent experiments and presented as mean ±SD. (∗: nonspecific bond)

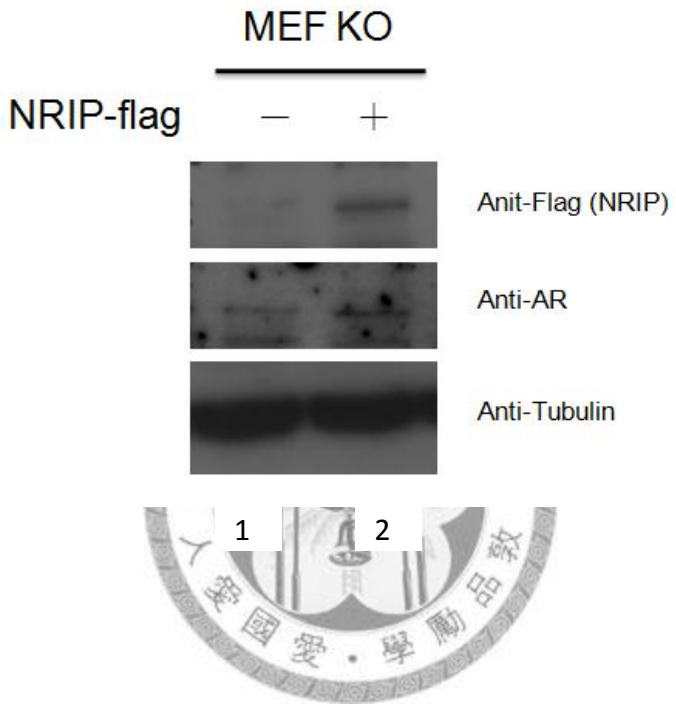
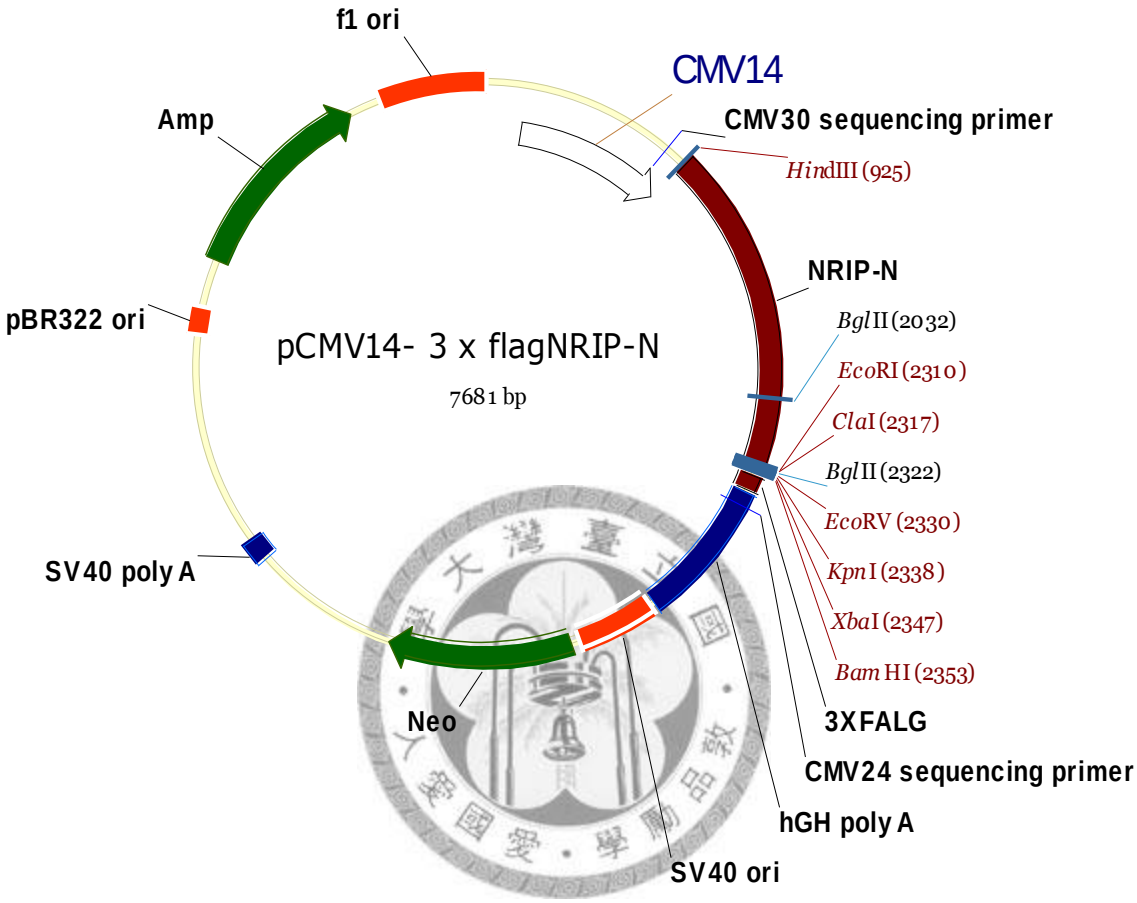


Figure 18. **Expression of NRIP can enhance AR protein level in MEF cell.**
The NRIP KO MEF cell was electroporated with pCMV14-3xflag-NRIP plasmid (lane2) or control vector (lane1). AR protein expression level was detected by Western blotting analysis with anti-AR antibody.

APPENDIX

Map 1



Description

The N fragment of NRIP was amplified by polymerase chain reaction using pCMV14-3xflag-NRIP as template. The amplified products were digested with HindⅢ and BamH. After purification, directly it ligated into HindⅢ and BamHⅠ digested pCMV14 vector.

Primer

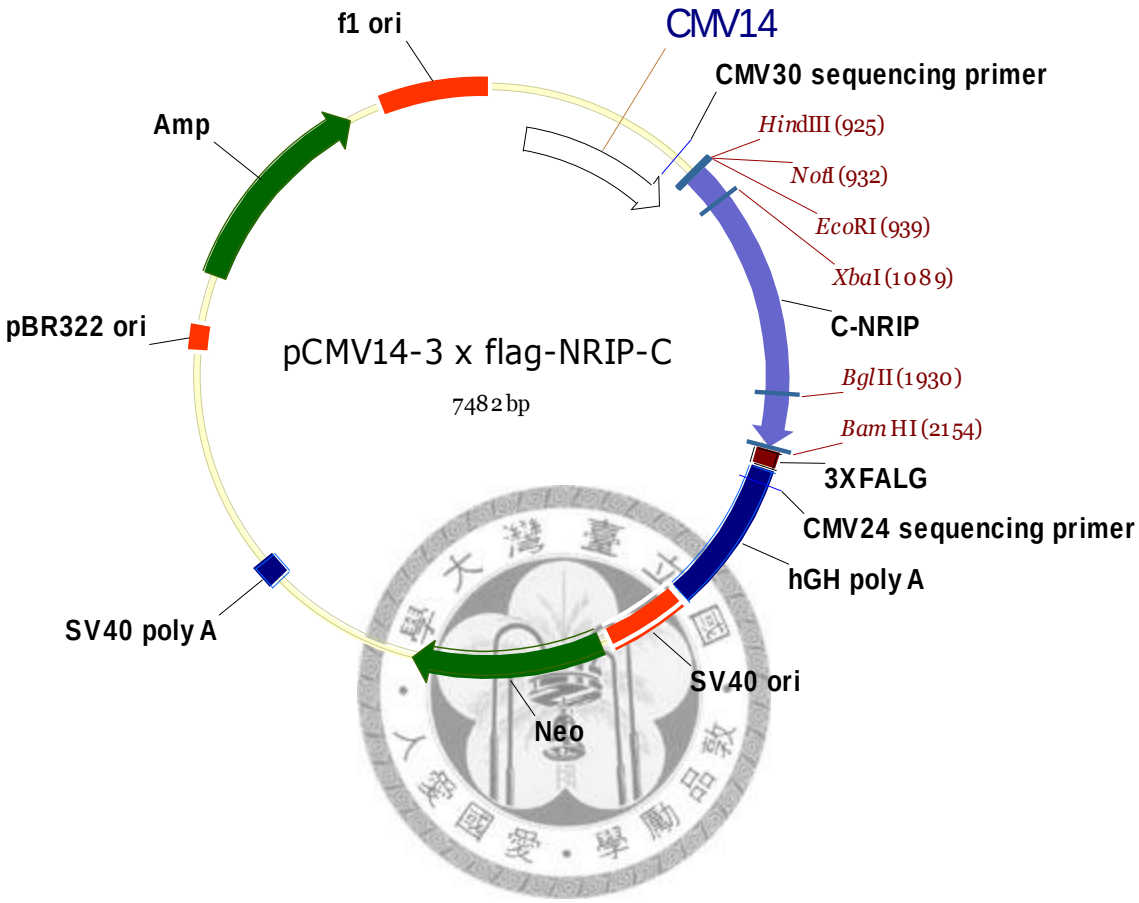
Forward primer:

5’-AAAAAAGCTTATGTCTCGGGGTGGCTCCT-3’

Reverse primer:

5’-AAAAGGATCCAGACTGATGATGTGTGTGGTGTC-3’

Map 2



Description

The C fragment of NRIP was amplified by polymerase chain reaction using pCMV14-3xflag-NRIP as template. The amplified products were digested with HindⅢ and BamH. After purification, it directly ligated into HindⅢ and BamHⅠ digested pCMV14 vector.

Primer

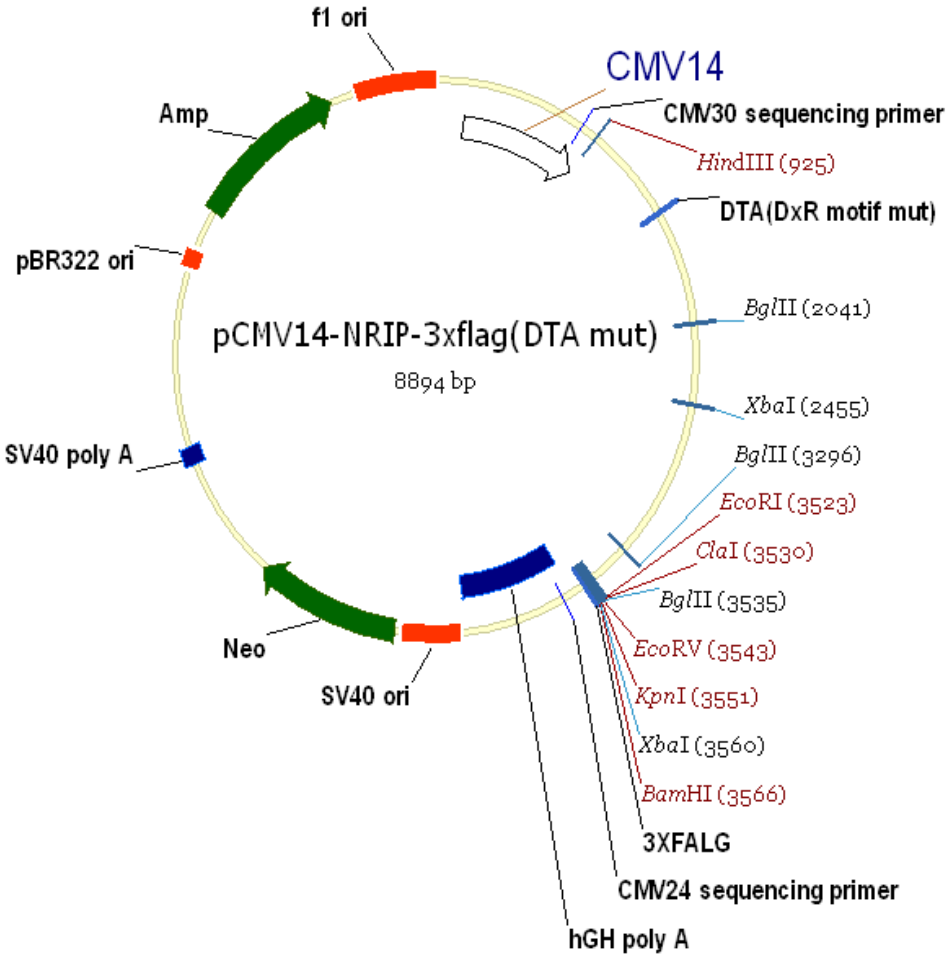
Forward primer：

5’-ATTGAATTCACCATGGATAACAATAATGAAAAGCTG-3’

Reverse primer：

5’-AAAAGGATCCTTCCTCATCCTCATTTTCATTCTC-3’

Map 3



Description

The plasmid was constructed by site-direct mutagenesis on the first WDXR motif (R172→A). pCMV14-3xflag-NRIP plasmid was used as the template to amplify the pCMV14-3xflag-NRIP-DTA plasmid by PCR method (described in material and method 2.4).

Primer

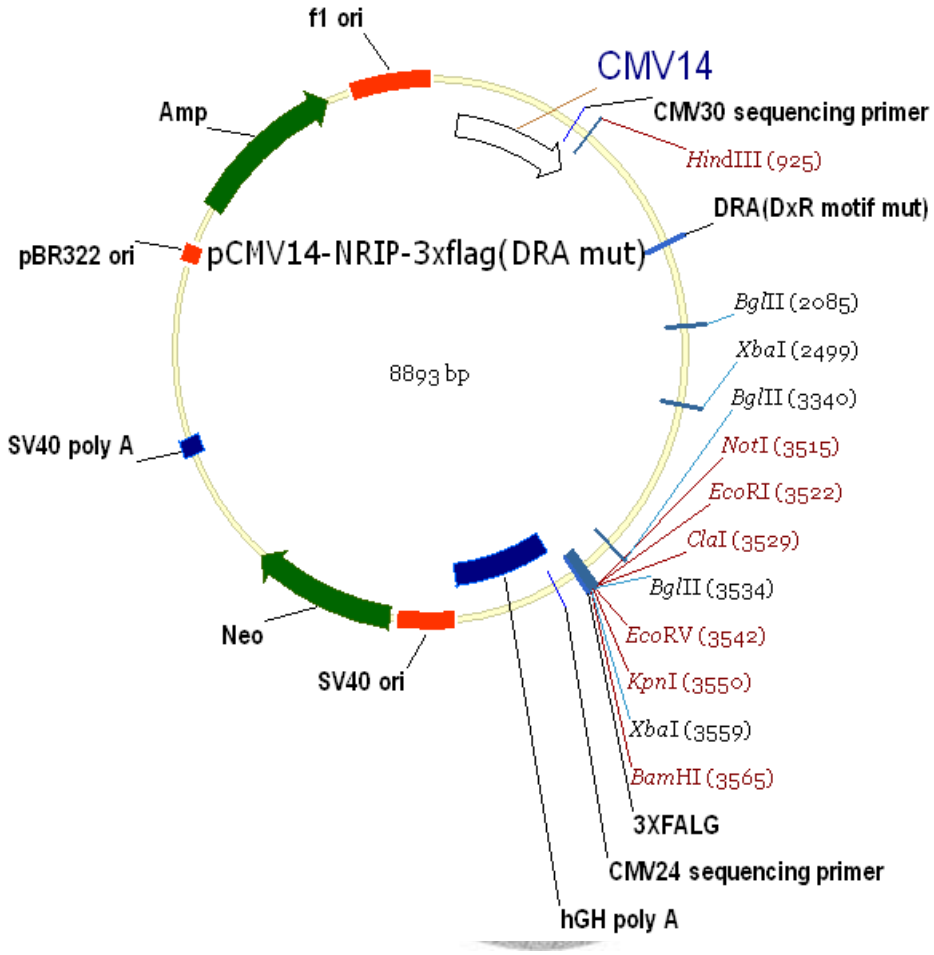
NRIP (DTA)-sense primer：

5'-CTGTTAGGTGGTTTGATACAGCCATCAAAACTAGCTGCACAAAAG-3'

NRIP (DTA)-antisense primer：

5'-CTTTTGTGCAGCTAGTTTTGATGCGTGTATCAA ACCACCTAACAG-3'

Map 4



Description

The plasmid was constructed by site-direct mutagenesis on the second WDXR motif (R222→A). pCMV14-3xflag-NRIP plasmid was used as the template to amplify the pCMV14-3xflag-NRIP-DRA plasmid by PCR method (described in material and method 2.4).

Primer

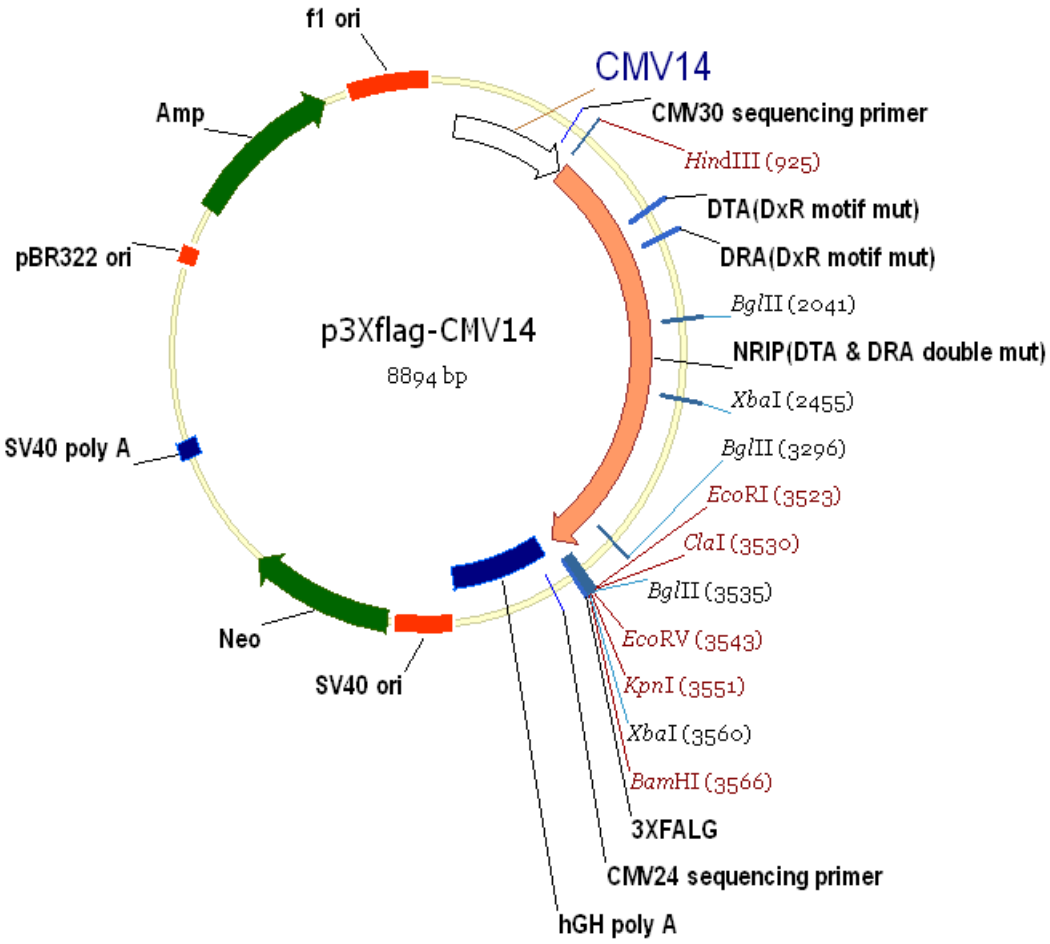
NRIP (DRA)-sense primer：

5'-GCTCAGTACGAATATATGATCGGGCAATGCTGGGCACAAGAGCTAC-3'

NRIP (DRA2)-antisense primer：

5'-GTAGCTCTTGTGCCCAGCATTCGCCGATCATACATTCGTACTGAGC-3'

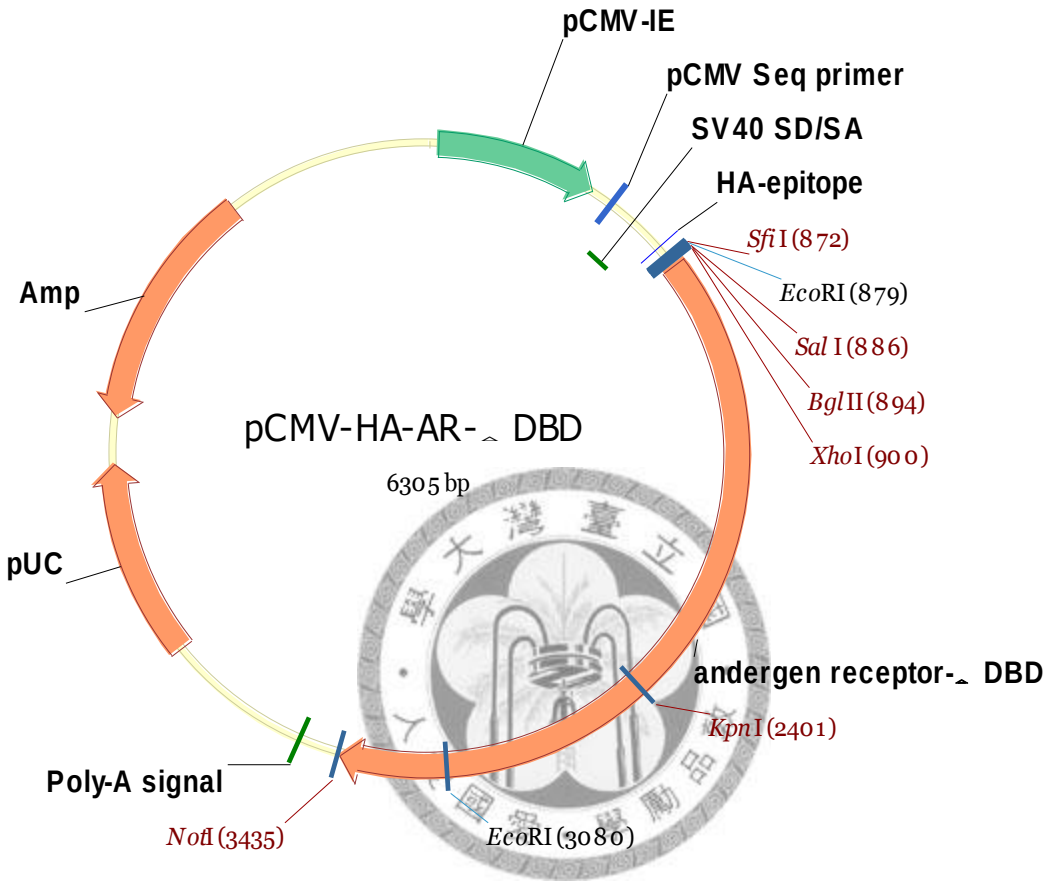
Map 5



Description

The double mutant WDXR motif plasmid was constructed by site-direct mutagenesis on the both WDXR motif (R172→A and R222→A). pCMV14-3xflag-NRIP-DTA plasmid was used as the template to amplify the pCMV14-3xflag-NRIP-DTA/DRA plasmid by PCR method with the designed DRA primers (described in material and method 2.4).

Map 6



Description

The plasmid was constructed by deleting internal DNA binding domain (DBD). pCMV-HA-AR was used as the template to amplify the pCMV-HA-AR-Δ DBD plasmid by PCR method (described in material and method 2.4).

Primer

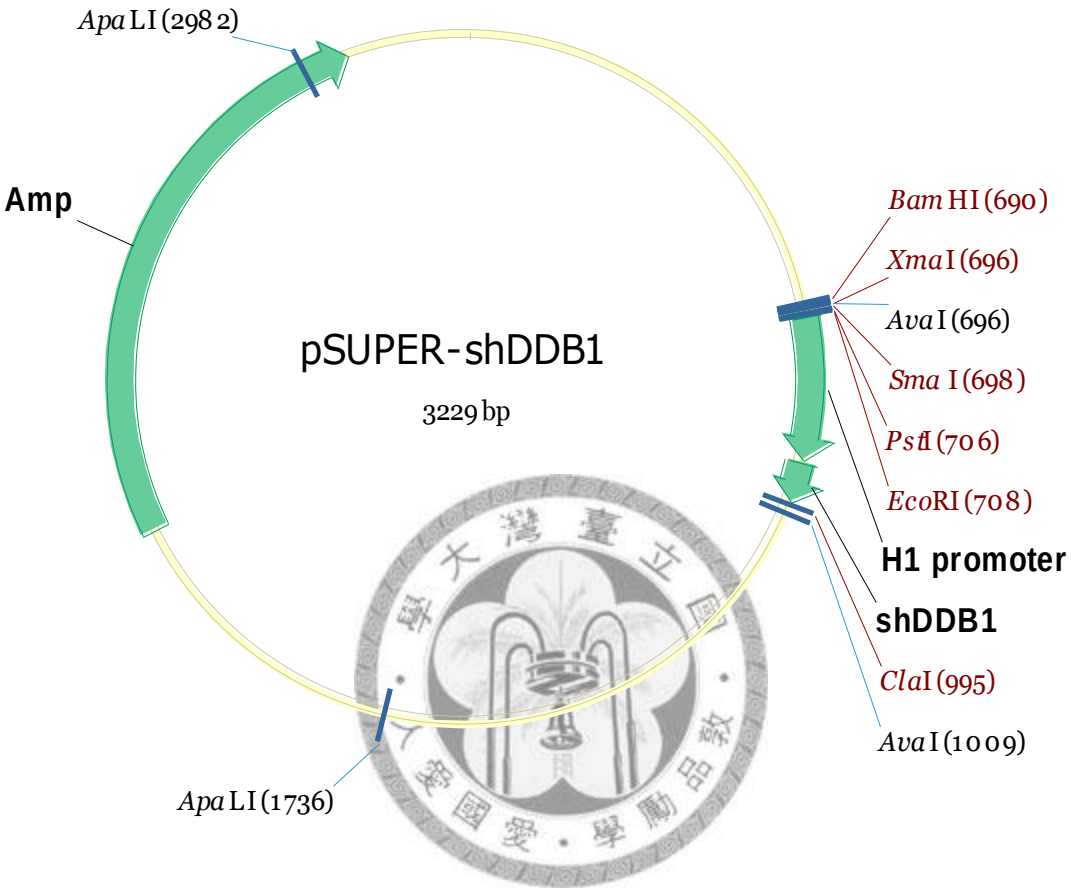
Sense primer：

5'-ACTTTCCACCCCAGAAGACCAAGAAACTTGGTAATCTGAAACTA-3'

Antisense primer：

5'-TAGTTTCAGATTACCAAGTTTCTTGGTCTTCTGGGGTGGAAAGT-3'

Map 7



Description

The gene-specific targeting sequences (5'-AGCATTGACTTACCAGGCA-3') were subsequently sub-cloned to the downstream of H1-RNA promoter between the Bgl Ⅱ and XhoI cutting sites in a pSUPER vector.

Primer

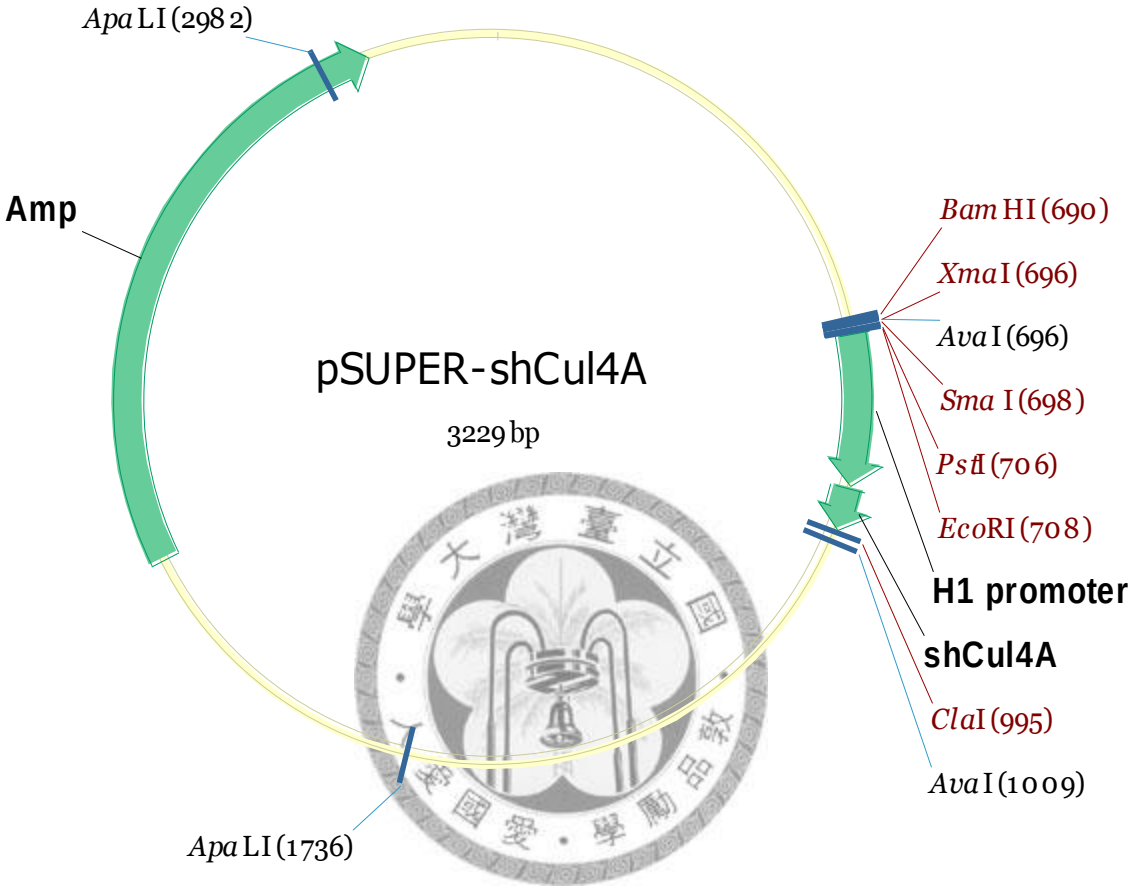
Sense primer：

5'-GATCCCCAGCATTGACTTACCAGGCATTCAAGAGATGCCTGGTAAGTCAATGCTTTTTTA-3'

Antisense primer：

5'-AGCTTAAAAAAGCATTGACTTACCAGGCATCTCTTGAATGCCTGGTAAGTCAATGCTGGG-3

Map 8



Description

The gene-specific targeting sequences (5'-GGAAGAGACTAATTGCTTA-3') were subsequently sub-cloned to the downstream of H1-RNA promoter between the Bgl Ⅱ and XhoI cutting sites in a pSUPER vector.

Primer

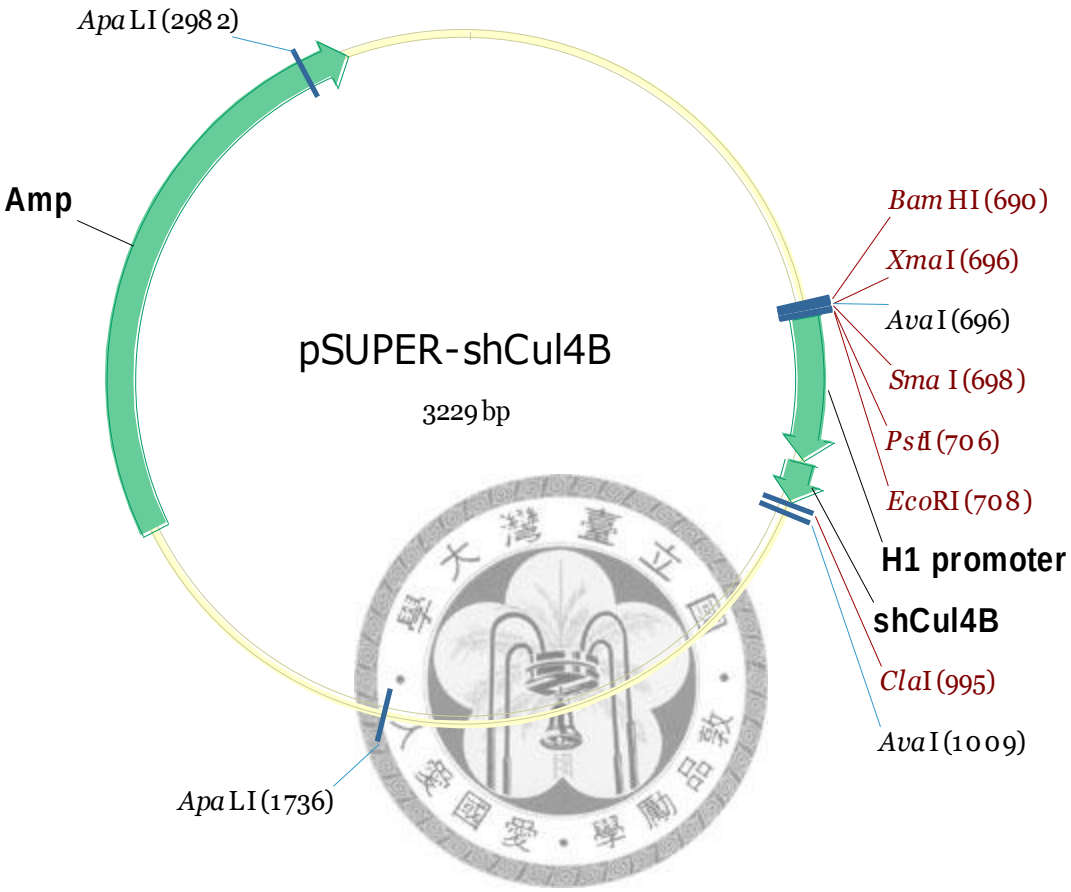
Sense primer：

5'-GATCCCCGGAAGAGACTAATTGCTTATTCAAGAGATAAGCAATTAGTCTCT TCCTTTTTA-3'

Antisense primer：

5'-AGCTTAAAAAGGAAGAGACTAATTGCTTATCTCTTGAATAAGCAATTAGTC TCTTCCGGG-3'

Map 9



Description

The gene-specific targeting sequences (5'-CGGAAAGAGTGCATCTGTA-3') were subsequently sub-cloned to the downstream of H1-RNA promoter between the Bgl Ⅱ and XhoI cutting sites in a pSUPER vector.

Primer

Sense primer:

5'-GATCCCCCGGAAAGAGTGCATCTGTATTCAAGAGATACAGATGCACTCTTTCCGTTTTTA-3'

Antisense primer:

5'-AGCTTAAAAACGGAAAGAGTGCATCTGTATCTCTTGAATACAGATGCACTCTTTCCGGGG-3'