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Dedication

I dedicate this dissertation to my dearest family

Yunseong Jeong

Alice Hee-jin Jeong

Ryan Seok-ho Jeong

And my parents and sisters

Sang-bock Shin

Seok-hee Kim

Ji-ye Shin

Ji-hee Shin

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THE NOVEL UPSTREAM REGULATOR OF FBXW7

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SCF^{FBXW7} is a tumor suppressor E3 ligase protein that targets numerous oncoproteins such as Cyclin E, c-Myc, c-Jun and MCL1. The deregulation of these proteins often leads to the proliferation of cancer cells. Thus, intracellular stability and functional activity of FBXW7 is critical for regulating cancer. However, there is a gap of knowledge about the intracellular signaling pathway or if there is another ubiquitin ligase that regulates FBXW7 stability. Here, I identify a novel mechanism of FBXW7 stability regulation which involves constitutive photomorphogenic 1 (COP1), AKT and CSN6 (i.e. the COP9 signalosome 6).

COP1 is an E3 ubiquitin ligase targeting important substrates such as, p53, 14-3-3-σ, and c-Jun. I found that COP1 binds to FBXW7 at the binding motif at V200 and P201. Interestingly, binding between COP1 and FBXW7 was dissociated by loss function of AKT. In addition, IGF-1 or EGF induced AKT1 directly regulates FBXW7 stability through phosphorylation of FBXW7 at T226 and S227. Moreover, phosphorylation of FBXW7 at T226 and S227 by AKT facilitates its interaction with COP1, and consequently induces ubiquitination of FBXW7 by COP1. Significantly, TSAA FBXW7 mutant reduced cell invasion, migration, proliferation and cell cycle progression. Thus, results define a novel signaling pathway for regulation of FBXW7 through the AKT-

COP1 axis which can be applied for therapeutic intervention in cancers overexpressing AKT and COP1.

CSN functions as an adaptor between substrate proteins and the 26S proteasome to facilitate proteasomal degradation of ubiquitinated proteins. In this study, I also found that CSN6 associates with, and causes the degradation of, FBXW7. Moreover, CSN6 regulates stabilization of Cyclin E and c-Jun through its negative effect on FBXW7, which in turn reduces ubiquitin-mediated protein degradation of Cyclin E and c-Jun. Therefore, CSN6 knockdown results in reduced cell migration, transformational activity, and tumor growth.

Together, my findings indicate the novel signaling pathway for regulation of FBXW7 through COP1, AKT or CSN6 which can be applied for therapeutic intervention in cancers overexpressing AKT and COP1 or Cyclin E and c-Jun.

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AMPK	AMP-activated protein kinase
AP1	Activator protein 1
APC	Anaphase-Promoting Complex
BAD	Bcl-2-associated death
CDK8	Cyclin-dependent protein kinase 8
C/EBP δ	CCAAT/enhancer binding protein-δ
COP1	Constitutive photomorpho- genesis 1
CPD	Cdc phosphodegrons
CREB	cAMP-response element-binding protein
CRL	Cullin RING ligase
CSN6	COP9 signalosome subunit 6
DDB	Damaged-DNA binding protein
DET1	De-Etiolated 1
E1A	Early region 1A
EGF	Epidermal growth factor
ER	Estrogen receptor
FGF	Fibroblast growth factor
G6Pase	Glucose-6-phosphatase
GFP	Green fluorescence protein
GSK3-β	Glycogen synthase kinase-β
GST	Glutathione S-transferase

HCC	Hepatocellular carcinoma
HECT	Homologous to the E6-AP carboxyl terminus
HER2	Human epidermal growth factor receptor 2
HGF	Human growth factor
HIV-1	Human immunodeficiency virus type-1
IGF-1	Insulin-like growth factor-1
IP	Immunoprecipitation
JAMM/MPN+	JAB1/MPN/MOV34 metalloenzyme
JNK	Jun N-terminal kinase
KLF5	Krüppel-like factor
MAPK	Mitogen-activated protein kinase
MDM2	Murine double minute
mTORC2	Mammalian target of rapamycin complex 2
MPN	Mpr1-Pad1-N-terminal
NEDD4-1	Neural precursor cell expressed, developmentally down-regulated 4-1
PCR	Polymerase chain reaction
PDGF-R	Platelet derived growth factor receptor
PDK1	Phosphoinositide-dependent kinase-1
PEPCK	Phosphoenolpyruvate carboxykinase
PH	Pleckstrin homology
PHLPP	PH domain leucine-rich repeat protein phosphatase
PI3K	Phosphatidylinositol 3-kinase

PI(3,4,5)P3	Phosphatidylinositol (3,4,5)-trisphosphate
PKB	Protein kinase B
PLK	Polo-like kinase
PPIse	Peptidyl-prolyl cis/trans isomerase
PP2A	Protein phosphatase 2A
PR	Progesterone receptor
PS	Presenilins
PTEN	Phosphatase and tensin homolog
RBX	RING box proteins
RING	Really interesting new gene
RIP1	Receptor-interacting serine/threonine-protein kinase 1
RNF8	Ring finger protein 8
SCF	Skp1-Cullin-F-box protein
Skp2	S-phase kinase-associated protein 2
shRNA	Small hairpin RNA
SIK2	Salt inducible kinase 2
TNBC	Triple negative breast cancer
TORC2	Transducer of regulated CREB activity 2
TSC2	Tuberous sclerosis 2
USP	Ubiquitin-specific protease
VCB	VHL/elongin C/elongin B
VEGF	Vascular endothelial cell growth factor

CHAPTER 1

INTRODUCTION

1. SCF^{FBXW7}

1-1 SCF complex in Ubiquitination

Ubiquitin is a small protein (~6kd) which can modify proteins to target them for the proteasome complex. The process of ubiquitination has multiple steps involving the E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ligase proteins. In the initial step, the E1 enzyme binds with both ATP and ubiquitin then activates ubiquitin proteins. Next the E3 ligase recognizes specific substrate proteins and the E2 enzyme transfers active ubiquitin to substrate proteins. In this cascade reaction, the E2 enzyme determines the fate of proteins dependent on which lysine residue, there are seven i.e. K6, K11, K27, K29, K33, K48 and K63, is linked to the construct ubiquitin chains [1]. For example, it is common that K48-linked ubiquitination is related with proteolysis thus, facilitating proteosomal degradation [2], whereas K63-linked ubiquitination is mainly related with regulation of signaling pathway and protein trafficking [3].

Although the E2 enzyme has a critical function in ubiquitination, the E3 ligase has the most important function thorough selectively recognizing substrate proteins. In general, the E3 ubiquitin ligase is classified either homologous to the E6AP C-terminus (HECT) domain group or to the really interesting new gene (RING) domain group [4]. HECT domain E3 ligases have a specific enzyme site to transfer ubiquitin from E2 to substrate proteins, whereas RING domain E3 ligases do not have catalytic functions but work as a scaffold which can bind both E2 and substrates to facilitate the ubiquitination process [3]. Some studies also verify the ubiquitination function of the ligase protein in multi-protein complexes such as Skp1-Cullin-F-box protein (SCF) [5], Anaphase-

Promoting Complex (APC) [6] and VHL/elongin C/elongin B (VCB) [7]. Among them, the SCF complex is the most commonly identified family of ubiquitin ligases. The SCF complex functions as a scaffold to ubiquitinate substrate proteins that are involved in various cellular regulations including cell cycle, tumorigenesis, and differentiation [5]. APC serves as an E3 ligase with Cdh1 protein in cell cycle regulation. Cdh1 contains a WD40 domain and functions as an adaptor between APC and substrate protein in cell cycle and facilitate ubiquitination [6]. VCB has Cullin-2 in the complex and also has a similar protein sequence as SCF and APC. Thus, based on structural similarity, VCB serves as an E3 ubiquitin ligase [7].

As individual E3 ligases such as HECT or RING type are important in cell cycle regulation, multi-complex E3 ligases also have critical role in cellular regulation. Most importantly, the SCF complex is the largest complex which can regulatevarious substrate proteins including p21, p27 [8], β-Catenin [9], Cyclin E [10] and mTOR [11]. Recent studies support that the SCF E3 ligases play a pivotal role in cancer regulation and also abnormal function of the SCF E3 ligases facilitate tumorigenesis. Therefore, to understand the cellular function of the SCF E3 ligases, more research studies need to be done.

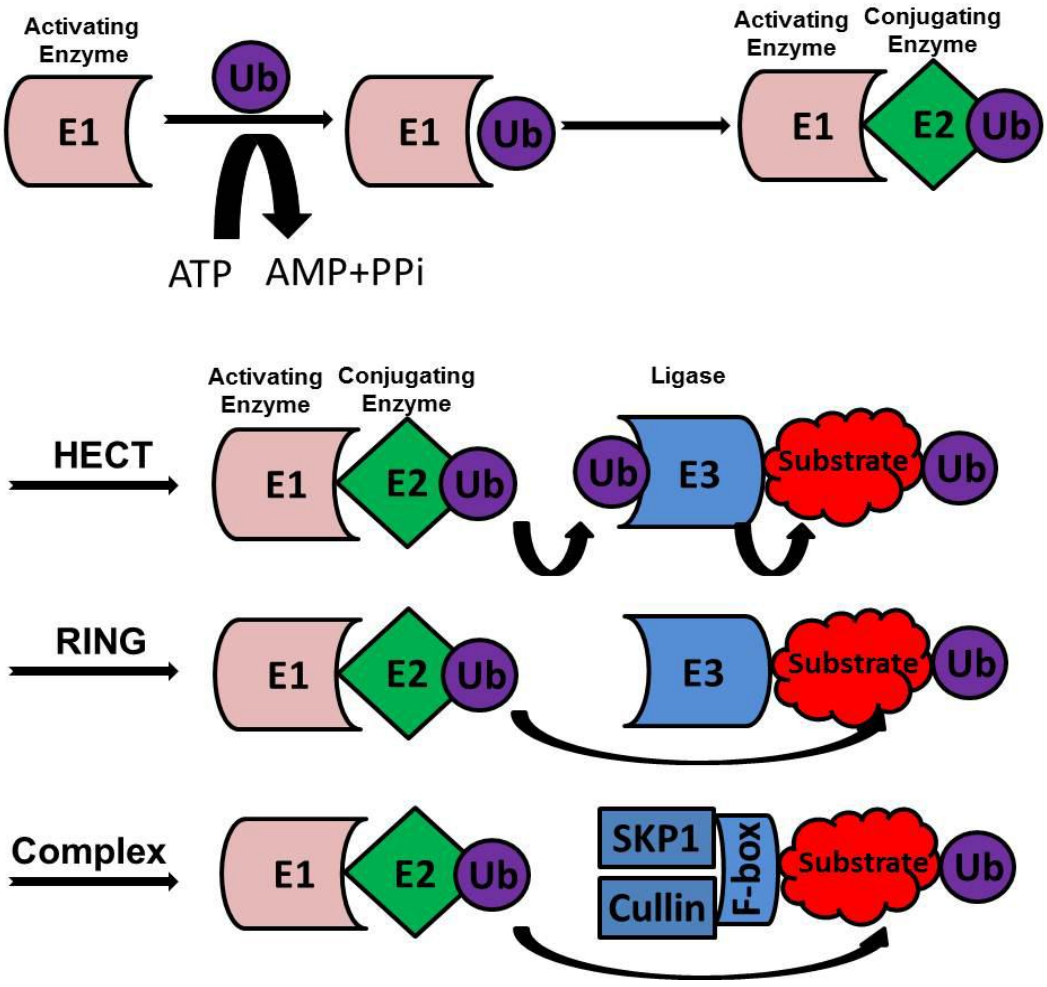

Figure 1. Ubiquitination Process. The E1 activating enzyme catalyzes the thioester bond between the c-terminus of ubiquitin and the cysteine of the E2 ubiquitin conjugating enzyme. In general, HECT domain containing E3 ligases have stronger catalytic activity than the RING domain E3 ligase. The E3 ligases directly transfer ubiquitin from the E2 enzyme to substrate proteins. However, RING domain containing E3 ligases bind both the E2 enzyme and substrate protein as a linker and transfer ubiquitin to substrates.

1-2 F-box protein FBXW7

The SCF E3 ligase complex is also known as Cullin RING ligase (CRL). Skp-1, Cullins, F-box proteins and RING box proteins (RBX) are the major components of the complex. RBX is a RING finger protein that activates ubiquitination through binding Cullin protein in a complex. F-box proteins are responsible for recognizing specific binding motifs of substrate proteins [12]. Skp2, FBXW7, and β-TrCP are classified as F-box proteins. Skp2 has been identified as an oncogenic protein [13] and β-TrCP has both oncogenic and tumor suppressor functions under different cellular conditions [14] whereas, FBXW7 is a well-defined tumor suppressor E3 ligase in SCF complex.

Importantly, SCF^{FBXW7} (FBXW7, CDC4, AGO and SEL10) is an adaptor protein of the SCF ubiquitin ligase complexes. FBXW7 contains a DD domain for dimerization, an F-box motif for ligase function and 7 tandem repeat WD40 domain which is responsible for binds with target proteins for ubiquitination and degradation. Alternative splicing generates three isoforms of FBXW7 α, β, and γ, which have different N-terminal structures. Also, each isoform has a different cellular localization; FBXW7 α aggregates in the nucleoplasm, FBXW7 β in the cytoplasm, and FBXW7 γ in the nucleolar [15]. Moreover, recent studies suggest that nuclear localized isoforms are involved in cancer regulation but cytoplasmic isoforms correspond to response for oxidative stress in the endoplasmic reticulum membrane [16]. The FBXW7 α isoform is ubiquitously and more dominantly expressed than the other isoforms. Moreover, FBXW7 α is responsible for ubiquitination and degradation of most oncogenic substrates such as c-Myc, c-Jun, Mcl-1, Notch, and Cyclin E [17]. In a similar manner, FBXW7 γ is also involved in c-Myc

protein degradation in the nucleolus [15]. However, cellular functions of FBXW7 β in tumorigenesis are still not clear and need to be investigated.

1-3 FBXW7 and kinase

E3 ubiquitin ligases recognize phosphorylated substrate proteins. In a similar manner, FBXW7 detects substrates through distinct sequences (T/S) PXX(S/T/E) called 'Cdc phosphodegrons (CPD)' after they got phosphorylated by kinase. Glycogen synthase kinase-3 (GSK3) is a well-known kinase that phosphorylates most substrate proteins of FBXW7 at serine or threonine site in CPD region then facilitate ubiquitination [18]. GSK3 uses 'priming phosphorylation' to detect and phosphorylate substrates. It is a unique functional character of GSK3 to identify substrates and bind them at specific residues Arg86, Arg180, and Lys205 in the C-terminal region [19]. Originally, GSK3 was identified as a protein kinase that phosphorylates glycogen synthase and phosphatidylinositol-3,4,5-trisphosphate, phosphoinositide-dependent kinase-1(PDK1). Additionally, AKT functions as a negative regulator of GSK3. It has been reported that inhibition of GSK3 possibly induces dephosphorylation of FBXW7 substrates c-Myc, Cyclin D1, and c-Jun. To correlate this, FBXW7 cannot detect non-phosphorylated substrates. And also, simultaneous mutations in the CPD region of substrate proteins causing a lost binding motif does not allow association of substrate protein with FBXW7 and thus hinders FBXW7 mediated ubiquitination. For example, Burkitt lymphoma patients commonly have a CPD mutation of c-Myc at T58 [20]. It suggest that phosphorylation of substrate protein is a critical event for FBXW7 mediated ubiquitination. However, most studies were focused on GSK3 and only the information about other kinase functions of FBXW7 substrate proteins is about cyclin-dependent protein kinase 8(CDK8). CDK8 is implicated in NOTCH degradation and ubiquitination.

Therefore, function of other kinase in FBXW7-mediated ubiquitination needs to be verified.

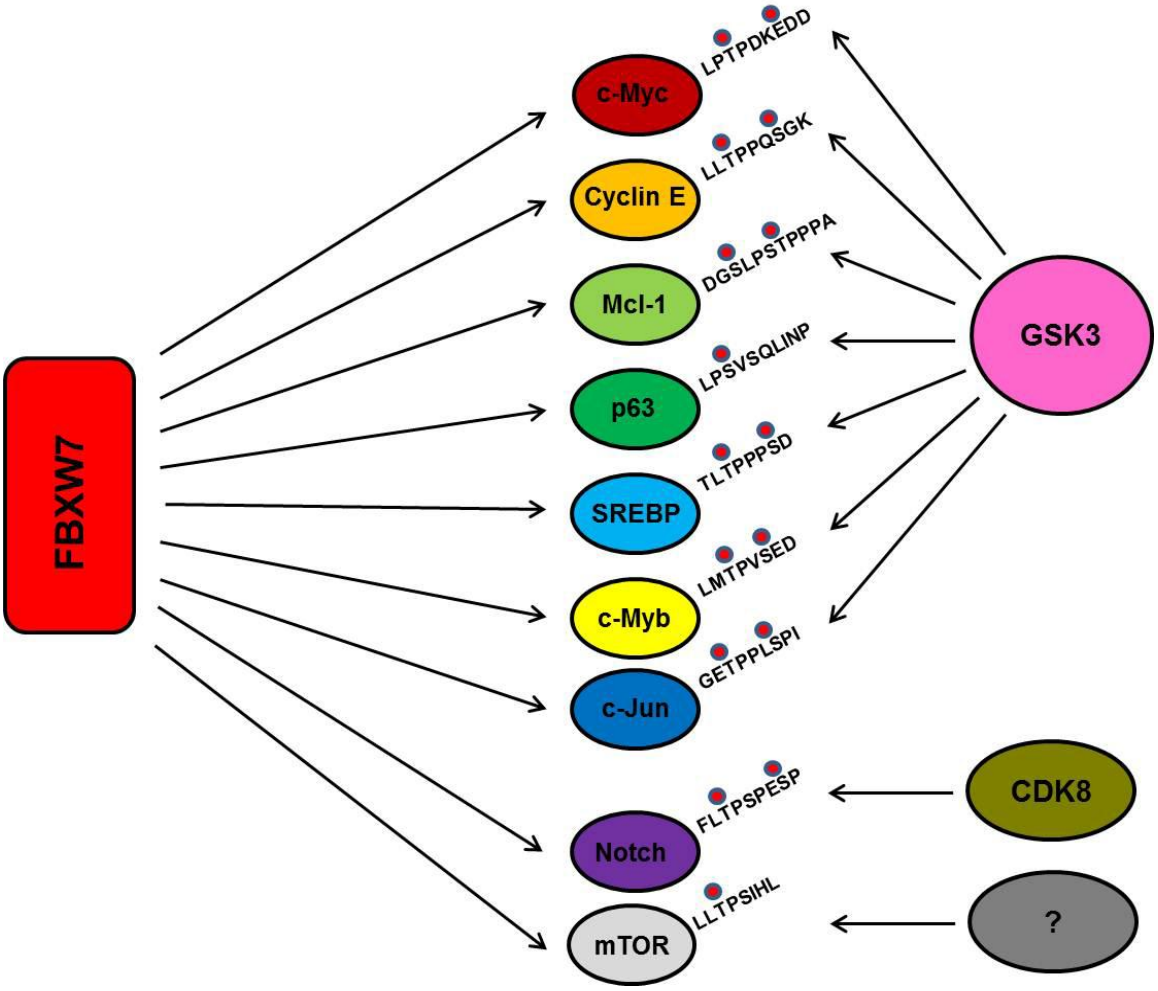

Figure 2. The substrates of FBXW7. Most of the substrates of FBXW7 contain a consensus phospho-degron motif (CPD). Followed by phosphorylation by specific kinase such as glycogen synthase kinase-3 (GSK3) or cyclin-dependent kinase 8(CDK8), FBXW7 recognizes the CPD of the substrate protein to facilitate ubiquitination.

1-4 FBXW7 in Cancer

The chromosomal localization of human FBXW7 (hCDC4) is 4q31.3, which is commonly deleted in many type of cancers. In the animal mouse model, targeted deletion of *FBXW7* gene caused genetic instability and tumor formation [21]. In addition, overall mutation rate of *FBXW7* is approximately 6% in various human cancers. Moreover, approximately 35% of bile-duct cancer, 31% of blood related cancer and 9% colon cancer show significantly high mutation rates of *FBXW7* [21]. FBXW7 mutations at Arg^{465} and Arg^{479} are critical to recognize substrate proteins for ubiquitination and degradation [21]. Due to genetic mutations or deletion of *FBXW7* in many cancers and also the fact that most substrates of FBXW7 are oncoproteins, FBXW7 was defined as a tumor suppressor protein. It has been reported that FBXW7 regulates various proteins that regulate diverse biological functions. For example, c-Jun [22], c-Myc [23], Krüppel-like factor (KLF5) [24], and c-Myb [25] are transcriptional factors that regulate cell proliferation and growth. In addition, Cyclin E regulates cell cycle progression [10], Mcl-1 inhibit apoptosis [26] and mTOR involved in cell growth [27]. Among several substrates, Cyclin E and c-Jun are the most studied substrate proteins.

Cyclin E is an activating subunit of CDK2 which promotesG1 to S phase of cell cycle progression and cell proliferation [28]. Cyclin E contains a dual CPD at both the N- and C- terminus and phosphorylation of Cyclin E at T62, T380, and S384 are related with its stability. Mutational study in each phosphorylation sites showed that T62 relatively low binding affinity with FBXW7 and mainly regulate its activity. However, T380 or T384 mutations completely lost binding ability with FBXW7 which then prevented FBXW7-mediated ubiqutination [29]. This suggests that T62 has an indirect effect on

Cyclin E turn over regulation but T380 and T384 sites have the pivotal function of FBXW7-mediated Cyclin E stability regulation.

c-Jun is a major component to form activator protein 1 (AP1) transcriptional factor. c-Jun protects UV- induced and TNFα -induced cellular apoptosis [30]. There are two kinases involved to regulate c-Jun stability and cellular function; Jun N-terminal kinase (JNK) and GSK-3 [31]. JNK phosphorylates c-Jun at S63 and S73 then activated JNK cascade to induce target gene expression. In contrast, GSK3 phosphorylate c-Jun at T239 and S243 then increased FBXW7-mediated c-Jun protein degradation. Interestingly, mono phosphorylation of either T239 or S243 fails to bind with FBXW7. It suggest that dual-phosphorylation of c-Jun is an important event to FBXW7-mediated ubiquitination.

1-5 Upstream regulator of FBXW7

Pin1 is a most recent verified upstream regulator of FBXW7. Pin-1 is a peptidyl-prolyl cis/trans isomerase (PPIase) that isomerizes substrate proteins through binding with phosphorylated Ser/Thr-Pro (pSer/Thr-Pro) motifs then changes protein structure [32]. Pin-1 binds with FBXW7 in a phosphorylation dependent manner. Pin-1 detects and binds with phospho-T205 FBXW7 and destabilizes FBXW7 through promoting self-ubiquitination of FBXW7. As results, Pin-1 mediated FBXW7 degradation contributes to tumorigenesis via stabilizing oncogenic substrate proteins of FBXW7 [33].

Polo-like kinase (PLK) regulates centriole duplication in mitosis result in involved cell cycle progression and embryonic development [34]. Cdk2/Cyclin E complex is required for initiation of centriole duplication thus Cyclin E expression level is important for regulating the cell cycle. It is known that FBXW7 is responsible for Cyclin E degradation. Interestingly, PLK2 phosphorylates FBXW7 at S25, S176, and S349 then degrades FBXW7 through destruction of homo-dimerization of FBXW7. As a result, PLK2 mediated FBXW7 degradation stabilizes Cyclin E in G1 toS phase and promotes cell cycle progression [34].

CCAAT/enhancer binding protein-δ (C/EBP δ) negatively regulates FBXW7 gene expression and increases breast cancer metastasis [35]. And also Presenilins (PS) is suppressed FBXW7 transcription then stabilizes Notch, one of the FBXW7 substrate proteins [36]. Early region 1A (E1A) derived from adenovirus, binds with FBXW7 and decreases ubiquitination of FBXW7 target oncogenic proteins [37].

2. Constitutive photomorphogenesis 1 (COP1)

2-1 COP1 in plants

COP1 (also known as RFWD2) is a RING domain containing E3 ligase protein which is identified as a suppressor of photomorphogenic development in Arabidopsis. COP1 contains three functional domains; RING-finger motif, coiled-coil domain, and a WD40 domain. Cellular localization of COP1 is critical for function as a photomorphonenic suppressor. In the dark, pleiotropic COP/DET/FUS genes increase nuclear accumulation of COP1 and nuclear localization signals (NLS) regulate its subcellular localization [38]. In a nucleus, through RING-finger motif, COP1 recognize and suppress HY5 which is a bZIP transcription factor that responsible for activation of genes that related with plant development [39]. However, in the light, the amount of nuclear localized COP1 decreases and COP1 losses its suppressor functions.

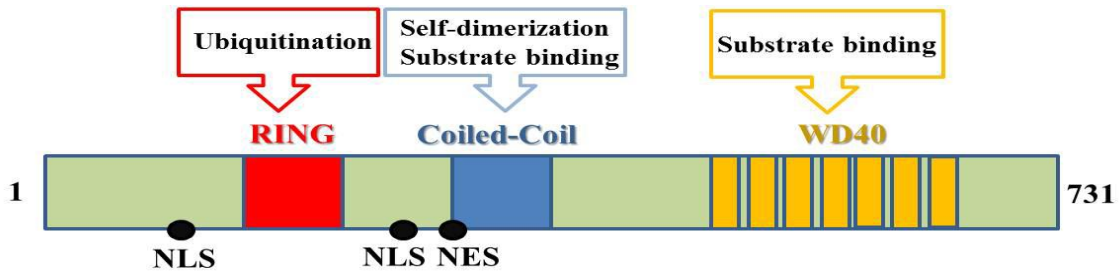

Figure 3. Structual domain of COP1. COP1 contains three different functional domains. The RING-finger domain is responsible for recruitment of the ubiquitination complex. The coiled-coil domain is for either self-dimerization of COP1 or substrate protein binding such as Suppressor of PHYA-105 (SPA) and De-Etiolated 1 (DET1). The 7-repeat WD40 domain function as a binding dock for substrate proteins such as HY5 and c-Jun.

2-2 COP1 substrates in mammal

COP1 is conserved from plants to mammals and recent studies reported that COP1 was involved with various cellular regulations in mammalian cells via ubiquitination of substrate proteins such as p53, 14-3-3σ, c-Jun, and MTA [40,41,42,43]. COP1 recognizes substrates through the consensus binding motif (D/E-D/E-X-X-X-V-P) in each substrate sequence [44].

COP1 is responsible for degradation of p53 independent from known p53 E3 ubiquitin ligases such as MDM2 and Pirh2 [45]. In addition, COP1 regulates 14-3-3σ ubiquitination followed by stabilization by COP9 signalosome subunit 6 (CSN6) [40]. Binding between c-Jun and the WD40 domain of COP1 increases turnover of the c-Jun protein [42]. Metastasis-associated protein 1 (MTA1) is a substrate protein of COP1 that also has feedback loop regulation with COP1 though increasing self-ubiquitination of COP1 [43]. Transducer of regulated CREB activity 2 (TORC2) is also known as cAMP-response element-binding protein (CREB) regulated transcription coactivator 2 (CRTC2) contains a COP1 binding motif. TORC2 phosphorylation by salt inducible kinase 2 (SIK2) at S171 regulates translocation of TORC2 in cytoplasm then COP1 binds with and facilitates ubiquitination [46]. COP1 regulates FOXO1 in an independent manner with known FOXO1 regulator CREB. COP1 decrease FOXO1 protein and also target gene expression such as glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) which are important molecules for gluconeogenesis [47]. The oncogene ETV1, 4, 5 are substrates of COP1 regulation. ETV also contains a COP1 binding motif and is negatively regulated by COP1. However, binding site mutations on ETV have significantly higher stability than wild-type ETV [48]. In fatty acid regulation,

pseudokinase Tribbles 3 (TRB3) recruits COP1 in adipose tissue under fasting conditions. Fatty acid oxidation is increased as a result of degradation of ACC by COP1 [49].

Substrate	Cellular function	Character	Reference
p53	Transcription factor	Tumor suppressor	[41]
14-3-3σ	Conserved regulatory molecules	Tumor suppressor	[40]
c-Jun	Transcription factor	Oncogene	[50]
MTA	Transcription factor	Oncogene	[43]
TORC2	Transcriptional coactivator	Gluconeogenic gene regulator	[51]
FOXO1	Transcription factor	Tumor suppressor	[47]
ETV1,4,5	Transcription factor	Oncogene	[48]
ACC	Lipid metabolism	Fatty acid synthesis regulator	[49]

Table 1. COP1 substrates

2-3 COP1 in cancer

COP1 is overexpressed in many type of cancer such as hepatocellular carcinoma (HCC), breast and ovarian cancer [52,53] and destabilized tumor suppressor proteins such as p53 and 14-3-3σ [40,41]. Thus, it is consider as an oncogenic E3 ligase protein. However, COP1 also recognizes and ubiquitinates oncoproteins as a substrate such as c-Jun and ETV [50].

Genetic studies support tumor suppressor function of COP1. Recent studies showed that partial or tissue specific loss of *COP1* in mice promotes tumor formation in several organs such as liver, lung, and kidney [48,50]. Cop1 has an important function in embryogenesis in mouse development. Therefore, homozygous $Cop1^{-/-}$ is embryonic lethal and non-fertile whereas heterozygous $Cop1^{+/-}$ mice are viable [54]. Although $Cop1^{hypo/hypo}$ mice showed less body weight than wild-type and also immunohistological studies showed that $Cop1^{hypo/hypo}$ mice have smaller organs than wild-type [50], there is no distinct phenotype difference between Cop1 deficient mice and wild-type mice. However, an interesting finding was that p53 expression level was not changed whereas c-Jun expression was increased in $Cop1^{+/-}$ mice. Both Western Blot analysis and imunohistochemical studies support that c-Jun protein steady-state in $Cop1^{+/-}$ mice was significantly increased compared to wild-type. However, there is no difference in c-Jun mRNA levels between $Cop1^{+/-}$ and WT mice [50]. In addition *Cop1* deficient mice with p53-null background showed that proliferation of cells occurred through stabilization of c-Jun proteins. COP1 control cell proliferation by regulating JNK pathway which can phosphorylate and activate c-Jun. *Cop1* deficient mice induced accumulation of non-

phosphorylated c-Jun proteins and as a result c-Jun mediated cell cycle progression was suppressed [50].

Although cellular functions of COP1 have been identified from plants to mammals, there are still debatable questions about their function in cancer regulation. Therefore, to verify cellular function of COP1 in regulating cancer, functional mechanisms or substrate proteins of COP1 in various signaling pathway need to be investigated.

3. The COP9 signalosome (CSN)

3-1 CSN and 26S proteasome complex

The COP9 signalosome (CSN) is a protein complex that was first identified in Arabidopsis. CSN implicated in the seedling process of plants [55]. CSN is composed of 8 subunits (CSN1-8), which are structurally similar to the proteasome complex. The proteasome complex is composed of a 20S core cylinder and a 19S lid. The 20S unit is responsible for the major function of the proteasome complex and it degrades ubiquitinated proteins. The 19S is regulatory particles serve as an adaptor between the proteasome complex and ubiquitinated proteins. The CSN has structural homology with the 19S proteasome lid [56]; thus it has been suggested that the CSN can guide ubiquitinated proteins to the 26S proteasome complex to facilitate their degradation of proteins [57]. Therefore, the function of CSN is linked to the ubiquitination pathway, which can regulate various aspects of the E3 ubiquitin ligase [58,59,60]. For example, either knock-down damaged-DNA binding protein (DDB), which is one of component of ubiquitin complex, or CSN1 with siRNA, can facilitate accumulation of p27 proteins [59].

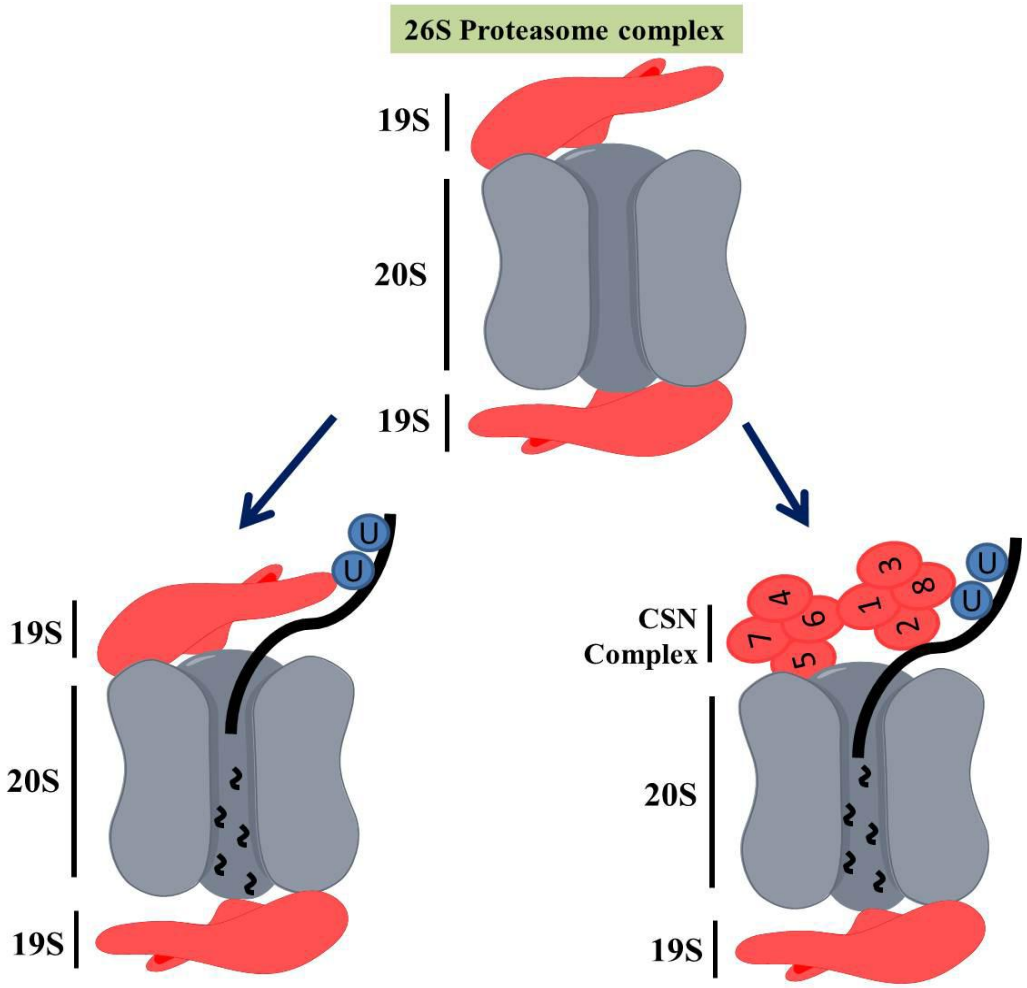

Figure 4. Structural and functional similarity between the 26S proteasome and the CSN. The 26S proteasome complex is composed of a 19S lid and a 20S core cylinder. Based on structural similarity, the CSN captures ubiquitinated proteins and functions as an adaptor protein like 19S lid. After binding with the 19S or CSN complex, proteins are transferred to the 20S core and then are degraded.

3-2 CSN5 and CSN6 contain MPN domain

Among the eight subunits of the CSN, six subunits (i.e. CSN1, 2, 3, 4, 7, and 8) contain Proteosome, COP9, and Initiation factor 3 (PCI) domain. Two subunits (CSN5 and 6) contain the Mpr1-Pad1-N-terminal (MPN) domain. Thefunction of CSN5 has been well defined since it is involved in regulating the stability of the Skp1, Cullin, F-box (SCF) complex through deneddylation of the Cullin protein [61,62]. Typically, both PCI and MPN domains are function as a scaffold. However, only CSN5 has a Jab1/MPN metalloenzyme (JAMM) motif in the MPN domain and have deneddylation function. CSN5 contains conserved sequence ($EX_nHS/THPX_7SX_2D$) in JAMM motif and this metalloprotease motif is analogous with zinc metalloproteases. The cullin protein is a one of the major components of the E3 ubiquitin ligase complex; therefore, the stability of cullin is critical for ligase function. Neddylation of cullin by CSN facilitates the SCF complex activity for ubiquitination. As previously reported, fission yeast CSN mutants lost deneddylation function for cullin proteins and inhibited ubiquitin ligase activity [63]. Interestingly, CSN6 also contains a MPN domain like CSN5, whereas CSN6 does not contain the JAMM motif. Although CSN6 and CSN5 both structurally closed, the role of CSN6 in SCF complex regulation remains elusive. Recent study showed that the CSN6 MPN domain is involved with binding between CSN6 and 14-3-3σ [40]. However, the unique function of CSN6 through MPN domain is not clear and needs to be verified.

3-3 Oncogenic function of CSN6

In a previous report, it was suggested that CSN6 has oncogenic function through binding with human immunodeficiency virus type-1 (HIV-1) viral protein R (Vpr) which facilitates virual infection in non- proliferating cells. Low expression of CSN6 suppressed cell cycle progression at the G2/M phase and therefore reduced cell proliferation and also increased HIV virus infected cell populations [64]. Based on some clues, recent studies strongly support the oncogenic function of CSN6.

Integrative Genomic Microarray Analysis (SIGMA) showed that CSN6 gene is amplified in more than 70% of breast cancer patient sample. Moreover, myeloma, glioblastoma, leukemia, and lung cancer patients showed higher levels of CSN6 expression. *In vivo* mouse model study suggested additional support for oncogenic function of CSN6. *Csn6*-knockout ($Csn6^{-/-}$) mice are embryonic lethal whereas *Csn6*-haplodeficient ($Csn6^{+/-}$) mice are viable. Interestingly, $Csn6^{+/-}$ mice have more p53 expression and less MDM2 expression than wild-type mice. And also, under DNA damage stimuli, $Csn6^{+/-}$ mice showed more p53-mediated apoptosis. These results suggest that CSN6 obtains a tumorigenic function by regulating the E3 ubiquitin ligase protein stability of MDM2 [65,66]. CSN6 stabilizes MDM2 through suppressing auto-ubiquitination activity of MDM2, an E3 ligase of p53. As a result, both p53 expression and it's tumor-suppressor function are decreased [65]. In addition, CSN6 is also involved in MDM2-mediated p53 degradation via HER2-Akt pathway. It has been known that HER2 mediated activation of Akt signaling pathway phosphorylates MDM2 at S166 and S186 which then facilitates p53 degradation in the nucleus. CSN6 was stabilized by activation of the Akt signaling pathway resulting in p53 being destabilized by MDM2. In

a similar manner, CSN6 destabilizes tumor suppressor protein 14-3-3σ by stabilizing the E3 ligase protein COP1. 14-3-3σ is positively regulated by p53 and COP1 is a known E3 ligase of p53. CSN6 directly binds with COP1 and stabilize COP1 expression result in COP1-mediated p53 degradation is increased and then 14-3-3σ expression is suppressed [40]. p57 Kip2, a member of the Cip/Kip family of cyclin-dependent kinase (CDK) inhibitor (CDI) is also regulated by CSN6. Mutations of p57 were commonly found in ovarian cancer, colorectal cancer, pancreatic cancer, lung cancer and breast cancer [67]. In addition, p57 binds with Cyclin/CDK complex protein in G1 phase and induces G1 cell cycle arrest. Therefore it was defined as a tumor suppressor protein. CSN6 interacts with both S-phase kinase-associated proteins 2 (SKP2), a known E3 ligase of p57, and p57 which then facilitates p57 degradation.

It has been confirmed that CSN6 has oncogenic function by regulating the cell cycle, cell proliferation, and tumor formation. Although some substrates of CSN6 were identified, more biological functions and substrates need to be identified.

4. AKT

4-1 Characteristic of AKT

AKT (also known as Protein Kinase B: PKB) is a serine/threonine kinase that has been identified as a homologue of the retroviral oncogene v-Akt [68]. AKT belongs to the AGC (PKA, PKG, PKC) family group which are regulated by another compound such as cyclic AMP, cyclic GMP, or lipid signaling [69]. AKT has three isoforms; AKT1, AKT2, and AKT3 which have conserved domains; the N-terminal pleckstrin homology (PH) domain, a central kinase domain (KD), and a carboxyl-terminal regulatory hydrophobic motif domain (HM). To activate AKT, PH domain serves as a phospholipid binding domain to integrate with PtdIns (3,4,5)P3 (PIP3) at the plasma membrane. PIP3 is a secondary messenger for AKT activation and it is activated by the phophatidylinositol 3 Kinase (PI3K) pathway. PI3K/AKT is activated by several growth factors and signaling stimulators such as insulin, insulin-like growth factor I (IGF-I), epidermal growth factor (EGF), vascular endothelial cell growth factor (VEGF), fibroblast growth factor (FGF), human growth factor (HGF), human epidermal growth factor receptor 2 (HER2), and platelet derived growth factor receptor (PDGF-R) [70]. Followed by membrane recruitment, both T308 in KD and S473 in the HM domain get phosphorylated by 3-phosphoinositide-dependent protein kinase (PDK1) and the mTOR complex 2 (mTORC2)[71]. In contrast, phosphatase and tensin homolog (PTEN), a lipid phosphatase, negatively regulate the PI3K/AKT signaling pathway by PIP3 dephosphorylation. In addition, serine/threonine phosphatase protein phosphatase 2A (PP2A) and PH domain leucine-rich repeat protein phosphatase (PHLPP) also negatively regulates AKT activity by dephosphorylating AKT on T308 and S473 [72,73].

4-2. AKT substrates in cancer regulation

AKT is involved in various cellular regulations through phosphorylation of substrate proteins. AKT recognize a specific sequence, R-X-RX-X-S/T, in substrate proteins and facilitates either activate or inhibitory regulation of them. Up to now, MDM2, mammalian target of rapamycin (mTOR), glycogen synthase kinase-3β (GSK3β) and Bcl-2-associated death promoter (BAD) are the well-documented AKT substrate proteins that are linked to cell proliferation, cell cycle regulation, apoptosis, and metabolism regulation. Phosphorylated MDM2 by AKT translocate from cytoplasm to nucleus. As a result, p53 ubiquitination is increased and p53 mediated cell cycle arrest, apoptosis, and DNA repair are negatively regulated by AKT [74]. AKT activates mTOR through either direct phosphorylation of mTOR or inhibitory regulation of mTOR inhibitors, tuberous sclerosis complex 2 (TSC2), and AMP-activated protein kinase (AMPK). Therefore, mTOR mediated cell proliferation and cell growth are positively regulated by AKT [75]. GSK3 phosphorylation by AKT inactivates GSK3 then increase cell proliferation and metabolism [76]. Lastly, AKT directly phosphorylates and negatively regulates BAD on S136. Followed by phosphorylation, BAD creates a tumor suppressor 14-3-3 σ binding site which then induces apoptosis [77].

Through modulating substrates, AKT is involved in cancer regulation. It has been reported that AKT1 is highly activated in many types of cancers including ovarian, breast, and prostate cancers [78]. In addition, AKT2 gene is over expressed and amplified in human ovarian and pancreatic carcinoma [79,80]. Moreover, amplification and overexpression of AKT3 was observed in triple negative breast cancer (TNBC) which have non-expression of the estrogen receptor (ER), progesterone receptor (PR)

expression, and the epidermal growth factor receptor 2 (HER2) [81]. Not only AKT but also regulators upstream AKT affect cancer regulation. PI3KCA, a positive regulator of AKT, gene copy number was increased in gastric cancer and expression level of active form of AKT was positively correlated with them. Also both somatic mutations of PI3KR1 and overexpression of AKT2 were founded in colon cancer and ovarian cancers [82]. In contrast, PTEN, a negative regulator of AKT, expression pattern is negatively correlated with active AKT in endometrial cancer samples [83]. Moreover, PTEN null cell injected nude mice form tumors and also Pten $^{+/-}$ mice are tumor prone [84].

Although many AKT related downstream substrates and their cellular functions are identified, there are still unidentified AKT functions and substrates. Therefore, verification of AKT on novel substrates and discovery of mechanisms is still required.

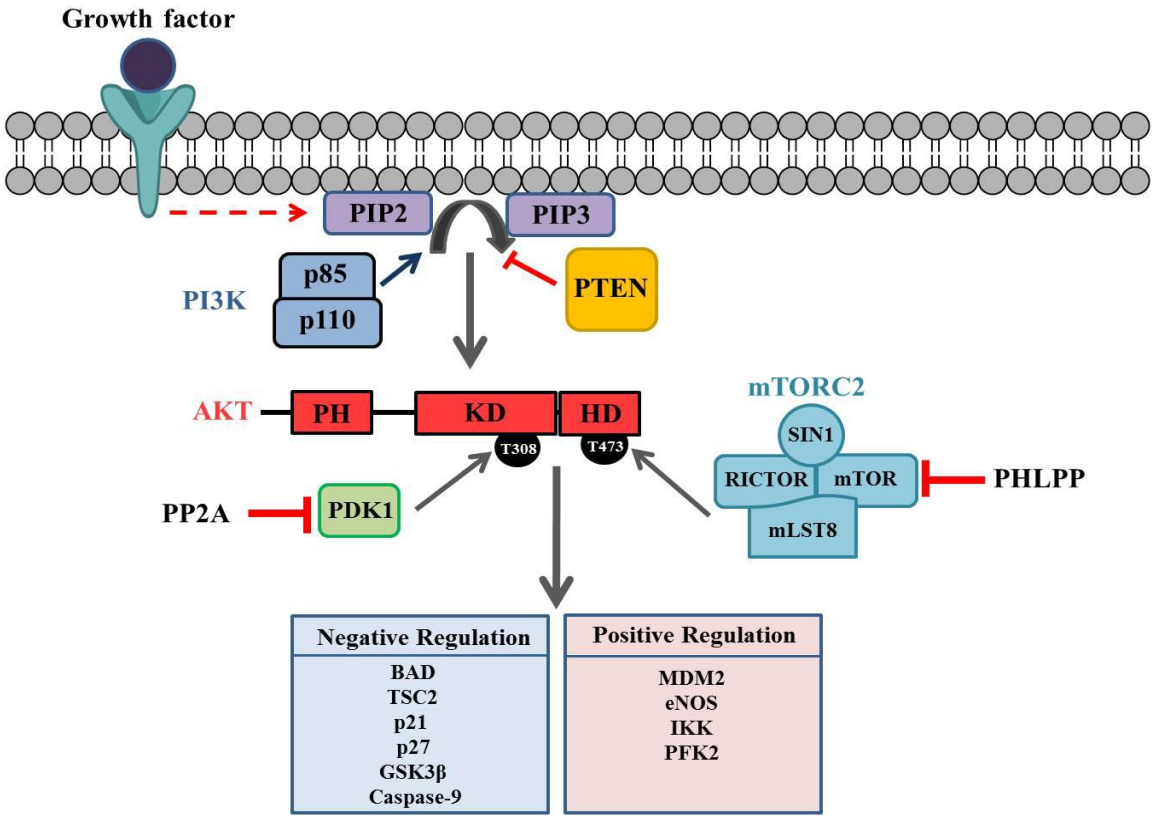

Figure 5. AKT signaling Pathway. Growth factor induced PI3 Kinase activation phosphorylates PIP2 and generates PIP3 phospholipid. PIP3 recruits AKT to the cytoplasmic membrane and binds through the PH domain of AKT. PDK1 phosphorylates AKT on T308 and mTORC2 phosphorylates S473 to activate AKT. Fully activated AKT regulates various cellular functions through phosphorylating substrate proteins. The AKT signaling pathway is also negatively controlled by other dephosphatases. PTEN inhibits AKT activation through dephosphorylating PIP3. PDK1 is negatively regulated by PP2A and mTORC2 also dephosphorylates by PHLPP.

CHAPTER 2

MATERIALS

AND

METHODS

2-1 Cell culture and reagents

HCT116 FBXW7$^{+/+}$ and FBXW7$^{-/-}$ cells were gifted to us by Dr. Bert Vogelstein (The Johns Hopkins University, Baltimore, MD) and cultured in McCoy's 5A medium. U2OS Myc-COP1 stable overexpressing cells have been described previously [85]. MDA-MB-453 WT and Dominant Negative Akt (DN-Akt) cells were gifted from Dr. Mien-Chie Hung (MD Anderson cancer center, Houston, TX). HEK 293, HEK 293T, U2OS, 3T3L1 and PC3 were purchased from ATCC and cultured in DMEM/F12 medium. All culture medium contained 10% fetal bovine serum (for HCT116, MDA-MB-231, HEK 293, HEK 293T, U2OS, PC3) or 10% bovine calf serum (for 3T3L1), 2 mM L-glutamine (Cellgro) and 1% antibiotic-antimycotic solution (Invitrogen). TNT system was purchased from Promega; MG132 and Cycloheximide (CHX) were obtained from Sigma. Ni-NTA agarose was purchased from Invitrogen. IGF-1 and EGF were obtained from Calbiochem. LY294002 was purchased from Cell Signaling. CIP was obtained from NEB. GST-Akt recombinant protein, Flag-peptide, and HA-peptide were purchased from Sigma. Agarose beads A, G were obtained from Santa Cruz Biotechnology. Antibodies: Flag (M2 monoclonal antibody, Sigma), actin (Sigma), Hemagglutinin (HA, 12CA5, Roche), tubulin (Sigma), c-Myc, Ubiquitin, GST and Cyclin E (Santa Cruz), and His, Akt, p-Akt (Cell signaling), FBXW7 (Invitrogen), Notch (Abcam), COP1 (Bethyl Laboratory), CSN6 (BIOMOL International).

2-2 Plasmids

The human *Csn6* gene was amplified by PCR and then subcloned either into pCMV5 with a Flag-tagged sequence or a GFP-tagged sequence or into pCDNA6 with a

Myc-tagged sequence. The PCR-generated Cyclin E DNA fragment was subcloned into pCMV5. shCSN6 was cloned into a pSilencer. His-ubiquitin WT, K48, K63 plasmids were kindly provided by Dr. Hui-Kuan Lin (M.D. Anderson Cancer Center), HA-FBXW7 WT plasmid was gifted from Dr. Xin Lin (M.D. Anderson Cancer Center) and Flag-c-Jun plasmid was a gift from Dr. Zhimin Lu (M.D. Anderson Cancer Center). The Flag- FBXW WT, ΔD, N-terminus only, WD40 and ΔF-box expressing vectors have been previously described [86]. Flag- FBXW7 TA, SA, TSAA, TSDD, VA, PA and VPAA mutants were generated using PCR-directed mutagenesis. Myc-COP1 WT, Flag-COP1 WT, N-terminus only, WD40 and ΔRING expressing vectors have been described [85]. shCOP1 was cloned into a pSilencer (Ambion). HA-CA-Akt, HA-DN-Akt and GST-WT-Akt expressing vectors were generated using PCR.

2-3 Immunoprecipitation and immunoblotting

Cells were lysed with lysis buffer (20 mM Tris [Fisher], 100 mM NaCl [Fisher], 0.5% Nonidet P-40 [USB Corp.], 0.5% Triton X-100 [Sigma], 1 mM EDTA [Fisher]). Fresh protease/phosphatase inhibitors (5 mM NaV, 1 mM NaF, 1 μM DTT, 0.1 mg/mL Pepstatin A, 1 mM PMSF, and 1,000× Complete Mixture Protease Inhibitor [Roche]) were added into the lysis buffer. Protein lysates were standardized, and equal amounts of proteins were subjected to immunoblot analysis. For immunoprecipitation, cells were lysed with lysis buffer and then the same amounts of proteins were directly pulled down with Flag (M2) agarose beads, or immunoprecipitated with specific antibody overnight at 4ºC; the antibody was then pulled down with Protein A/G beads (Santa Cruz) for 3 hrat 4ºC, and finally Western blot analysis was performed.

2-4 *In vitro* binding assay

The indicated T7 promoter containing plasmid DNAs were reacted with a TNT system (transcription/translation reactions) for eukaryotic *in vitro* translation. Recombinant proteins were incubated in binding buffer and then immunoprecipitated overnight with specific antibody at 4ºC; the antibody was then pulled down with Protein A/G beads (Santa Cruz) for 3 hr at 4ºC, and finally Western blot analysis was performed.

2-5 Protein turnover assay

Cells were transfected with the indicated plasmids for 48 hr and were then treated with 200 μg/ml of cycloheximide (CHX) for the indicated times. Cells were collected at each indicated point in time and lysed with a protease/phosphatase inhibitor contained in cell lysis buffer, as previously described [87]. After the protein was standardized, equal amounts of protein were subjected to Western blot analysis with the indicated antibody.

2-6 *In vivo* ubiquitination assay

HEK293T, HCT116 WT, and HCT116 FBXW7$^{-/-}$ cells were co-transfected with His-tag containing plasmids for 48 h. Before the cells were harvested, they were treated with 5 μg/ml of MG132 (Sigma) for 6 h. PBS-washed cells were lysed with denaturing buffer (6 M guanidine-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM imidazole); the cell lysates were then incubated with Ni-NTA agarose beads for 3 hr at room temperature. Western blot analysis was then performed with the indicated antibody.

2-7 *In vitro* ubiquitination assay

Flag-FBXW7 and Flag-COP1 recombinant proteins were generated by TNT system (Promega, L1170). 200 pmol His_6-ubiquitin, 2 pmol UBE1 (Biomol International),

10 pmol E2 (UbcH5a/5b), 2 mmol/L ATP, and active GST-Akt recombinant proteins were mixed and then incubated for 1 hr at 37°C.

2-8 Generation of stable transfectants

HEK293T cells were co-transfected with the indicated lentiviral plasmid DNAs Akt-lentiviral shRNA-1(5' CCGGCGTGCCATGATCTGTATTTAACTCGAGTTAAA TACAG ATCATGGCACGT TTTTG-3'), shRNA-2(5'-CCGGGGACAAGGACGGG CACATTAAC TCGAGTTAATGTGCCCGTC CTTGTCCTTTTT-3'), CSN6-lentiviral shRNA-1(5'- CCG GCGGAGTGACTGGGAGTGTTTCTCGAGAAACACTCCCAGT CACTCCGGTTTTTG-3'), CSN6-lentiviral shRNA-2(5'- CCGGCCTATGACCAAGC ACACAGATCTCGAGAT CTGTGTGCTTGGTCATAGGTTTTTG-3'), control shRNA and packaging (deltaVPR8.9) and envelope (VSV-G) plasmids to make lentiviral particles through the viral packaging process. Virus-containing supernatants were collected and filtered, then MDA-MB-231, 3T3L1 and HEK 293 cells were infected with lentiviral particles, either shLuciferase or target shRNA with 8 μg/ml of polybrene. After infection, cells were selected with 2-4 μg/mL of puromycin for 2 weeks.

2-9 *In vitro* kinase assay

In vitro kinase assay was performed as described [88]. Flag-FBXW7 WT, T226A, S227A and TSAA mutants were generated by TNT system. Each protein was incubated with γ32 ATP (Perkin-Elmer) and active GST-Akt recombinant proteins at 30 °C for 30 min. Kinase activity was analysed by Western-Blotting and then gels were dried and

imaged using a phosphoimager cassette (Molecular Dynamics) and a Typhoon Trio variable mode imager. Images were processed using Image Quant 5.1 software.

2-10 Wound healing, Trans-well migration assay, Soft agar colony formation assay, Invasion assay, FACS analysis

A. Wound healing assay: the same amounts of HCT116 FBXW7$^{-/-}$ or MDA-MB-231 cells were plated into a 12-well plate and then cultured until confluence. After making a scratch with tip, plates were placed into a Microscope incubator for 30hr and images were captured at each time point.

B. Trans-well migration assay: 0.6x10^{5} HCT116 FBXW7$^{-/-}$ or MDA-MB-231 cells were plated onto a transwell membrane with 0.5% FBS containing culture media on top and add 10% FBS media on bottom then incubated for 12hr. Wiped top of the membrane and cells were fixed and stained with Crystal violet.

C. Soft agar colony assay: 2.5 x 10^{3} MDA-MB-231 cells were suspended in 0.35% agarose containing complete media and then were seeded onto 0.7% agarose that contained a complete media bottom layer. Cells were cultured in agarose, and every 3 days, culture medium was added onto the plate; this continued for 4 weeks. Colonies were stained with 0.5 mg/ml of *p*-iodonitrotetrazolium violet (Sigma) and were counted with use of a light microscope.

D. Invasion assay: 0.6x10^{5} HCT116 FBXW7$^{-/-}$ cells were plated onto a matrigel Boyden chamber with 0.5% FBS containing culture media on top and add 10% FBS media on the

bottom then incubated for 48hr. Wiped top of the membrane and cells was fixed and stained with Crystal violet.

E. FACS analysis: HCT116 FBXW7$^{-/-}$ cells were transfected with indicated FBXW7 expressing vectors then 0.5x10^{6} cells were stained with Propidium Iodide (PI) for 30 min at RT. Cell cycle distribution was analyzed by flow cytometry.

2-11 Nude mice experiment

A total of 5 × 10^{6} cells, either shControl or shCSN6 3T3L1 cells, were injected into the flanks of 6-week-old *nu/nu* mice, which were then monitored for 6 weeks. Tumor volume was measured twice a week, and solid tumors were collected, weighed, and immunohistochemically analyzed with use of indicated antibodies.

2-12 Immunohistochemical analysis

Tumor tissues were fixed and embedded. Slide sections were then incubated overnight with the indicated primary antibody (1:100–1:200 dilutions) at 4°C. Hematoxylin staining was used for counterstaining. After the slides were stained, they were scanned and analyzed by an ACIS III image analyzer (DAKO).

2-13 Human tumor samples

Gene expression profiles of 278 patients of the GSE20194 cohort with stage I, II, or III breast cancer were retrieved from the Gene Expression Omnibus database and correlated with their corresponding clinical profile. These patients had not received any treatment at the time of sample collection. In addition, the samples from these patients contained fine needle aspirates with minimal contamination of normal tissues. Therefore,

this cohort was very reliable and appropriate for our analysis. Patients were then divided into 4 quartiles according to their CSN6 mRNA levels with use of Nexus Expression 3.0 software (BioDiscovery). The high CSN6 quartile was compared with the low CSN6 quartile with use of Gene Set Enrichment Analysis (Broad Institute, Massachusetts Institute of Technology). Overall and recurrence-free survival curves were built by using Graph Pad Prism v5.0d (GraphPad). A logrank test was used to compare the survival curves. To examine the frequent overexpression of CSN6 in multiple common types of cancer, we used the Oncomine database and analysis tools. N represented the total number of patients analyzed for each type of cancer. A 60% increase in CSN6 mRNA when compared with corresponding normal tissue was used as our standard. A patient was considered as having CSN6 overexpression only if their level of tumor CSN6 mRNA was at least 60% higher than that of her normal breast tissue.

CHAPTER 3

COP1regulates FBXW7 stability in an AKT dependent manner

A. RATIONALE

FBXW7 is a major tumor suppressor protein that ubiquitinates several oncogenic substrate proteins. Therefore, stability and activity of FBXW7 is critical to suppress tumorigenesis. However, only a few studies report that FBXW7 expression level or activity is regulated by other molecules. Kinase was suggested one of possible regulator of FBXW7. Kinases such as PLK2 [89] and PKC [90] regulate FBXW7 through controlling dimerization or cellular localization. It suggests that FBXW7 could be affected by kinase reaction. However, only a few kinases were verified to control FBXW7 and also signaling inducer to phosphorylate FBXW7 is unknown. It is a common phenomenon for the E3 ubiquitin ligases to detect phosphorylated substrates. Therefore, it is highly possible that phosphorylated FBXW7 could be detected by other E3 ubiquitin ligases as a substrate.

The goal of this study is identify a novel kinase which can regulate FBXW7 stability and also verify whether other E3 ubiquitin ligases are involve in regulation of FBXW7 stability.

B. RESULT

3-1. FBXW7 is a substrate of AKT

To investigate a novel kinase for FBXW7 and biological conditions, I analyzed possible kinase consensus sequences of FBXW7 protein with NetPhos program. I found that FBXW7 has AKT consensus binding sequence at T226 and S227 (Fig.6A). *In vitro* kinase assay with ^{32}P labeled FBXW7 and recombinant AKT proteins clearly showed that mutations at T226 and S227 in FBXW7 proteins have less phosphorylation compared with WT FBXW7 (Fig.6B). I also examined whether AKT phosphorylates *in vivo* FBXW7 with dual phospho-FBXW7 (T226 and S227) antibody. Indeed, I detected phosphorylated FBXW7 in WT but not in T226A/S227A mutants (Fig.6C). IGF1 and EGF are known growth factors which activate the AKT signaling pathway [91]. Thus, I further investigated whether they can induce FBXW7 phosphorylation through AKT activation. Both IGF1 and EGF induced endogenous FBXW7 phosphorylation followed by AKT activation. Interestingly, I observed that endogenous FBXW7 proteins were destabilized whereas substrate proteins of FBXW7, Cyclin E, and c-Myc, expression levels were significantly increased according to IGF1 and EGF treatment (Fig.7). In contrast, PI3K inhibitor LY 294002 reversed both endogenous FBXW7 and phosphorylated FBXW7 expression levels under IGF1 treatment (Fig.8A). In addition, I verified the effect of IGF1 on FBXW7 phosphorylation with WT and T226A/S227A mutants. As a result, IGF1 induced FBXW7 phosphorylation in WT but not in T226 and S227 mutants (Fig.8B). Together, these results suggest that FBXW7 is a substrate of AKT kinase and both IGF1 and EGF work as a biological inducer to phosphorylate

FBXW7 followed by AKT activation then increase Cyclin E and c-Myc stability through destabilizing FBXW7.

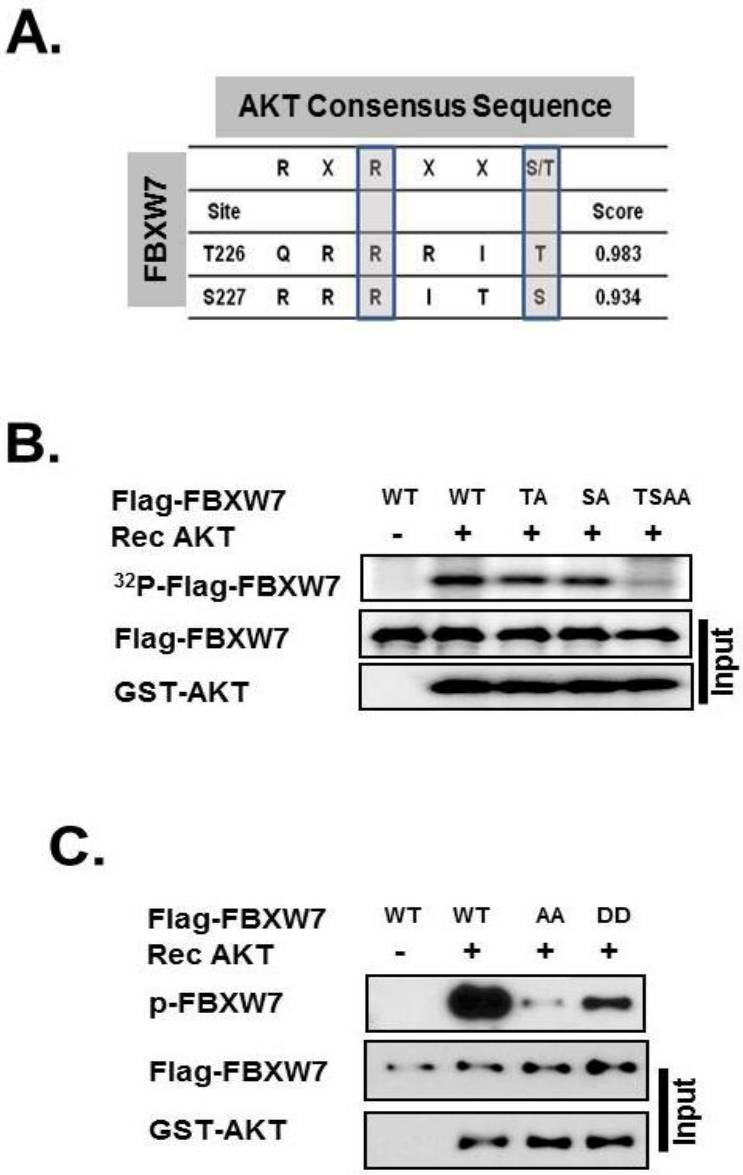

Figure 6. AKT phosphorylates FBXW7. (A) AKT consensus site in human FBXW7. (B-C) Flag-FBXW7 proteins were made using a TNT system for *in vitro* kinase assay. Indicated Flag-FBXW7 proteins were incubated with recombinant active AKT1 protein and subjected to SDS-PAGE analysis.

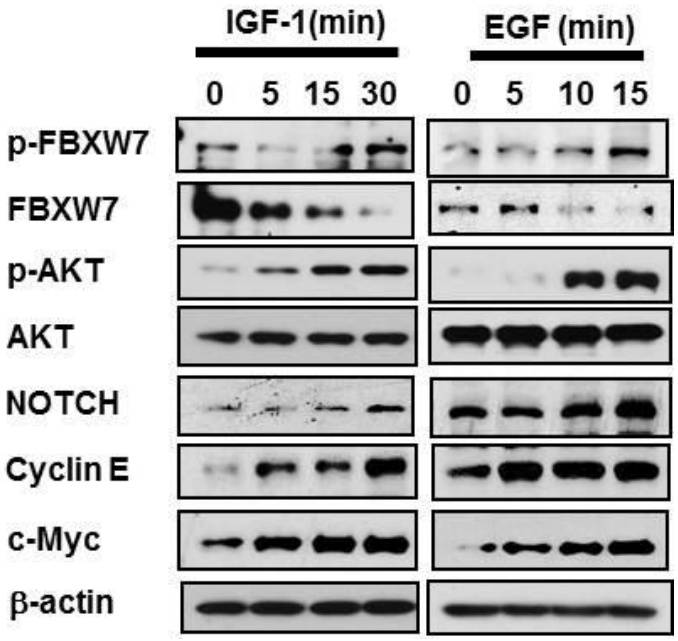

Figure 7. IFG-1 and EGF induce FBXW7 phosphorylation. HEK 293 cells were serum starved for 24hr then treated with 100ng/ml IFG-1 or 50ng/ml EGF. Same amount of proteins were immunoblotted with T226/S227 dual phospho-FBXW7 antibody.

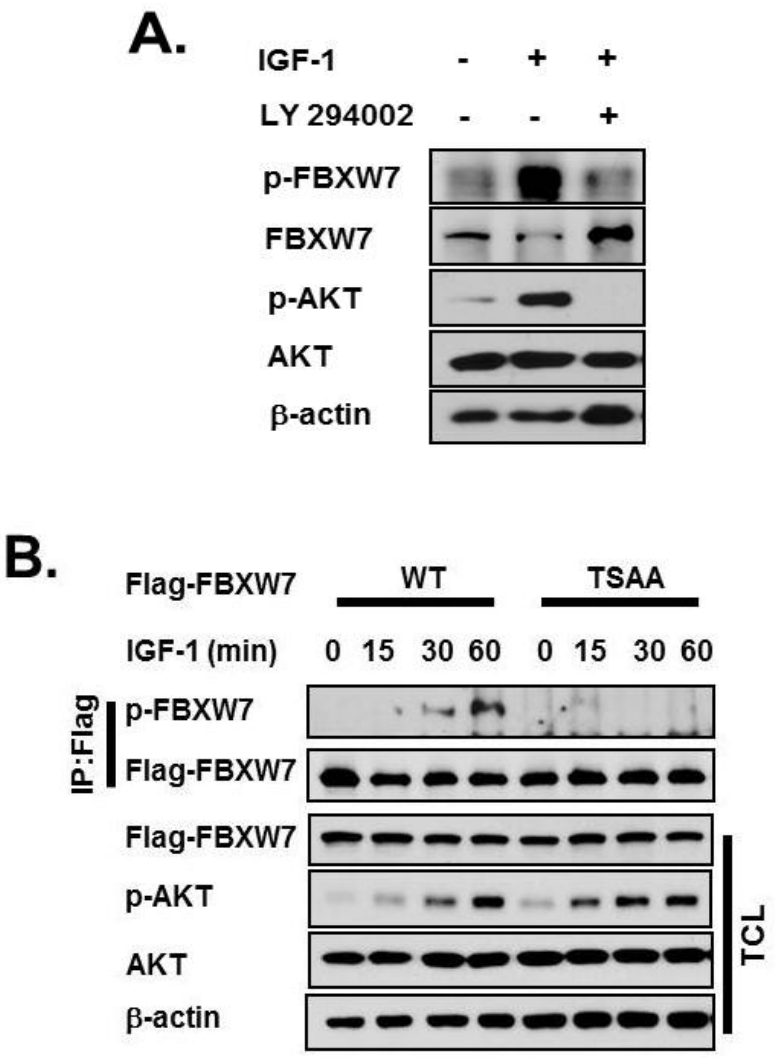

Figure 8. PI3K/AKT inhibitor LY294002 and phospho-dead mutant FBXW7 inhibit IGF-1 induced FBXW7 phosphorylation. (A) HEK 293 cells were serum starved for 24hr and treated with 100ng/ml IGF-1 for 1hr with or without 20μM LY294002 for 6 hr before harvesting. (B) HEK 293 cells were transfected with Flag-tag WT FBXW7 or T226A/S227A FBXW7 plasmids and treated with 100ng/ml IGF-1 for indicated time after 24hr serum starvation. Equal amount of cell lysates were immunoprecipitated with anti-Flag and immunoblotted with anti-phospho FBXW7 antibody. IP: imunoprecipitation; TCL: total cell lysates.

3-2. AKT affects FBXW7 stability

To further investigate whether FBXW7 phosphorylation by AKT regulates FBXW7 stability, I tested steady-state expression of FBXW7 using *de novo* protein synthesis inhibitor cycloheximide (CHX). Turnover rate of FBXW7 protein in shAKT HEK 293 cell was decelerated compare with control shRNA cell (Fig.9). Consistent with these studies, increased dosage of LY294002 stabilized WT FBXW7 expression levels but failed to increase T226A/S227A in FBXW7 mutants (Fig.10). To investigate the underlying mechanisms of AKT-mediated FBXW7 destabilization, I examined whether AKT increased FBXW7 degradation through the proteasome complex. Indeed, AKT increase FBXW7 ubiquitination through K48-linked polyubiquitination (Fig.11). However, T226A/S227A FBXW7 mutant was rescued from AKT mediated ubiquitination (Fig.12A). FBXW7 ubiquitination levels were significantly decreased with DN AKT (Fig.12B). In contrast, CA AKT increased FBXW7 ubuquitination but LY294002 reversed FBXW7 ubiquitination level (Fig.13A). Moreover, endogenous FBXW7 ubiquitination levels were increased by IGF1 treatment (Fig.13B). I therefore conclude that AKT regulates FBXW7 stability through K48-linked proteostomal degradation system.

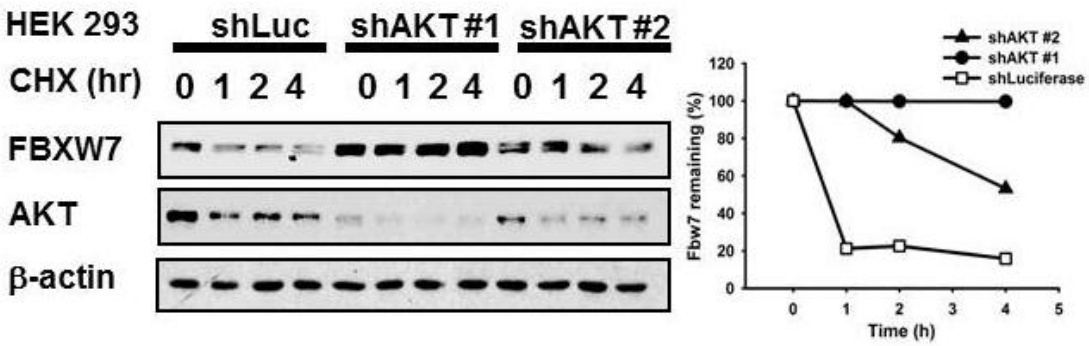

Figure 9. AKT have negative impact on FBXW7 stability. HEK 293 cells were infected with lentiviral shLuciferease or shAKT and were then treated with cycloheximide (CHX) for 0, 1, 2, 4 hr. Cell lysates were subjected to Western Blot analysis.

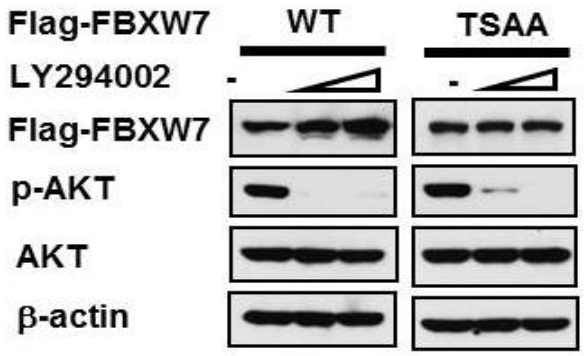

Figure 10. Phospho-dead mutant FBXW7 is not regulated by PI3K/AKT inhibitor. HEK 293 cells were transfected with WT or T226A/S227A FBXW7 plasmids for 48 hr then 10μM or 20μM LY294002 were treated for 6 hr before harvesting. Immunoblot analysis was performed.

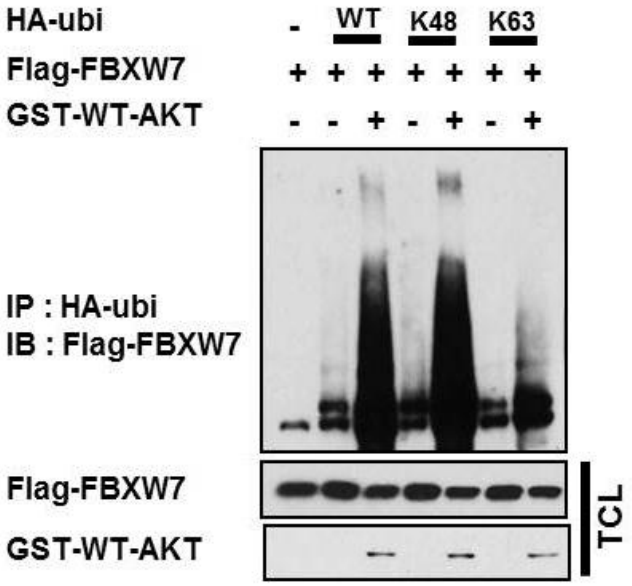

Figure 11. AKT regulates FBXW7 stability through K48-linked proteostomal degradation. HA- Ubiquitin WT, K48 or K63 plasmids were transfected into HEK 293 cells with Flag- FBXW7 and GST-WT-AKT. 48hr after transfection, cells were treated with 10 μM MG132 for 6 hr. Western blot analysis with Flag-antibody was performed followed by immunoprecipitate with HA antibody .

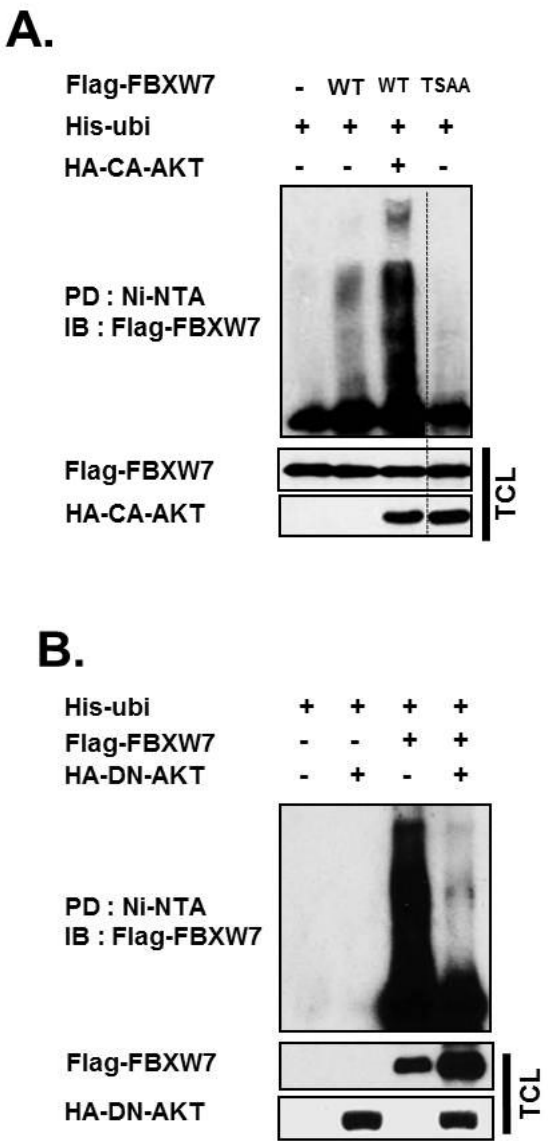

Figure 12. AKT ubiquitinates FBXW7. (A) HEK 293 cells were transfected with His-Ubiquitin WT, HA-CA-AKT and Flag-FBXW7 WT or T226A/S227A plasmids. (B) HEK293 cells were transfected with His-Ubiquitin WT, HA-DN-AKT and Flag-FBXW7 WT plasmids. All cells were treated with 10 μM MG132 for 6 hr then subjected to pull-down with Ni-NTA. Denatured cell lysate were subjected for immunoblot with Flag antibody. PD: pull-down; Ni-NTA: nickel nitrilotriacetic acid.

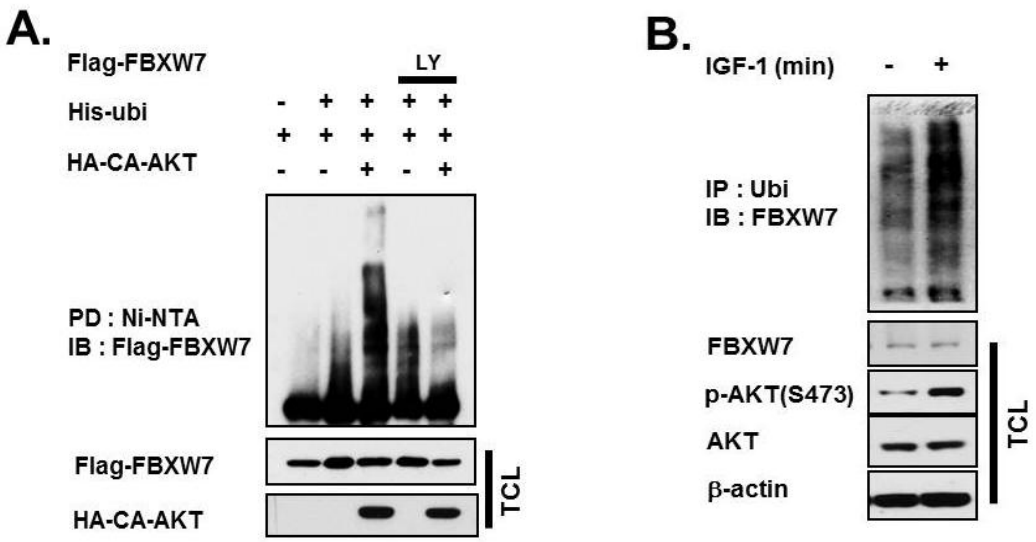

Figure 13. IGF-1 and LY294002 affect AKT-mediated FBXW7 ubiquitination. (A) HEK 293 cells were transfected with His-Ubiquitin WT, HA-CA-AKT and Flag-FBXW7 WT plasmids. Cells were treated with 20μM LY 294002 for 6 hr and subjected to pull-down with Ni-NTA. All cells were denatured cell lysate were subjected for immunoblot with Flag antibody. (B) HEK 293 cells were serum starved for 24hr then treated with 100ng/ml IGF-1 for 1hr then immunoblot with FBXW7 after IP with Ubiquitin antibody. All cells were treated 10 μM MG132 and for 6 hr before harvesting.

3-3. COP1 interacts with FBXW7

In a previous figure, I found that AKT regulates FBXW7 stability through proteasome complex. However, AKT is not an E3 ubiquitin ligase protein. Thus, to investigate a possible E3 ubiquitin ligase protein, I analyzed possible E3 ligase binding motifs of the FBXW7 protein. I found that FBXW7 has an evolutionary conserved COP1 binding sequence (Fig.17). First, I confirmed both *in vivo* endogenous binding and *in vitro* binding between FBXW7 and COP1 (Fig.14 A, B). Domain mapping studies suggest that the FBXW7 N-terminus binds with C-terminus of COP1 (Fig.15). In addition, COP1 interacts with FBXW7 through the WD40 domain which is the substrates binding domain (Fig.16). In further investigation, I examined the binding affinity to COP1 with WT FBXW7 and V200A/P201A, a COP1 binding site mutant of FBXW7. As a result, WT FBXW7 associated with endogenous COP1 but V200A/P201A mutant FBXW7 failed to bind with COP1 (Fig.17).

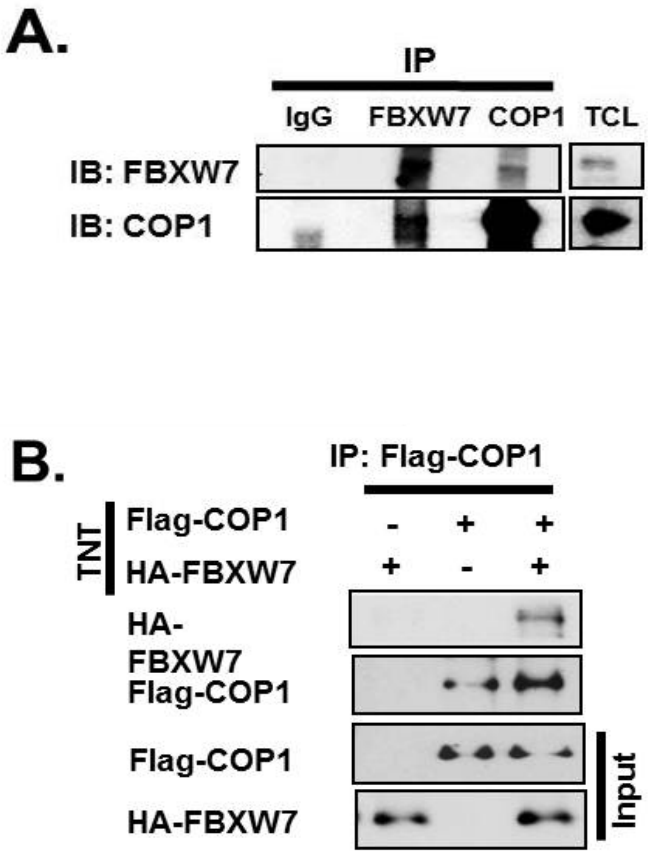

Figure 14. *In vivo* and *in vitro* binding between FBXW7 and COP1. (A) HEK 293T cells were harvested after MG132 treatment for 6hr then IP and IB with indicated antibody were performed. (B) Flag-COP1 and HA-FBXW7 were translated using an *in vitro* transcription/translation system (TNT). Flag-COP1 proteins were immunoprecipitated with anti-Flag and then immunoblott analysis was performed with the indicated antibodies.

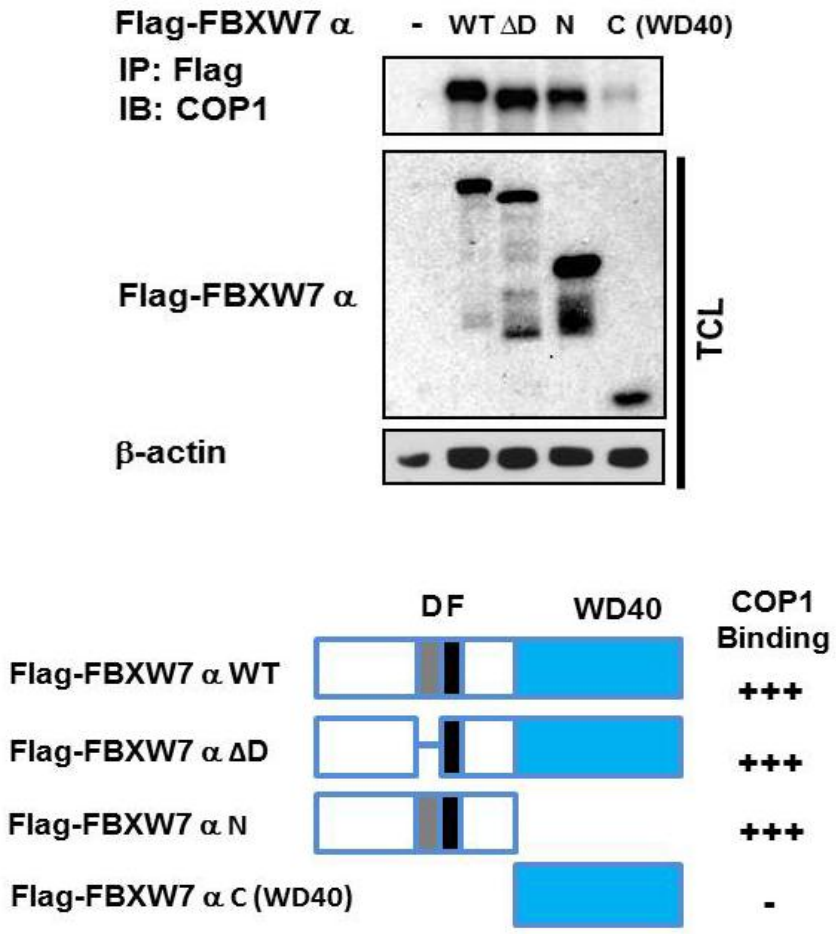

Figure 15. FBXW7 interacts with COP1 through N-terminal domain. WT, dimerization domain deletion (ΔD), N-terminus only or C-terminus only Flag-FBXW7 constructs were transfected into HEK 293T cells. After 48 hr, cells were treated with 10 μM MG132 and for 6 hr before harvesting. Cell lysates were immunoprecipitated with Flag-beads and Western Blot analysis was performed with COP1 antibody.

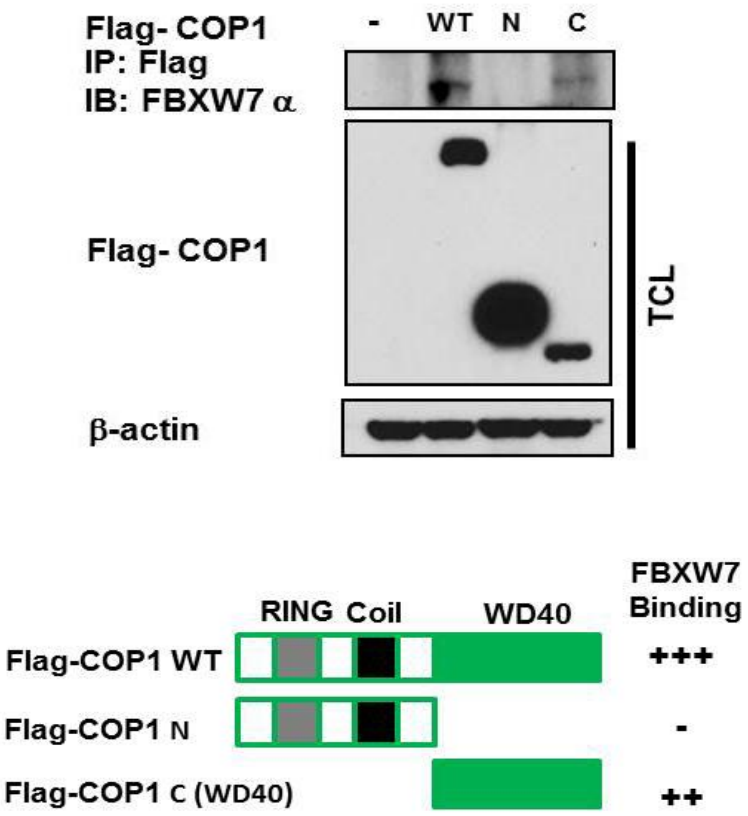

Figure 16. COP1 interacts with FBXW7 through WD40 domain. WT, N-terminus only or WD40 domain Flag-COP1 were transfected into HEK 293T cells. After 48 hr, cells were treated with 10 μM MG132 and for 6 hr before harvesting. Cell lysates were immunoprecipitated with Flag-beads and WesternBlot analysis was performed with FBXW7 antibody.

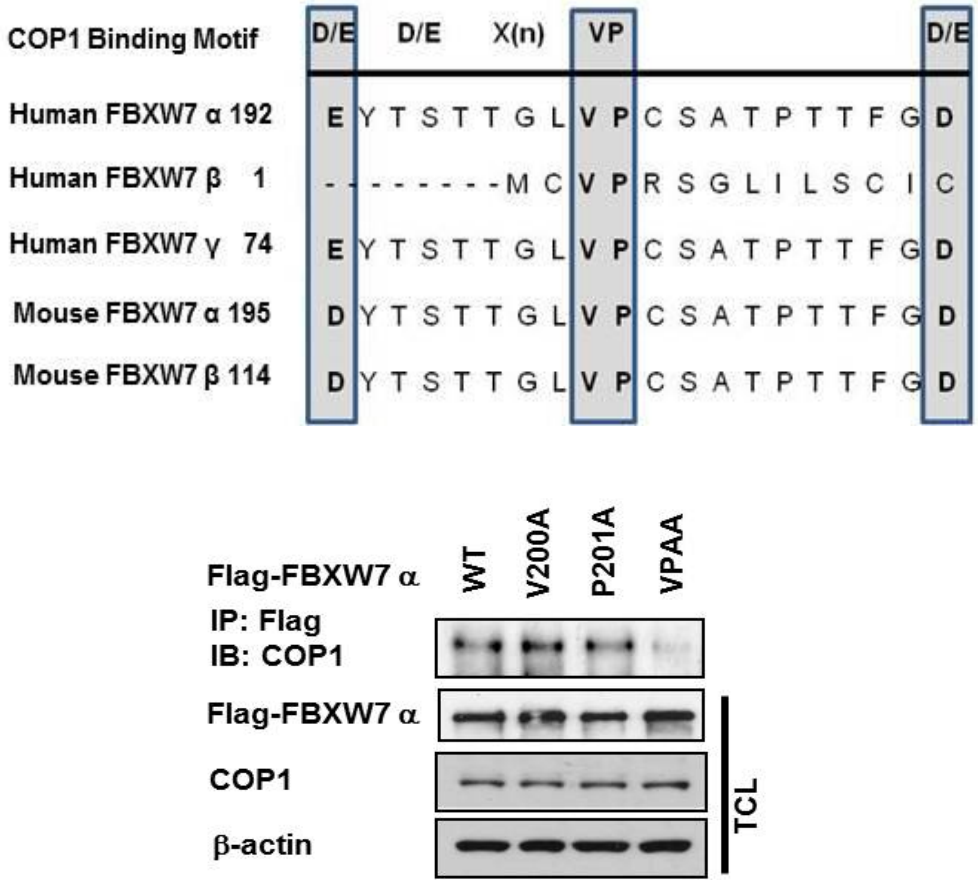

Figure 17. FBXW7 has evolutionary conserved COP1 binding motif at V200 and P201. WT, V200A, P201A or V200A/ P201A Flag-FBXW7 constructs were transfected into the cell then cell lysates were subjected for IP with Flag beads and IB with COP1 antibody. All cells were treated with 10 μM MG132 and for 6 hr before harvesting.

3-4. COP1 work as an E3 ligase of FBXW7

To further confirm negative regulation of COP1 on FBXW7, I verified the steady-state expression of FBXW7. Steady-state of all isoforms of FBXW7 (α, β, γ) was decreased by overexpression of COP1 in HEK293 cell (Fig.18A). In addition, I verified the negative impact of COP1 on FBXW7 regulation with cycloheximide (CHX). Turn-over rate of FBXW7 proteins was increased by stable overexpression of COP1 (Fig.18B). In contrast, FBXW7 turn-over rate was significantly decreased by knock-down COP1 (Fig.18C). Interestingly, WT FBXW7 but not V200A, P201A and V2001/P201A FBXW7 mutants turn-over rate were regulated by COP1 (Fig.19). To further investigate the cellular mechanisms of COP1 mediated FBXW7 destabilization, I examined whether COP1 functions as an E3 ubiquitin ligase of FBXW7. FBXW7 degradation by COP1 was rescued with proteasome inhibitor MG132 treatment. Overexpression of COP1 increased FBXW7 poly-ubiquitination whereas shCOP1 decreased FBXW7 ubiquitination level (Fig.20A, B). Moreover, V2001/P201A FBXW7 rescued from COP1 mediated ubiquitination compare with WT FBXW7 (Fig.21). I also examined the effect of COP1 on FBXW7 ubiquitination with WT, Ring Mut, and the truncated form (N-terminus, C-terminus) of COP1. Only WT COP1 induced FBXW7 polyubiquitination (Fig.22). *In vitro* ubiquitination assay with TNT products also clearly show that COP1 increased FBXW7 polyubiquitination (Fig.23). To confirm that FBXW7 ubiquitination is not by self-ubuquitination, I examine effect of COP1 on FBXW7 with FBXW7ΔF, which destroyed its auto-ubiquitination activity. As I expected, overexpression of COP1 increased FBXW7ΔF ubiquitination whereas shCOP1 significantly decreased FBXW7

ubiquitination (Fig.24). Thus, I conclude that FBXW7 is a novel substrate of COP1 and COP1 works as an E3 ubiquitin ligase of FBXW7.

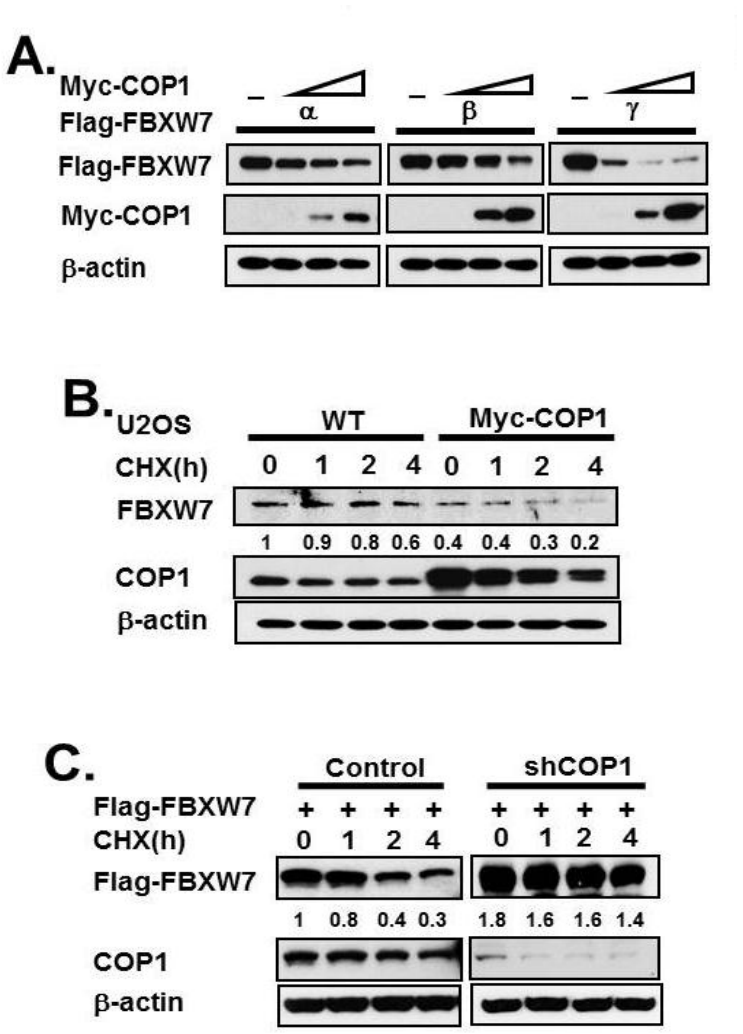

Figure 18. COP1 negatively regulates FBXW7 stability. (A) Equal amounts of HEK 293T cells were transfected with Flag-FBXW7 (α, β, γ) and different amount of Myc-COP1 plasmids. (B) Control vector or Myc-COP1 stable overexpressing U2OS cells were treated with CHX for indicated time. (C) Control shRNA or COP1 shRNA infected HEK 293 cells were treated with CHX for indicated time. Cell lysates were immunoblotted with indicated antibodies.

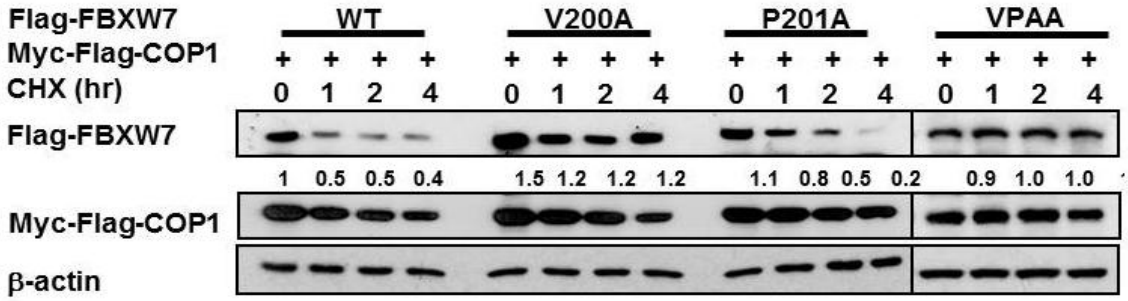

Figure 19. FBXW7 mutants at COP1 binding motif rescued by negative regulation of COP1. WT, V200A, P201A or V200A/ P201A Flag-FBXW7 constructs were co-transfected with Myc-Flag-COP1 plasmid into the HEK 293 cell then subjected for Western Blot analysis followed by CHX treatment at different time points.

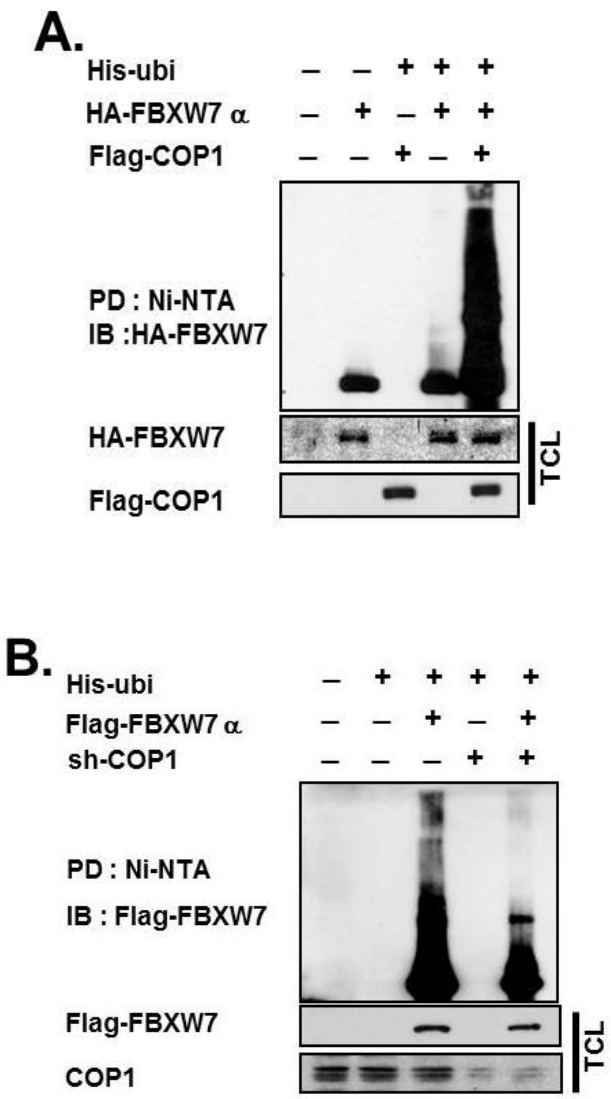

Figure 20. COP1 ubiquitinates FBXW7. (A) HEK 293T cells were co-transfected with His-Ubiquitin, HA- FBXW7 and Flag- COP1 constructs. (B) HEK 293T cells were co-transfected with His-Ubiquitin, Flag - FBXW7 and shCOP1 constructs. Cell lysates were pulled down (PD) with Ni^{++} NTA beads and then immunoblotted (IB) with indicated antibodies. All cells were treated with 10 μM MG132 for 6 hr before harvesting.

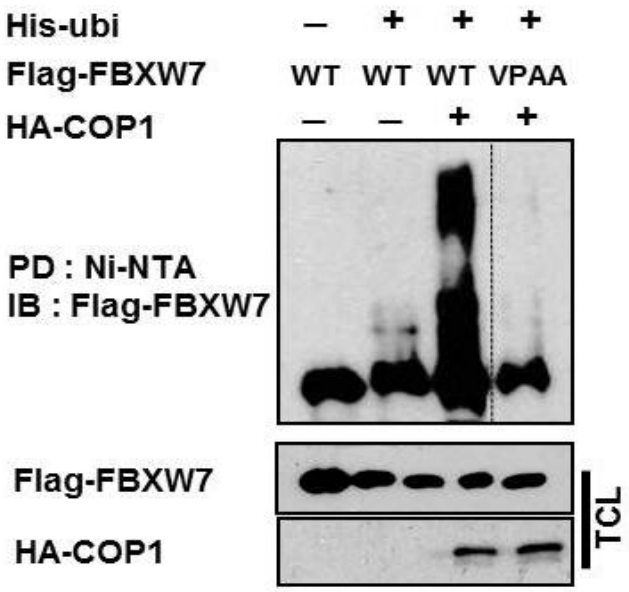

Figure 21. COP1 fail to ubiquitinates FBXW7 mutants at COP1 binding motif. HEK 293T cells were co-transfected with His-Ubiquitin, Flag-FBXW7 (WT, 200A/201A) and COP1 constructs for 24 hr and treated with MG132 for 6 hr. Cell lysates were pulled down (PD) with Ni^{++} NTA beads and then immunoblotted (IB) with indicated antibodies.

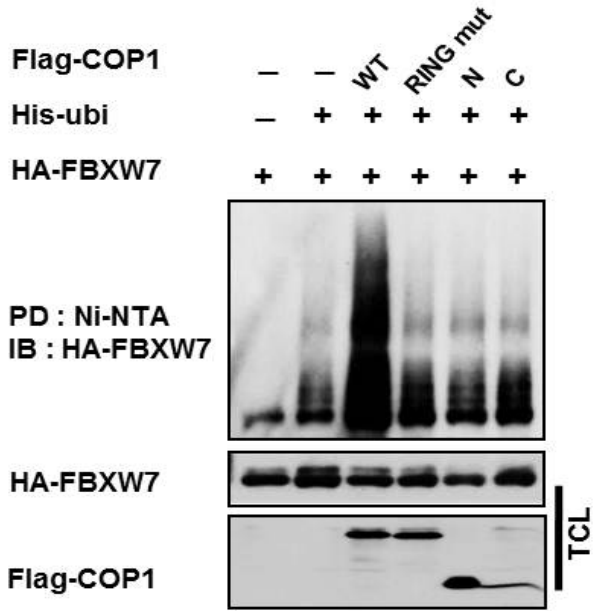

Figure 22. RING domain mutated COP1 fail to ubiquitinates FBXW7. His-Ubiquitin, Ha-FBXW7 and WT, Ring-mutant, N-terminus only or C-terminus only Flag-COP1 plasmids were transfected into cells. After MG132 treatment for 6 hr, cell lysates were PD with Ni-NTA and IB with HA-FBXW7.

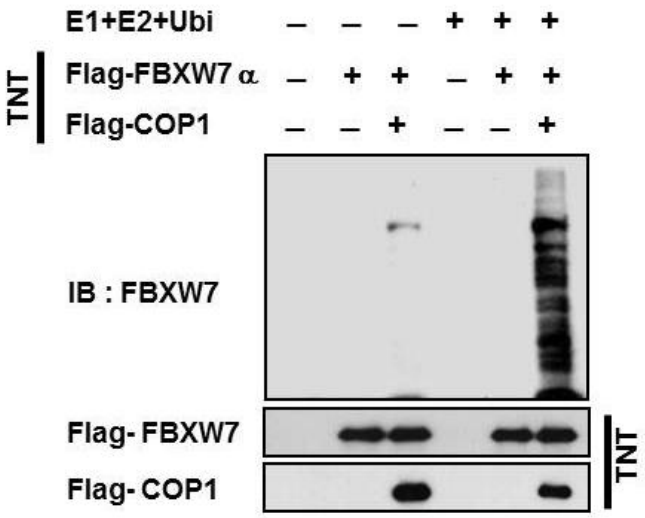

Figure 23. *In vitro* ubiquitination of FBXW7 by COP1. Flag-COP1 and Flag-FBXW7 were translated using an *in vitro* transcription/translation system (TNT). *In vitro* ubiquitination reaction was performed with recombinant E1, E2 and Ubi and then immunoblotting was performed with FBXW7 antibody.

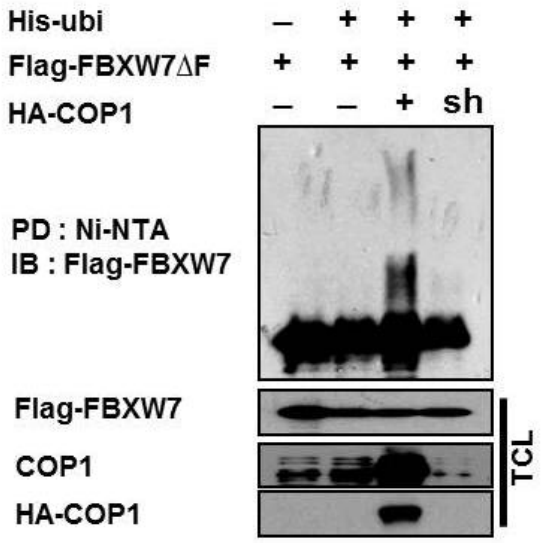

Figure 24. COP1 ubiquitinates FBXW7 via proteasomal pathway. HEK 293T cells were transfected with His-Ubiquitin, F-box domain deleted Flag-FBXW7 (ΔF), HA-COP1 and shCOP1 constructs. Cell lysates were subject to pulldown with Ni-NTA beads then immunoblot was performed with Flag antibody.

3-5. COP1 regulates FBXW7 in an AKT dependent manner

I found that AKT phosphorylates FBXW7 and COP1 is a novel E3 ligase of FBXW7. However, I still needed to verify whether AKT and COP1 collaborate to regulate FBXW7. Interestingly, I observed that the association between COP1 and FBXW7 is significantly decreased by CIP (Calf-intestinal alkaline phosphatase) treatment (Fig.26A). This suggests that any kinase possibly involves binding between COP1 and FBXW7. Thus, I examined whether AKT was related with COP1 and FBXW7 binding. Indeed, AKT bound not only with FBXW7 but also with endogenous COP1 (Fig.25A). In addition, I confirm *in vitro* association between AKT and FBXW7 (Fig.25B). Next, I verified whether AKT affects binding between COP1 and FBXW7 with MDA-MD-453 WT and DN AKT stable cell lines. Compared with WT AKT, DN-AKT expressing cells showed less FBXW7 binding with COP1 (Fig.26B). In addition, I examined the binding affinity between COP1 with WT, T226A, S227A, T226A/S227A, and T226D/S227D FBXW7. T226A/S227A FBXW7 showed less binding with COP1 whereas T226D/S227D FBXW7 showed more binding with COP1 (Fig.26C). To further investigate whether AKT phosphorylation affect COP1 mediated FBXW7 regulation, we tested steady-state of FBXW7 under COP1 overexpression condition. As a result, steady-state of phosphorylation site mutants of FBXW7 (T226A, S227A, T226A/S227A) were stable compare with WT FBXW7 (Fig.27A). However, steady-state of T226D/S227D FBXW7 was decreased in COP1 overexpression compared with WT FBXW7 (Fig.27B). Not only steady-state but also ubiquitination of FBXW7 was affected by phosphorylation by AKT. Poly-ubiquitination level of FBXW7 was increased upon IGF1 treatment condition (Fig.28A). In a similar manner, COP1 mediated endogenous FBXW7

ubiquitination was significantly increased under IGF1 treatment (Fig.28B). Moreover, *in vitro* kinase assay clearly show that COP1 induced FBXW7 ubiquitination *in vitro* system. Importantly, phosphorylated FBXW7 by *in vitro* kinase reaction with AKT showed high level of ubiquitination compare with non-phosphorylated FBXW7 (Fig.29). To confirm effect of AKT in COP1 mediated FBXW7 ubiquitination, we examine poly-ubiquitination level of FBXW7 with AKT knock-down condition. Compare with control knock-down group, FBXW7 have less ubiquitination level in shAKT group (Fig.30). In addition, phosphor-dead form of FBXW7 (T226A/S227A) showed less ubiquitination whereas phospho-mimic form of FBXW7 (T226D/S227D) showed much higher ubiquitination level when COP1 was overexpressed (Fig.31). Together, I conclude that phosphorylation status of FBXW7 by AKT is a critical condition for COP1 mediated FBXW7 polyubiquitination and degradation.

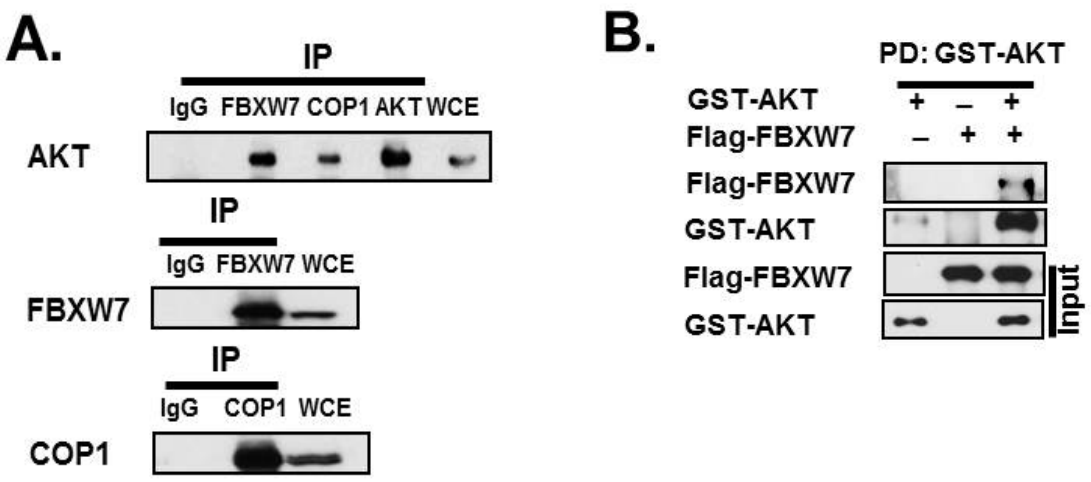

Figure 25. FBXW7 binds with AKT both *in vivo* and *in vitro* condition. (A) HEK 293T cells were treated with MG132 for 6 hr and equal amount of cell lysate were used for IP and IB with indicated antibodies. (B) GST-AKT and Flag-FBXW7 were co-transfected into the HEK 293T cells. GST-AKT was pull-down with GST beads and then Western blot was performed with indicated antibodies.

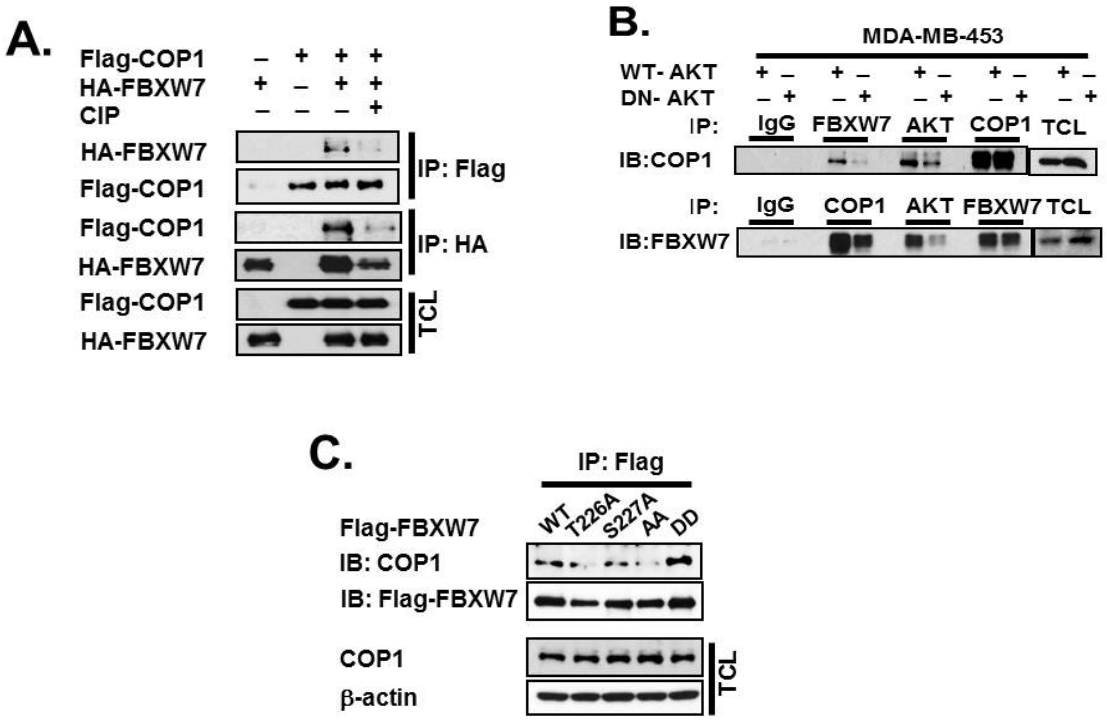

Figure 26. AKT facilitates FBXW7 binding to COP1. (A) HEK 293 cells were co-transfected with Flag-COP1 and HA-FBXW7 plasmids then IP and IB were performed after CIP treatment for 1 hr. (B) WT or Dominant Negative AKT (DN-AKT) expressing MDA-MB-453 cells were treated with MG132 for 6 hr. Equal amounts of cell lysate were subject to IP and IB with indicated antibodies. (C) WT, T226A, S227A, T226A/S227A Flag-FBXW7 were transfected into HEK 293 cells and then immunoblotting was performed and followed by immunoprecipitation with Flag antibody.

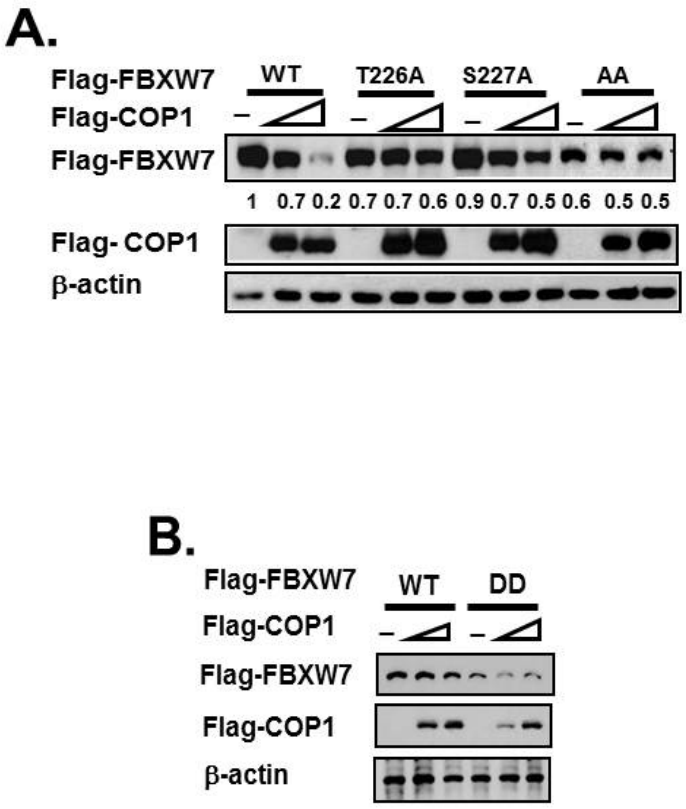

Figure 27. AKT facilitates COP1 mediated FBXW7 degradation. (A) HEK 293T cells were co-transfected with WT, T226A, S227A, T226A/S227A Flag-FBXW7 and Flag-COP1 plasmid for 48hours and performed Western Blot analysis. (B) HEK 293T cells were co-transfected with WT, T226D/S227D Flag-FBXW7 and Flag-COP1 plasmid for 48 hr and immunoblotting was performed.

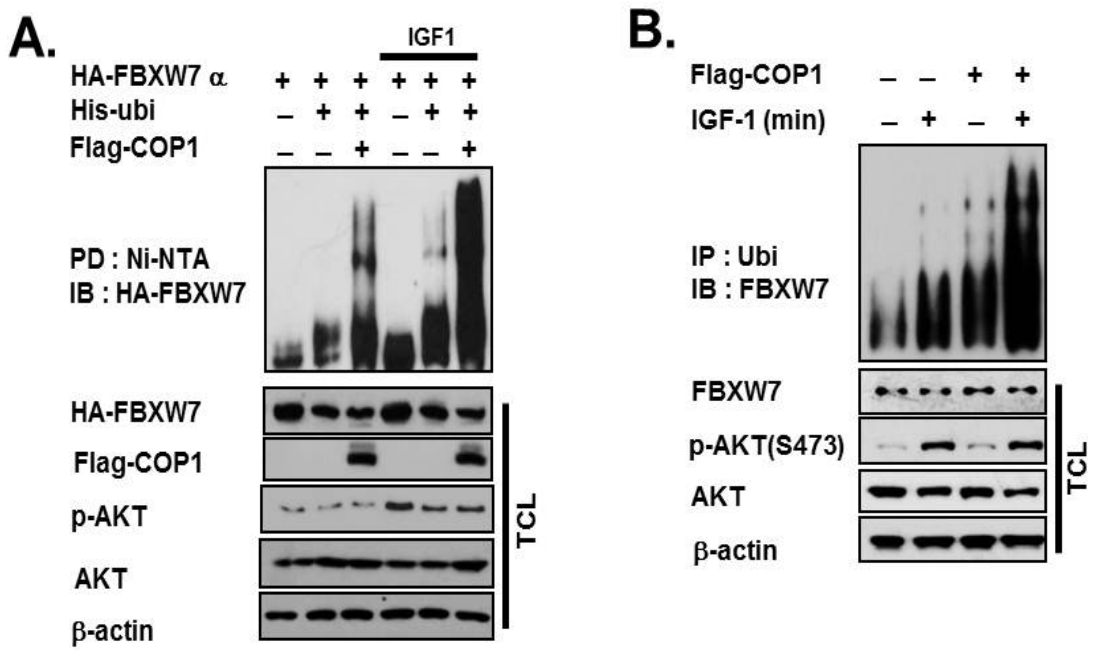

Figure 28. IGF-1 increases COP-mediated FBXW7 ubiquitination. (A) HEK 293 cells were co-transfected with His-ubiquitin, HA-FBXW7 and Flag-COP1 plasmids. Cells were treated with 100ng/ml IGF1for 1 hr followed by 24hr serum starvation then subjected to nickel beads purification and Western-blot analysis. (B) HEK 293 cells were transfected with Flag-COP1. After 24 hr serum starvation, cells were treated with 100 ng/ml IGF1 for 1 hours then IP with ubiquitin antibody and IB with FBXW7 were performed. All cells were treated with 10μM MG132 for 6 hr before harvesting.

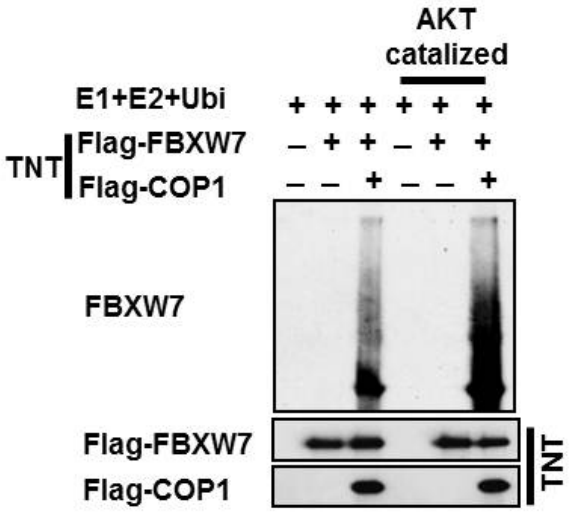

Figure 29. AKT facilitates COP-mediated FBXW7 ubiquitination *in vitro* condition.

Flag-COP1 and Flag-FBXW7 proteins were made using an *in vitro* Transcription/Translation system (TNT). Indicated proteins were incubated with E1, E2, ubiquitin, and recombinant active AKT1 for 30 min and subjected to SDS-PAGE analysis.

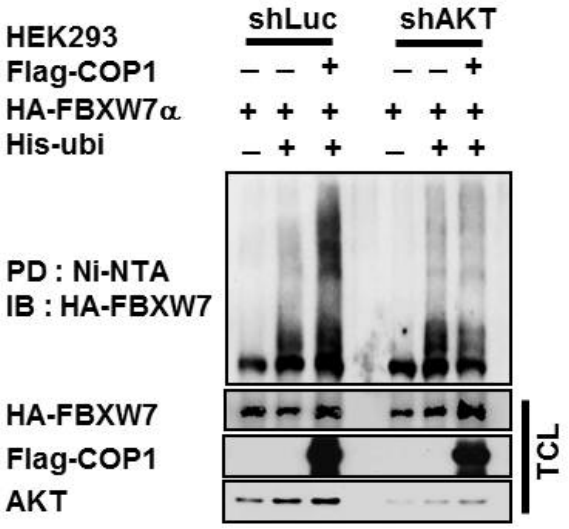

Figure 30. Knock-down AKT is failed to induce COP1-mediated FBXW7 ubiquitination. HEK 293 cells were infected with lentiviral shLuciferease or shAKT and then co-transfected with His-ubiquitin, Ha-FBXW7, and Flag-COP1 plasmids for 48 hr. Equal amounts of cell lysate were subject to pull-down with Ni-NTA and immunoblotting was performed. All cells were treated with 10μM MG132 for 6 hr before harvesting.

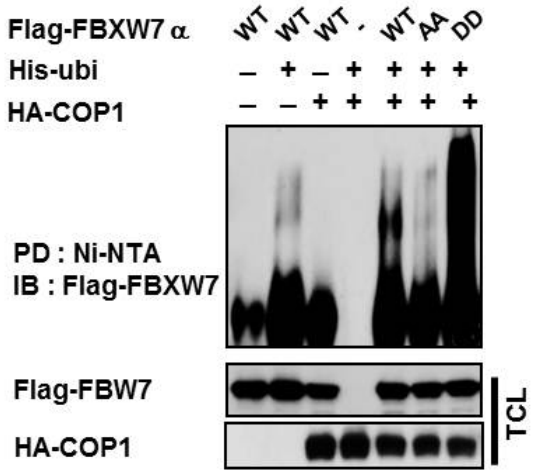

Figure 31. FBXW7 phosphorylation status is critical for COP1-mediated FBXW7 ubiquitination . WT, T226A/S227A, T226D/S227D Flag-FBXW7 were co-transfected with His-Ubi and Ha-COP1 into HEK 293 cells. Denatured cell lysates were subjected to nickel beads purification for *In vivo* ubiquitination assay and immunoblot analysis. All cells were treated with 10 μM MG132 for 6 hr before harvesting.

3-6. FBXW7 phosphorylation is deregulated during tumorigenesis

To clarify the effect of COP1-Akt-FBXW7 axis regulation in tumorigenesis, I performed invasion and migration assay with both WT and mutant phosphorylation sites of FBXW7. Phospho-dead mutant form of FBXW7 (T226A/S227A) transfected cell showed less invasion and migration activity compare with WT FBXW7 transfected cell. However, phospho-mimic form of FBXW7 (T226D/S227D) transfected cell showed significantly higher invasion and migration activity (Fig.32A, B). In addition, I also examined cell proliferation activity with a Wound-healing assay. T226A/S227A FBXW7 showed much less cell proliferation whereas T226D/S227D FBXW7 showed higher cell proliferation activity (Fig.32C). To further understand about cell cycle distribution in different phosphorylation status of FBXW7, I performed FACS analysis. Compared with Vector control and WT FBXW7 transfected group, the T226A/S227A FBXW7 group showed that more cells in G0 phase and also significantly less cells in S phase (Fig.33A, B).

Thus, I concluded that the phosphorylation status of FBXW7 by AKT is a pivotal factor for FBXW7 stability and tumorigenesis. High phosphorylation of FBXW7 increased carcinogenic activity such as cell migration, invasion, and proliferation by regulating the cell cycle.

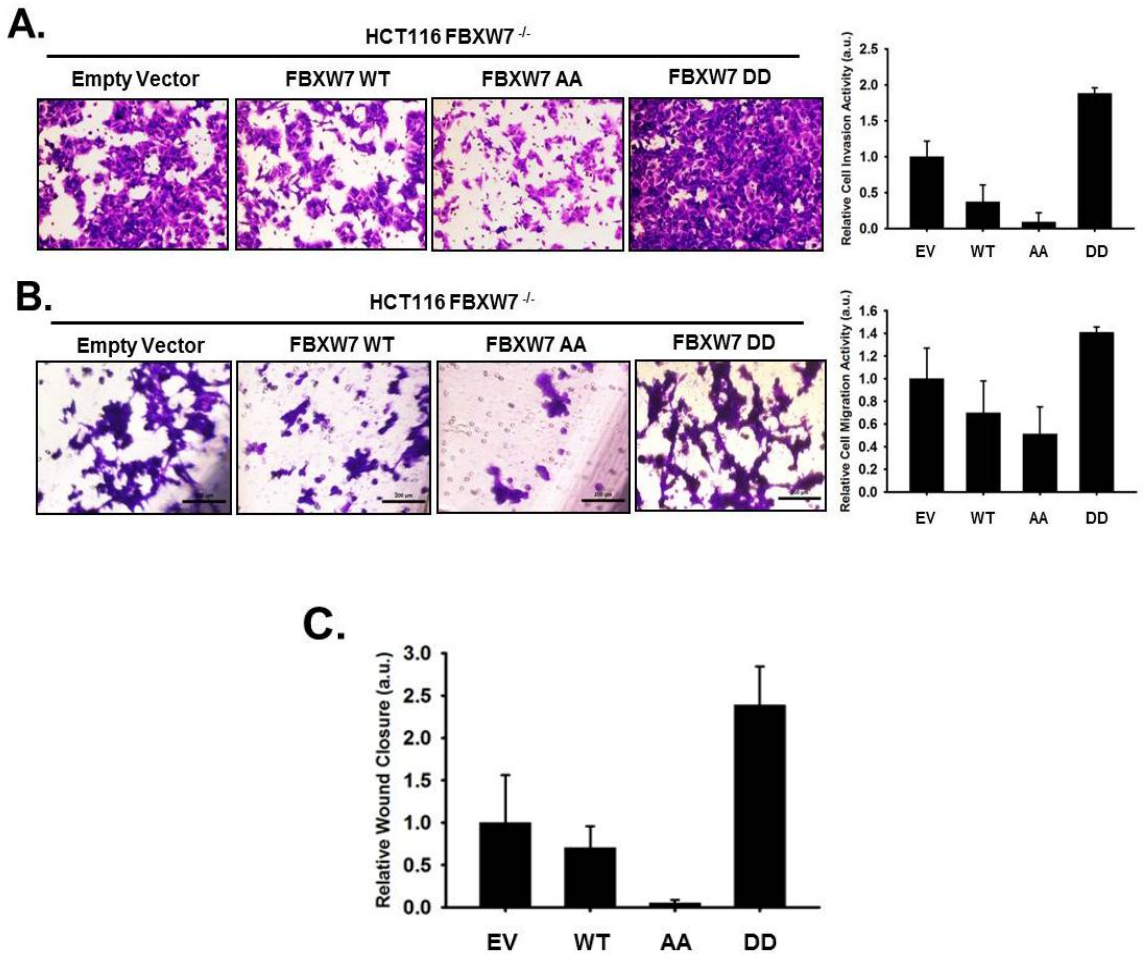

Figure 32. AKT/COP1 mediated FBXW7 regulation has impact on tumorigenesis. HCT116 FBXW7 $^{-/-}$ cells were transfected with Vector control, WT, T226A/S227A or T226D/S227D Flag-FBXW7 constructs. (A) Matrigel invasion assay (B) Transwell migration assay (C) Wound healing assay was performed. Activity was quantitated and presented as a bar graph.

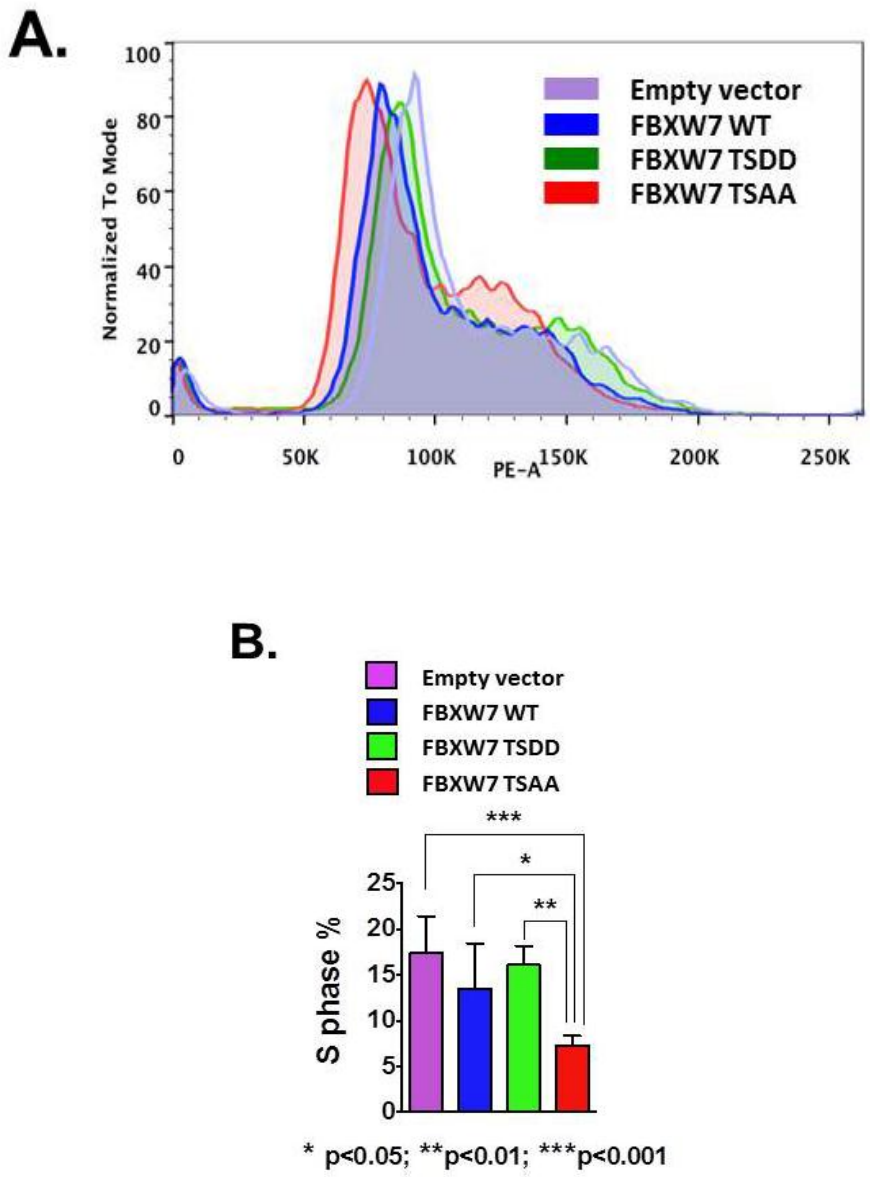

Figure 33. FBXW7 phosphorylation status is critical for cell cycle regulation. (A) (B) HCT116 FBXW7 $^{-/-}$ cells were transfected with Vector control, WT, T226A/S227A or T226D/S227D Flag-FBXW7 constructs and then a flow cytometry assay was performed.

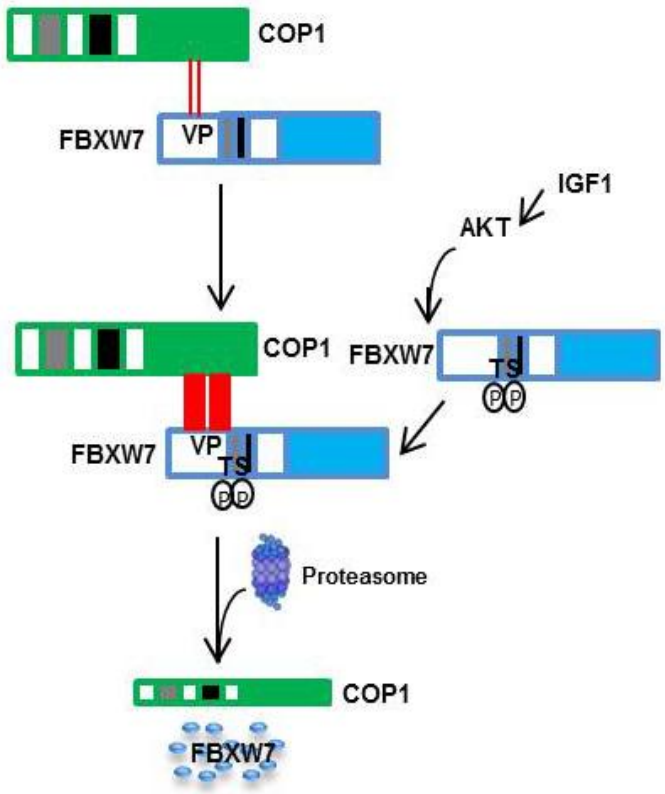

Figure 34. Working model of AKT-COP1-FBXW7 axis regulation. Binding affinity between COP1 and FBXW7 through COP1 binding motif is increased by AKT-mediated FBXW7 phosphorylation. COP1 facilitates ubiquitination and degradation of phosphorylated FBXW7 via the proteasome complex.

CHAPTER 4

CSN6 negatively regulates FBXW7's degrading activity toward Cyclin E and c-Jun

RATIONALE

CDC4 (FBXW7) is a one of the components of the SCF complex which is responsible for recognizing substrate proteins for ubiquitination. Activity of the SCF complex is regulated by CSN5 mediated deneddylation process on the Cullin protein which is another compound of SCF complex. It have been reported that in the nonfunctional SCF complex, FBXW7 can be targeted for auto-ubiquitination [92]. Moreover, another study reported that CSN inhibits FBXW7 by suppressing conformational activity [61]. It suggests that CSN is involved in FBXW7 stability regulation. However, CSN5 is the only subunit of the CSN complex that affects the SCF complex and functions of the other subunits are remains unclear. CSN6 is an only subunit which have same functional domain with CSN5. Therefore, it is highly possible that CSN6 have some impact on FBXW7 regulation.

The goal of this study is identify biological function of CSN6 on FBXW7 regulation and also verify whether CSN6 can affect FBXW7 substrates Cyclin E and c-Jun stability.

RESULTS

4-1. CSN6 negatively regulates FBXW7 stability through ubiquitination

To investigate whether CSN6 impacts FBXW7 which is a part of the SCF complex, I first analyzed the interaction between CSN6 and FBXW7 with cell lysates that were cotransfected with FBXW7 and CSN6. Indeed, FBXW7 was able to associate with transfected CSN6 as proven by the co-immunoprecipitation assay (Fig. 35). Endogenous interaction between FBXW7 and CSN6 was also confirmed. As a CSN6-associated protein, it is possible that FBXW7 is regulated by CSN6. The FBXW7 family has three isoforms—α (nuclear), β (cytoplasmic), and γ (nucleolar)—each with distinct subcellular localization [29]. To determine whether all three isoforms are regulated by CSN6, I examined the steady-state expression of FBXW7 isoforms in the presence of increasing amounts of CSN6. Indeed, overexpression of CSN6 can reduce the steady-state expression of FBXW7 isoforms in a dose-dependent manner (Fig. 36 A). In keeping with these results, the steady-state expression of FBXW7 increased when CSN6 was knocked down (Fig.36 B).

I also examined the turnover of FBXW7 in the presence of cycloheximide (CHX) when cotransfected with increasing amounts of CSN6 or shCSN6. The FBXW7 protein turnover rate increased in the CSN6 overexpression group when compared with the control group. In contrast, in the CSN6 knockdown group, the FBXW7 protein turnover rate was significantly decreased (Fig. 36 C). Furthermore, the proteasome inhibitor MG132 rescued CSN6-mediated FBXW7 downregulation, suggesting the involvement of proteasome-mediated degradation (Fig.37 A). I verified the effect of CSN6 on FBXW7

ubiquitination and degradation in denaturing conditions. Overexpression of CSN6 increased FBXW7 polyubiquitination in a dose-dependent manner (Fig.37.B), whereas CSN6 knockdown decreased ubiquitination of FBXW7 (Fig.37.C). These results indicate that CSN6 is a negative regulator of FBXW7.

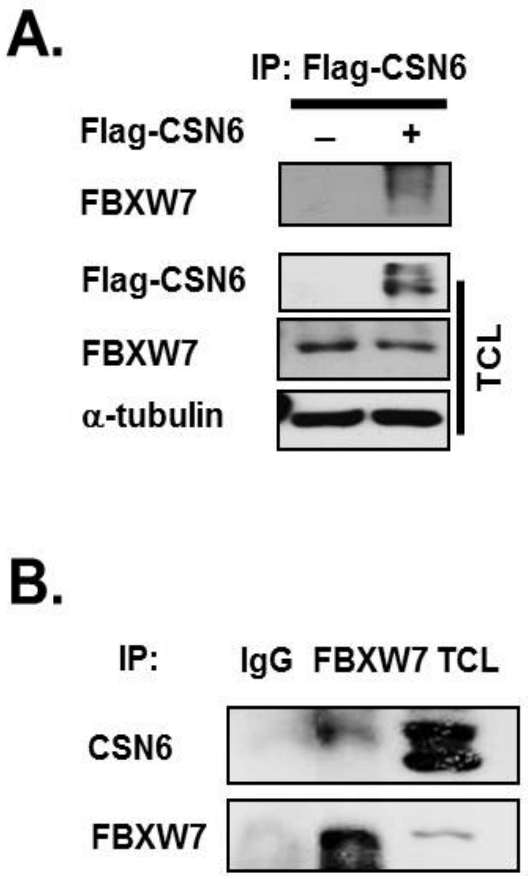

Figure 35. CSN6 interacts with FBXW7. (A) HEK 293T cells were transfected with Flag-CSN6 plasmid and equal amount of cell lysates were immunoprecipitated with anti-Flag and then immunoblotted with anti-FBXW7 antibody. (B) Endogenous FBXW7 was immunoprecipitated with anti-FBXW7 and immunoblotted with indicated antibodies. All cells were treated with 10 μM MG132 for 6 hr before harvesting.

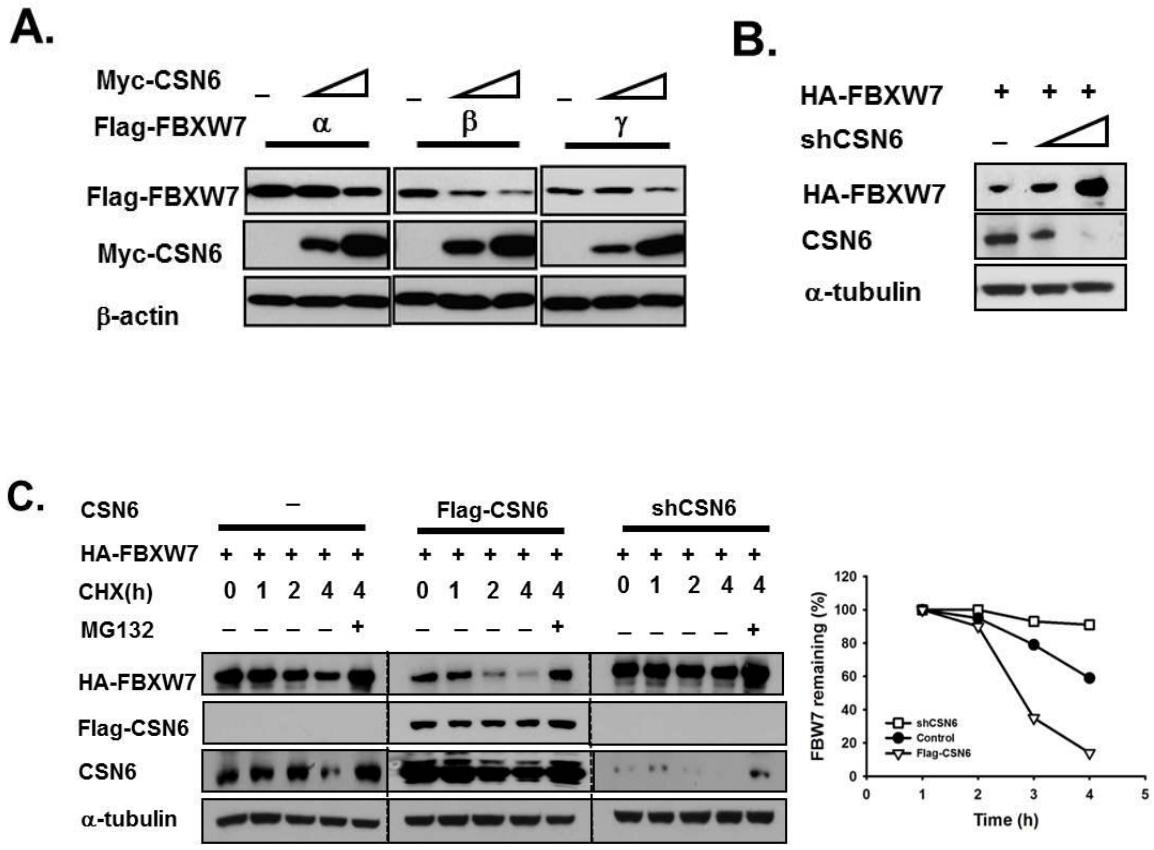

Figure 36. CSN6 negatively regulates FBXW7 stability. (A) Equal amounts of HEK 293T cell lysate which were transfected with Myc-CSN6 and Flag-FBXW7 α, β, γ plasmids, were immunoblotted with indicated antibodies. (B) HEK 293T cells were transfected with HA-FBXW7 and shCSN6 constructs and then subjected to Western Blot analysis. (C) HEK 293T cells were cotransfected with the HA-FBXW7, Flag-CSN6 and shCSN6 plasmids and then treated with cycloheximide (CHX) for the indicated times. Cell lysates were immunoblotted with FBXW7.

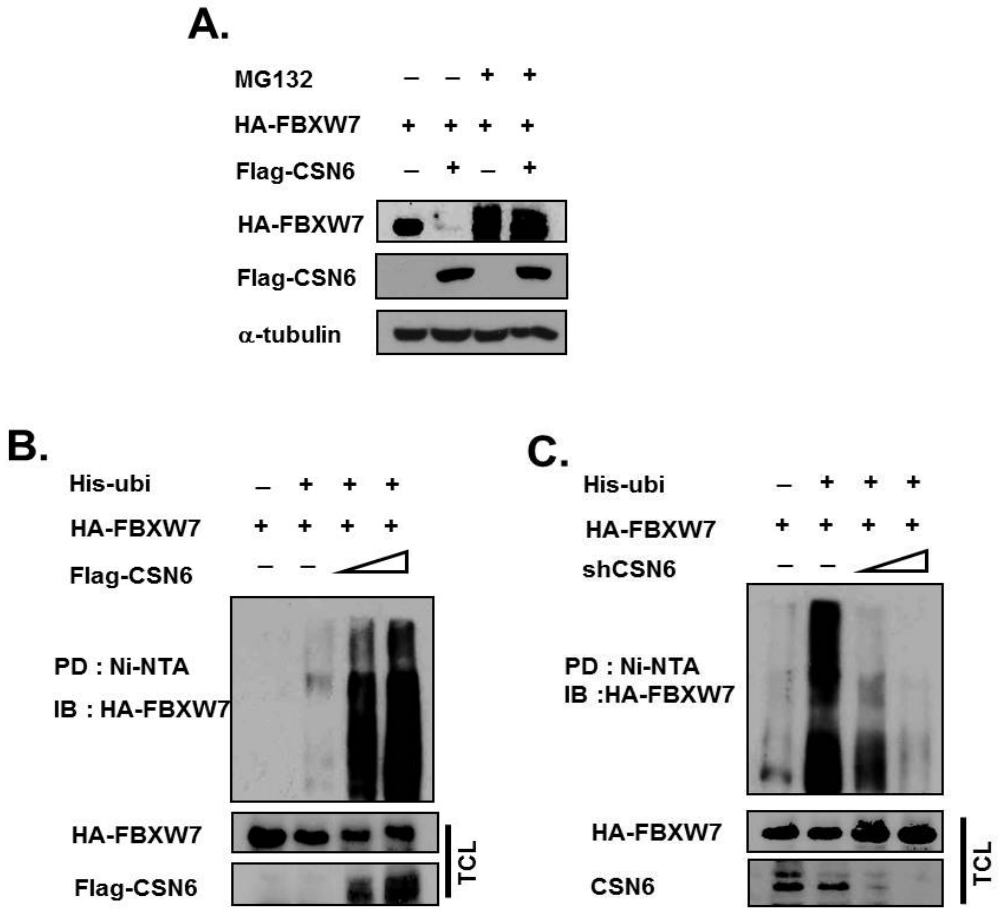

Figure 37. CSN6 degrades FBXW7 via the proteasome. (A) HEK 293T cells were cotransfected with Flag-CSN6 and HA-FBXW7 plasmids for 48 hr then treated with DMSO or MG132 for 6 hr before harvest. Equal amounts of cell lysates were immunoblotted with the indicated antibodies. (B) Cells were transfected with His-ubiquitin, HA-FBXW7 and Flag-CSN6 constructs. (C) Cells were transfected with His-ubiquitin, HA-FBXW7 and sh-CSN6 constructs. Cell lysates were pulled down (PD) with Ni++ NTA beads and then immunoblotted (IB) with anti-HA. All cells were treated with MG132 for 6 hr before harvest.

4-2. CSN6 interacts with and regulates Cyclin E and c-Jun

Cyclin E and c-Jun have been reported as substrate proteins of FBXW7 [93]. To further determine the impact of CSN6 in regulating FBXW7 stability, I examined whether CSN6 affects FBXW7-mediated Cyclin E and c-Jun regulation. Interestingly, endogenous CSN6 was associated with both endogenous Cyclin E and c-Jun (Fig.38 A). Also, transfected CSN6 interacted with Cyclin E and c-Jun (Fig.38 B). Domain mapping studies suggest that the CSN6 N-terminus (aa 1-184) conatins Cyclin E or c-Jun binding domain (Fig.39). Consistent with these studies, I confirmed *in vitro* binding between CSN6 and Cyclin E or c-Jun using *in vitro* translated products (Fig.40 A, B). I have demonstrated that CSN6 destabilized FBXW7. Because FBXW7 tightly regulates both Cyclin E and c-Jun protein stability, I then investigated whether CSN6 affects FBXW7-mediated Cyclin E and c-Jun degradation. Both Cyclin E and c-Jun proteins were stabilized by the expression of CSN6 in a dose-dependent manner (Fig.41A). In contrast, CSN6 knockdown reduced the steady-state expression of Cyclin E and c-Jun (Fig.41B). Moreover, the turnover rate of both Cyclin E and c-Jun was significantly decreased in the CSN6 overexpression group (Fig.42A, B), whereas the turnover rate of both proteins was increased in the CSN6 knockdown group (Fig.43A, B). Together, these results suggest that the CSN6-FBXW7 axis regulates the stability of Cyclin E and c-Jun.

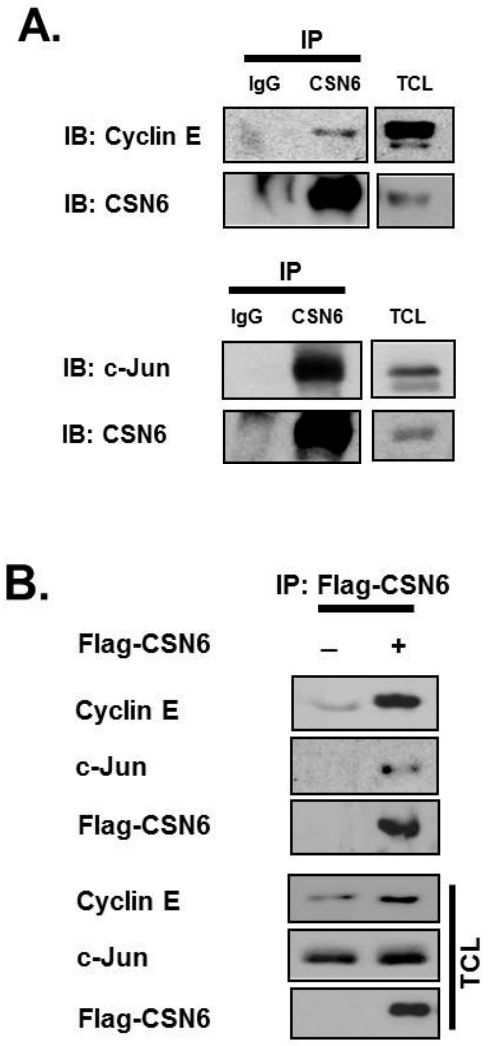

Figure 38. CSN6 interacts with both Cyclin E and c-Jun. (A) HEK 293T cells were subjected to immunoprecipitation (IP) with IgG and CSN6 antibodies and then immunoblotted (IB) with the indicated antibodies. (B) Equal amounts of HEK 293T cell lysates which were transfected with indicated plasmids, were immunoprecipitated with anti- Flag and then immunoblotted with the indicated antibodies. Cells were treated with 10 μM MG132 for 6 hr before harvesting.

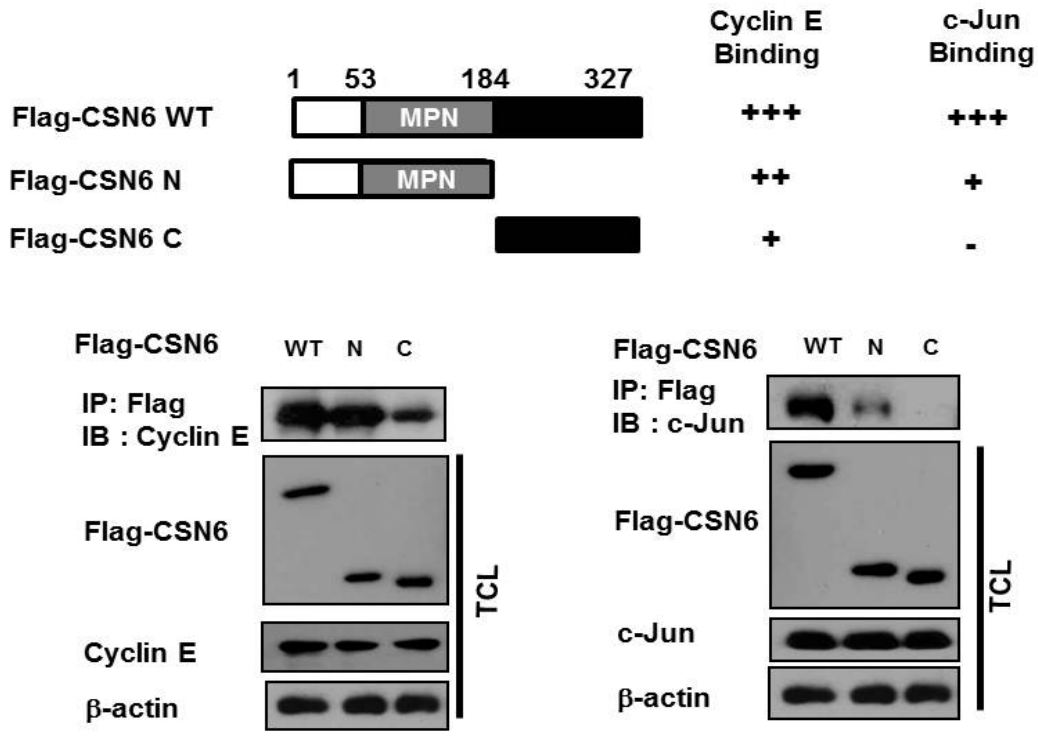

Figure 39. CSN6 binds with Cyclin E and c-Jun through N-terminus. (A) Flag-CSN6 WT, Flag-CSN6 N-terminus (aa 1-184), or C-terminus (aa 184-327) was transfected into HEK 293T cells. Equal amount of cell lysates were immunoprecipitated with anti-Flag and immunoblotted with anti-Cyclin E or c-Jun. Cells were treated with 10 μM MG132 for 6 hr before harvesting.

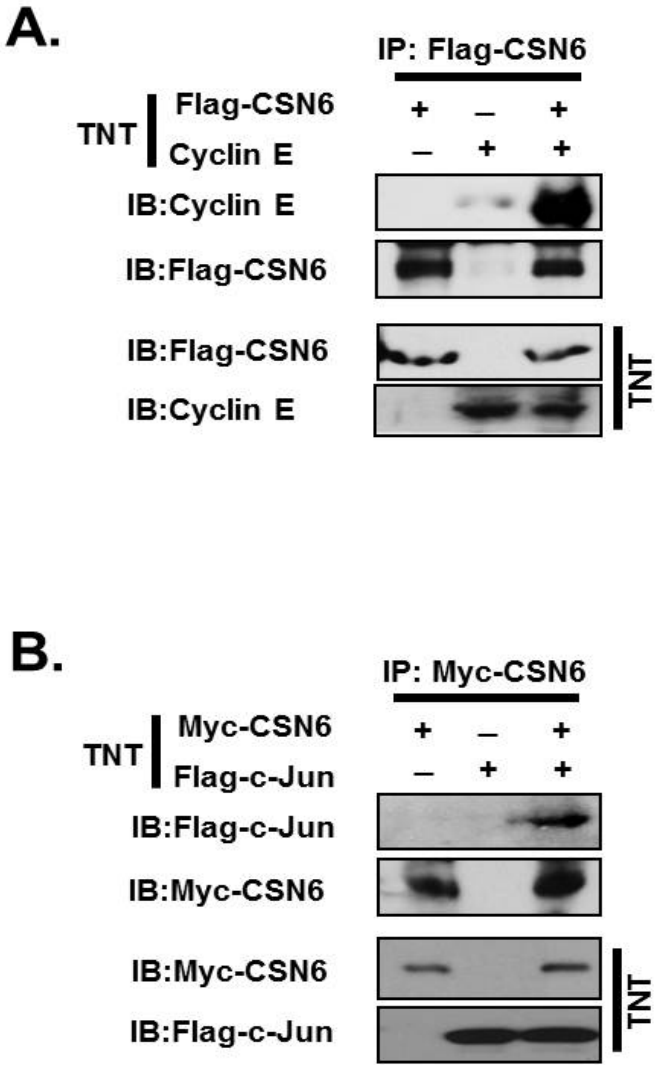

Figure 40. CSN6 binds with either Cyclin E or c-Jun *in vitro*. Myc-CSN6, Flag-CSN6, Flag-c-Jun or Cyclin E proteins were translated using *in vitro* TNT. (A) Flag-CSN6 proteins were immunoprecipitated with anti-Flag and then immunoblotted with Cyclin E antibodies. (B) Myc-CSN6 proteins were immunoprecipitated with anti-Myc and then immunoblotted with c-Jun antibodies. 10 μM MG132 was added into the mixture 6 hr before IP.

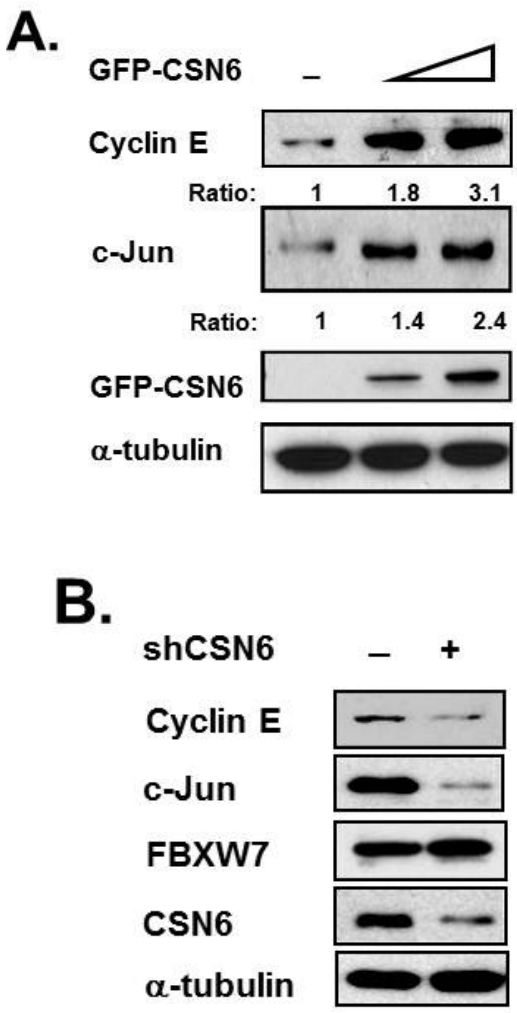

Figure 41. CSN6 stabilizes Cyclin E and c-Jun. (A) HEK 293T cells were transfected with different amount of GFP-CSN6 plasmids. Equal amounts of the indicated cell lysates were immunoblotted with Cyclin E or c-Jun antibodies. (B) HEK 293T cells were transfected with different amounts of shCSN6 constructs and then subjected to Western Blot analysis.

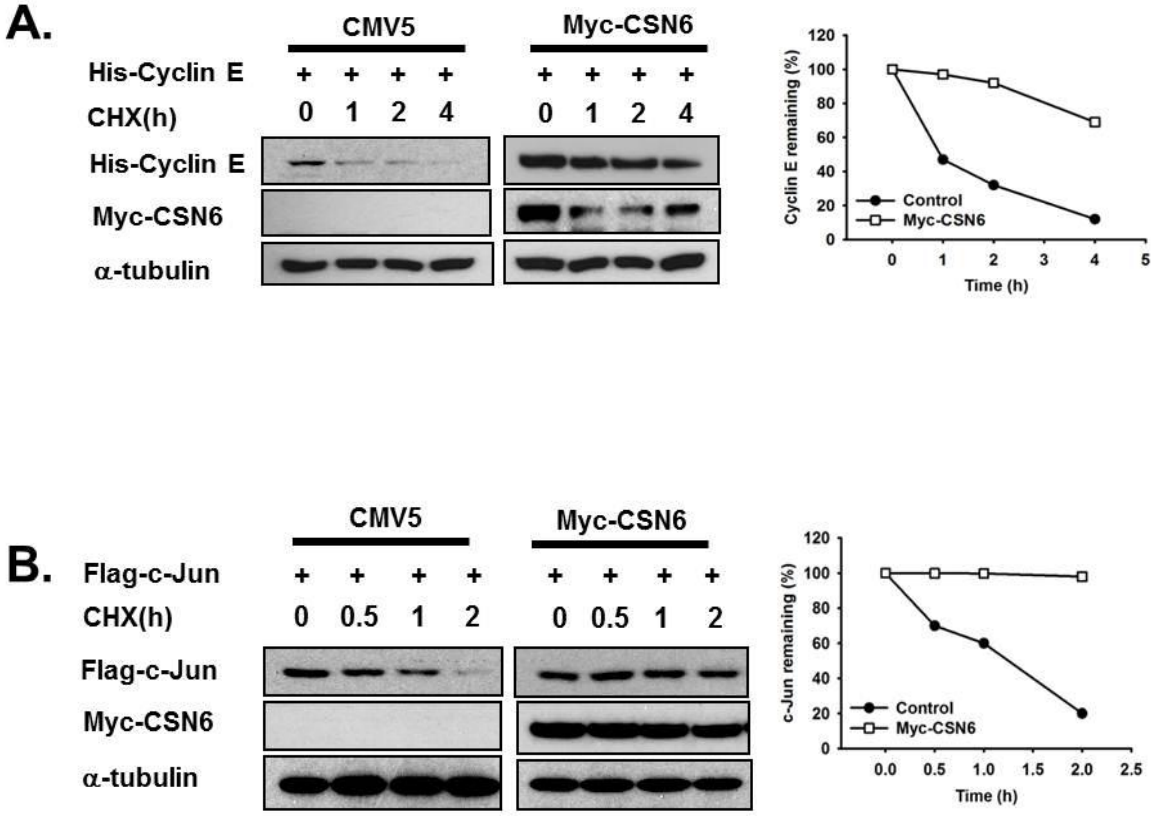

Figure 42. CSN6 expression leads to decreased turnover of c-Jun and Cyclin E. (A) HEK 293T Cells were cotransfected with Myc-CSN6 and His-Cyclin E plasmids. (B) HEK 293T Cells were cotransfected with Myc-CSN6 and Flag-c-Jun plasmids. After 48hr transfection, cell were treated with cycloheximide (CHX) for the indicated times. Equal amounts of cell lysate were immunoblotted with Cyclin E or c-Jun antibodies.

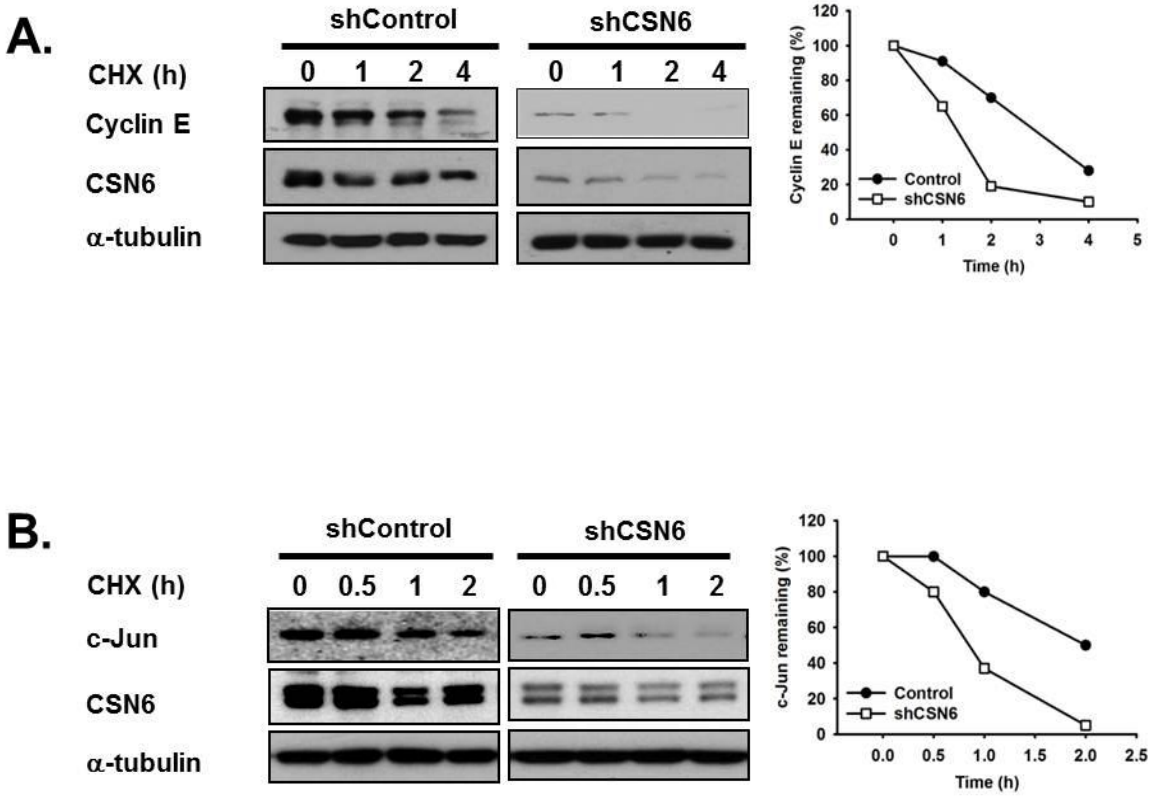

Figure 43. CSN6 knockdown increased turnover of c-Jun and Cyclin E. (A) (B) HEK 293T Cells were transfected with shCSN6. After 48 hr transfection, cells were treated with cycloheximide (CHX) for the indicated times. Equal amount of cell lysates were immunoblotted with Cyclin E or c-Jun antibodies.

4-3. CSN6-mediated stabilization of Cyclin E and c-Jun stability is FBXW7-dependent

To further confirm whether positive regulation of CSN6 on Cyclin E and c-Jun is FBXW7-dependent, I verified the steady-state expression of Cyclin E and c-Jun proteins in HCT116 FBXW7$^{+/+}$ and HCT116 FBXW7$^{-/-}$ cells (Fig.44 A,B). I demonstrated that knockdown of CSN6 decreased the steady-state expression of Cyclin E and c-Jun proteins in HCT116 FBXW7$^{+/+}$ but not in HCT116 FBXW7$^{-/-}$ cells (Fig.44 A). In addition, I confirmed the positive impact of CSN6 on Cyclin E and c-Jun regulation using *de novo* protein synthesis inhibitor cycloheximide (CHX). Turn-over rate of both Cyclin E and c-Jun proteins was decreased by CSN6 expression in HCT116 FBXW7 $^{+/+}$ cells, whereas the turnover rate of both proteins was accelerated by CSN6 knockdown (Fig.44 B). In contrast, protein stability of Cyclin E and c-Jun was not changed by CSN6 expression level through ectopic expression or knockdown in HCT116 FBXW7$^{-/-}$ cells (Fig.44 B). I therefore conclude that CSN6-mediated regulation of Cyclin E and c-Jun is through FBXW7.

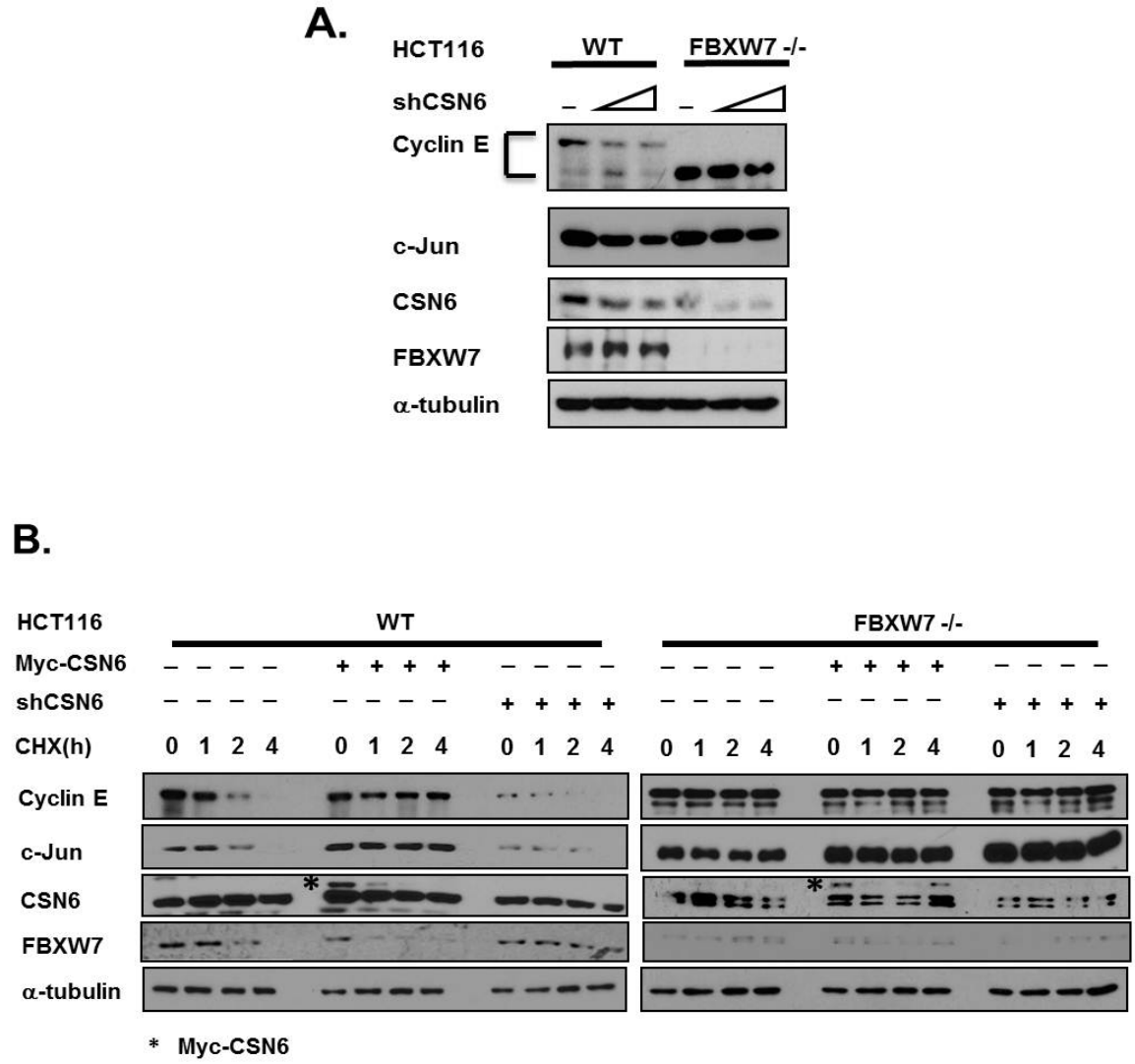

Figure 44. CSN6 regulates Cyclin E and c-Jun in an FBXW7-dependent manner. (A) shCSN6 constructs were transfected into WT HCT116 or FBXW7$^{-/-}$ HCT116 cell. (B) WT HCT116 or FBXW7$^{-/-}$ HCT116 cell were transfected with Myc-CSN6 or shCSN6 plasmid and then treated with cycloheximide (CHX) for the indicated times. Equal amounts of cell lysates were immunoblotted with Cyclin E or c-Jun antibodies.

4-4. CSN6 reduces Cyclin E and c-Jun ubiquitination via suppressing FBXW7

To investigate the underlying mechanisms of CSN6-mediated Cyclin E and c-Jun stabilization, I examined whether CSN6 decreased Cyclin E and c-Jun degradation by suppressing FBXW7-mediated polyubiquitination activities. Increasing amounts of CSN6 antagonized FBXW7-mediated ubiquitination of Cyclin E in a dose-dependent manner (Fig.45 A), whereas CSN6 knockdown enhanced FBXW7-mediated ubiquitination of Cyclin E (Fig.45 A). Consistently, I observed that CSN6 significantly reduced Cyclin E ubiquitination in HCT116 FBXW7$^{+/+}$ cells (Fig.46). HCT116 FBXW7$^{-/-}$ cells served as a negative control to show that CSN6-mediated reduction of Cyclin E ubiquitination requires the presence of FBXW7. In a similar manner, overexpression of CSN6 clearly suppressed FBXW7-mediated c-Jun ubiquitination (Fig.46 A), whereas CSN6 deficiency enhanced FBXW7-mediated c-Jun ubiquitination (Fig.46 B). Again, I observed that CSN6 significantly reduced c-Jun ubiquitination in HCT116 FBXW7$^{+/+}$ cells (Fig.48). Also, HCT116 FBXW7$^{-/-}$ cells served as a negative control to show that CSN6-mediated reduction of c-Jun ubiquitination requires the presence of FBXW7. Together, CSN6 decreased Cyclin E and c-Jun ubiquitination by suppressing FBXW7's ubiquitinating activity.

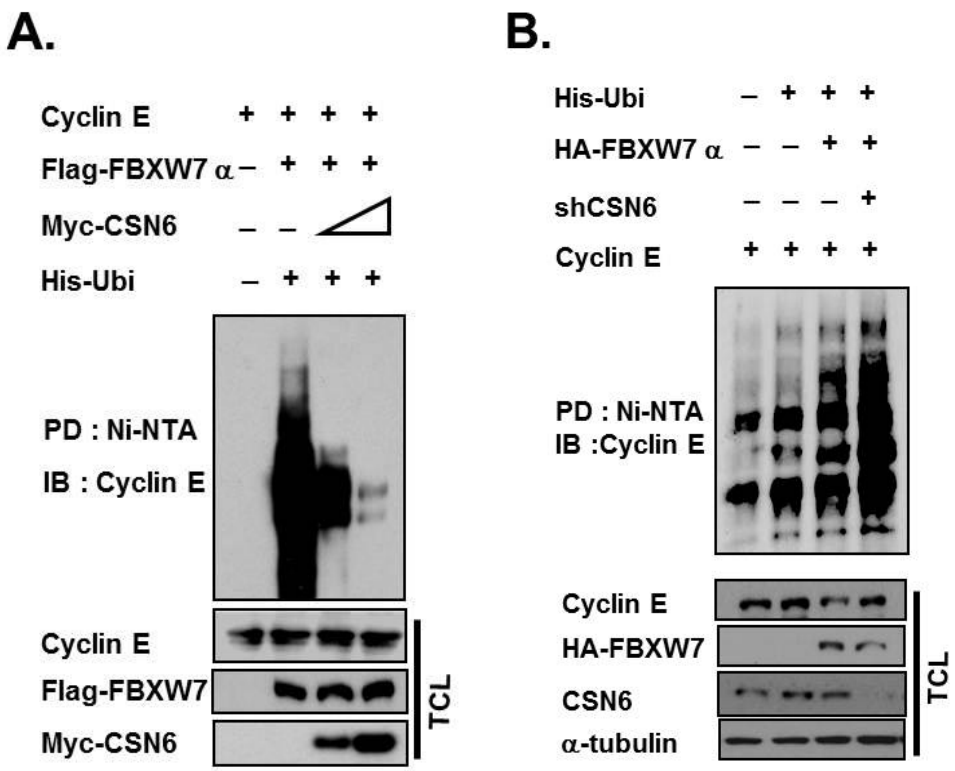

Figure 45. CSN6 reduces Cyclin E ubiquitination. (A) HEK 293T cells were cotransfected with Cyclin E, Flag-FBXW7, Myc-CSN6 and His-ubiquitin plasmids. (B) HEK 293T cells were transfected with Cyclin E, HA-FBXW7, shCSN6 and His-ubiquitin plasmids. Cell lysates were pulled down with Ni^{++} NTA beads and then immunoblotted with anti-Cyclin E. Cells were treated with 10 μM MG132 for 6 hr before harvesting.

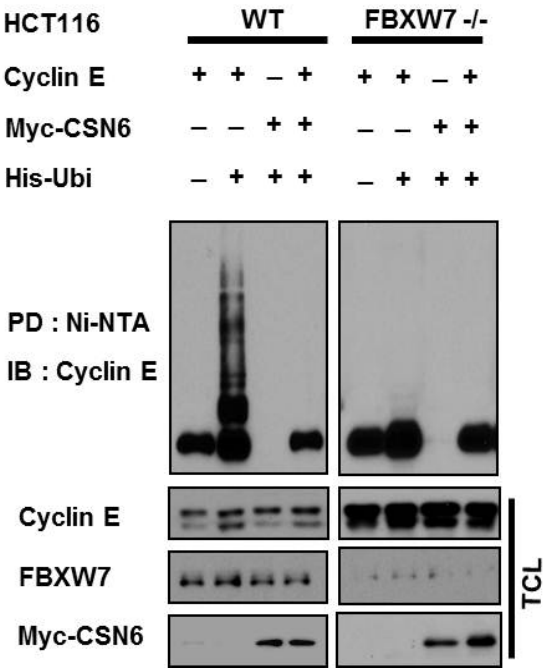

Figure 46. CSN6 regulates Cyclin E ubiquitination through FBXW7. WT HCT116 or FBXW7$^{-/-}$ HCT116 cell were transfected with Cyclin E, Myc-CSN6 and His-ubiquitin plasmids. Cell lysates were pulled down with Ni^{++} NTA beads and then immunoblotted with anti-Cyclin E. Cells were treated with 10 μM MG132 for 6 hr before harvesting.

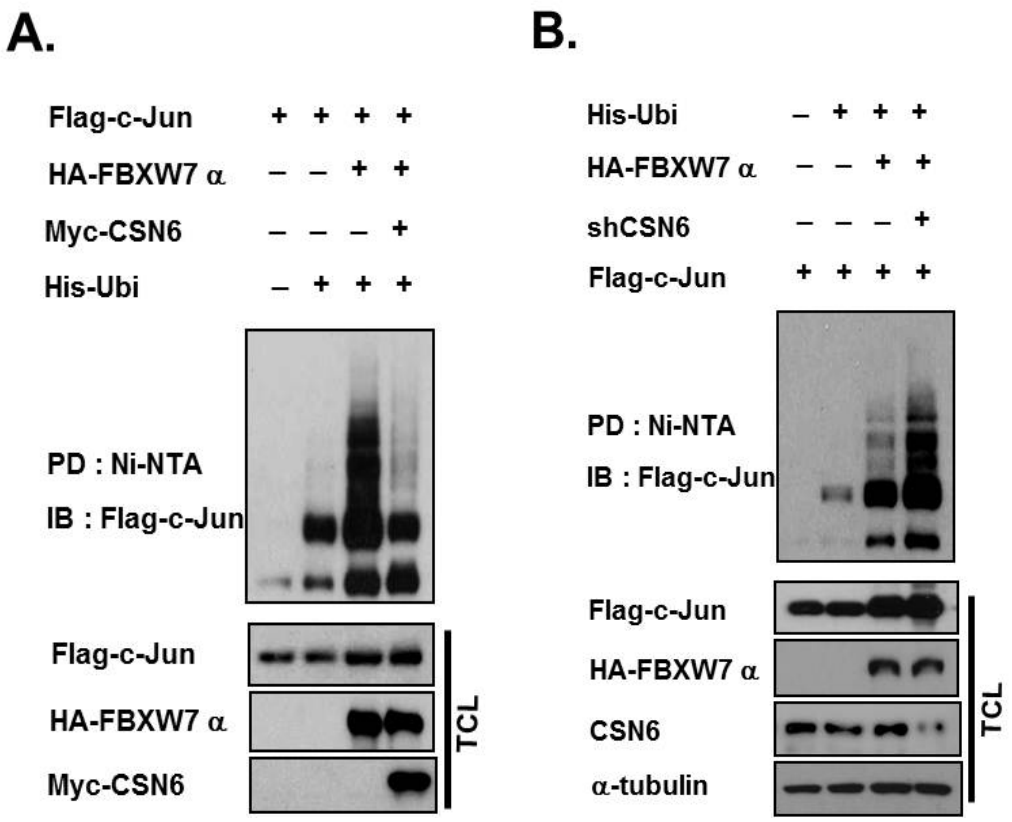

Figure 47. CSN6 reduces c-Jun ubiquitination. (A) HEK 293T cells were cotransfected with Flag-c-Jun, HA-FBXW7, Myc-CSN6 and His-ubiquitin plasmids. (B) HEK 293T cells were transfected with Flag-c-Jun, HA-FBXW7, shCSN6 and His-ubiquitin plasmids. Cell lysates were subject to pull-down with Ni^{++} NTA beads and then immunoblotted with anti-Flag. Cells were treated with 10 μM MG132 for 6 hr before harvesting.

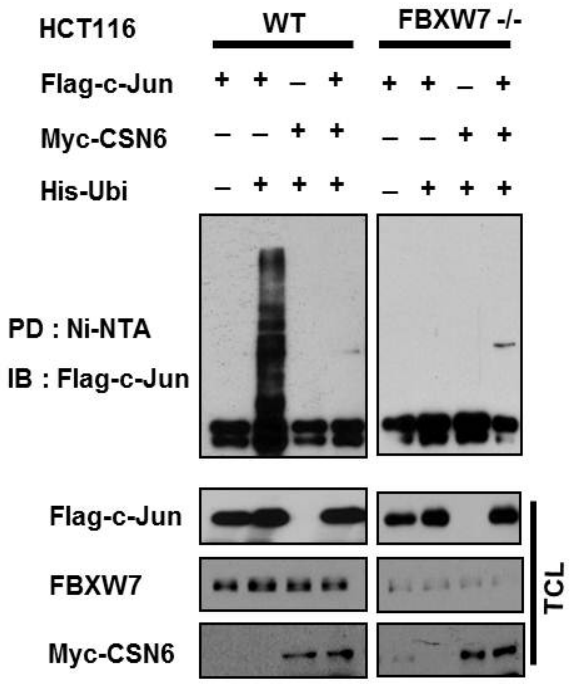

Figure 48. CSN6 regulates c-Jun ubiquitination through FBXW7. WT HCT116 or FBXW7$^{-/-}$ HCT116 cell were transfected with Flag-c-Jun, Myc-CSN6 and His-ubiquitin plasmids. Cell lysates were subject to pull-down with Ni^{++} NTA beads and then immunoblotted with anti-Cyclin E. Cells were treated with 10 μM MG132 for 6 hr before harvesting.

4-5. CSN6 knockdown hinders cell migration and transformational activity

I screened breast cancer cell lines and found that the CSN6 expression positively correlates with the expression of Cyclin E and c-Jun (Fig.49 A, B). Because Cyclin E and c-Jun proteins are critical oncoproteins that have tumorigenic functions, I investigated whether the positive impact of CSN6 on Cyclin E and c-Jun promotes cell characteristics involved in carcinogenesis. I determined with use of the wound-healing assay that depletion of CSN6, by shRNA, decreased migration of MDA-MD-231 cells (Fig.50 A). In addition, the trans-well assay confirmed that the migration rate of MDA-MD-231 cells was significantly decreased in the CSN6 knockdown group compared with the control group (Fig.50 B). Flow cytometric analysis indicates that the CSN6 knockdown group has an increased sub-G1 population but a reduced S-phase population (Fig.50 C). I also performed an anchorage-independent colony formation assay and found that MDA-MB-231 cells with CSN6 knockdown showed inhibited formation of anchorage-independent growth of colonies (Fig.50 D). This data illustrates that CSN6 plays an important role in oncogenic signaling including migration, cell proliferation, and transformation. The biological significance of CSN6's positive impact on c-Jun and Cyclin E expression may contribute to the above-described oncogenic signaling activities.

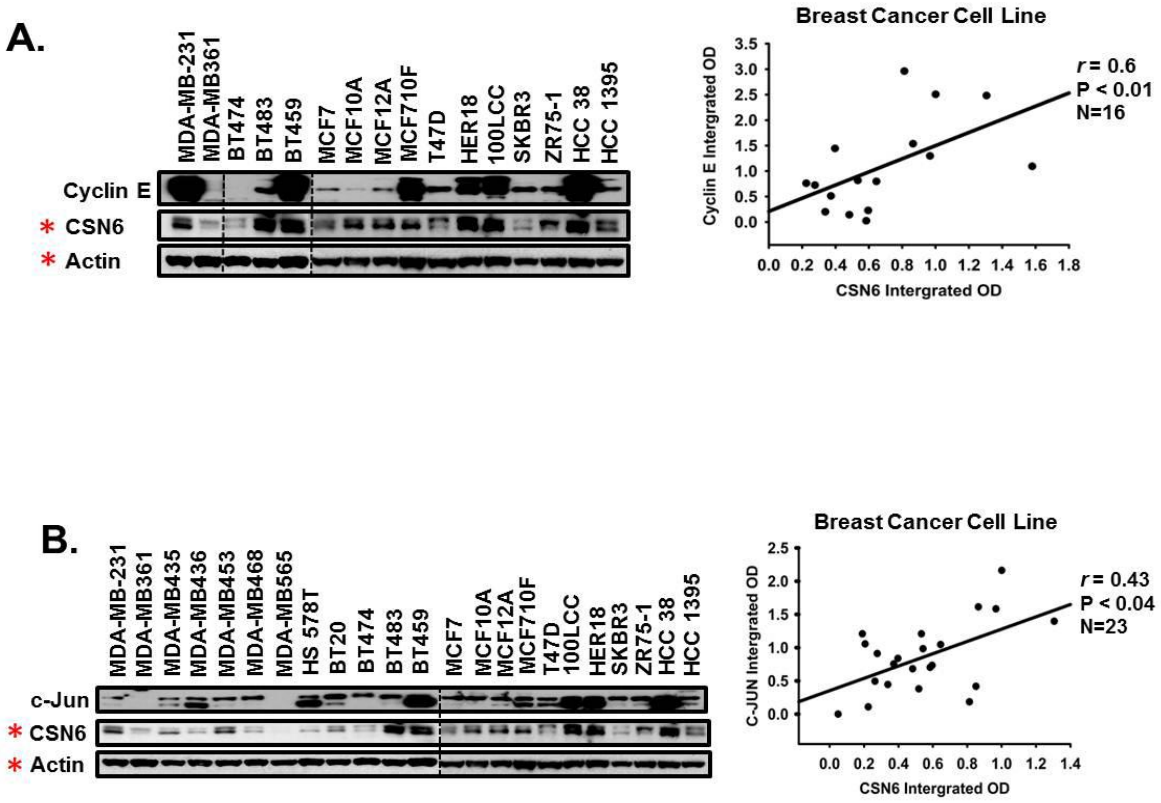

Figure 49. CSN6 expression correlates with the expression of Cyclin E and c-Jun in breast cancer cell lines. Equal amounts of cell lysate from each cell line were immunoblotted with the Cyclin E, c-Jun or CSN6 antibodies.

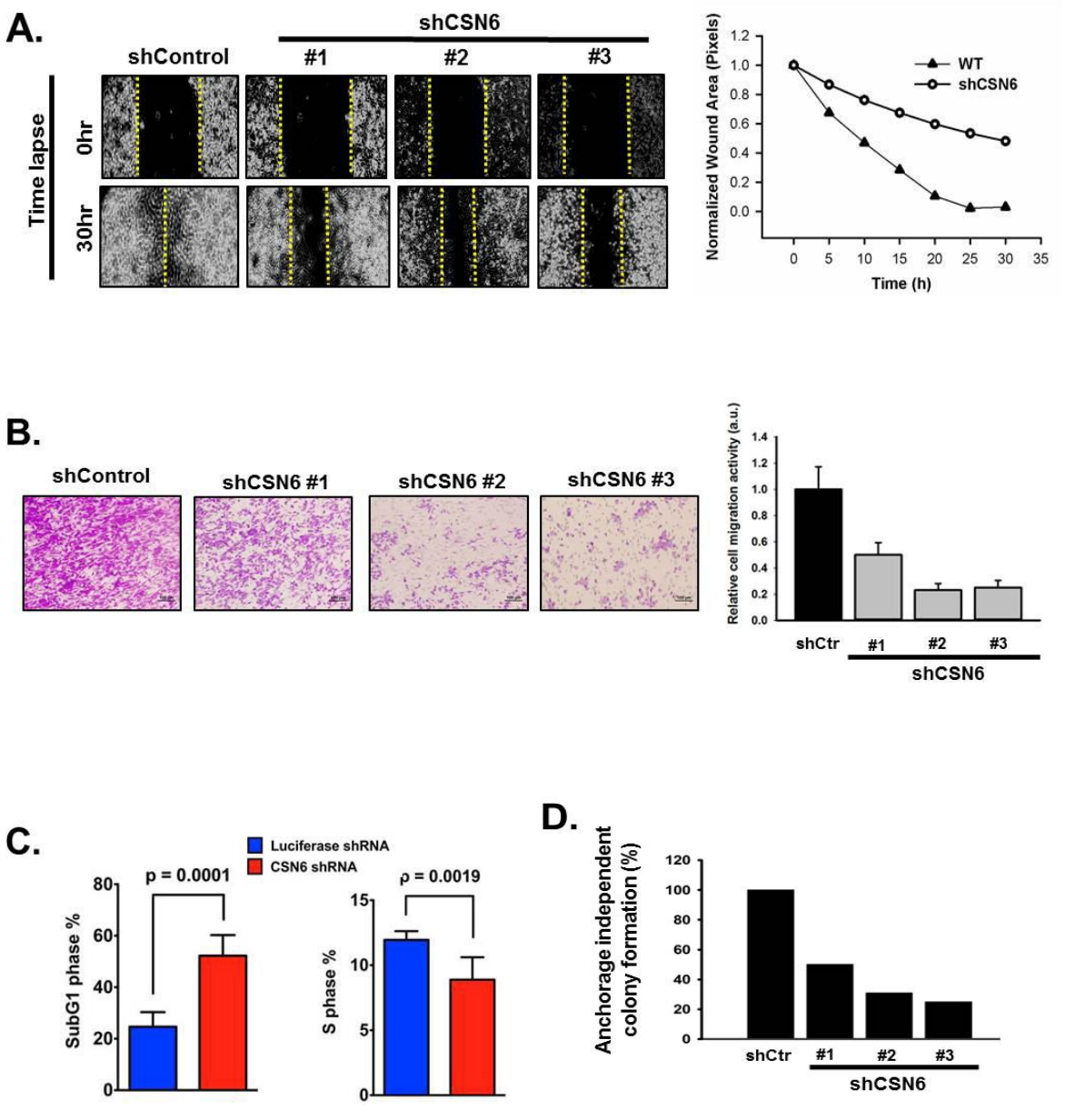

Figure 50. CSN6 knockdown decreases tumorigenesis. MDA-MB-231 cells were infected with either lentiviral expressing luciferase control or shCSN6. (A) Wound healing assay (B) Cell migration assay (C) Flow cytometry assay (D) Anchorage-independent colony formation assay were performed.

4-6. Role of CSN6 in tumorigenesis

To understand the contribution of the CSN6-c-Jun axis to human tumorigenesis, I analyzed the gene expression profiles of 278 patients with stages I, II, or III breast cancer and found a positive correlation between CSN6 and c-Jun target genes (Fig.51 A). Moreover, GeneSet Enrichment Analysis demonstrated that CSN6 positively correlated with expression of c-Jun target genes (Fig.51 B). It has also been reported that CSN6 is overexpressed in breast and thyroid cancers [94]. To further demonstrate CSN6's role in cancer, I found that CSN6 was overexpressed in many types of human cancers including liposarcoma after performing human clinical cancer sample analysis based on the Oncomine cancer microarray database and The Cancer Genome Atlas (TCGA) (Fig.52).

Liposarcoma is known to overexpress c-Jun. To investigate the contribution of CSN6 in tumorigenesis, I determined the tumor formation rate with use of a liposarcoma xenograft model. 3T3L1 cells can form liposarcoma with elevated c-Jun expression. I established 3T3L1 cell lines with CSN6 knockdown by infection with lentiviral CSN6 shRNA. Stable CSN6 knockdown 3T3L1 cells demonstrated high ubiquitination levels of c-Jun and Cyclin E (Fig.53 A). CSN6 knockdown 3T3L1 cells were injected into the flanks of nude mice. Xenografted tumor volume was significantly decreased in the shCSN6 3T3L1 cell–injected group compared with the shRNA control group (Fig.53 A). Hematoxylin and eosin (H&E) staining clearly showed a morphological difference between the control and shCSN6 xenografts. shControl 3T3L1 cells would form tumors whereas shCSN6 3T3L1 cells differentiated into lipid cells, based on H&E staining (Fig.53 A). Furthermore, immunohistochemical staining of tumors obtained from this study indicated that both Cyclin E and c-Jun protein levels are downregulated in

shCSN6-expressing tumors (Fig.53 B). Also, the proliferation marker Ki67 was found to be expressed less in shCSN6 tumors (Fig.53 B).

I also examined the effect of CSN6 on human cancer survival by identifying and reviewing the medical records of breast cancer patients. Importantly, Kaplan-Meier analysis showed that high levels of CSN6 expression correlated with poor overall and recurrence-free survival in a cohort of breast cancer patients (Fig.54). Thus, CSN6's positive impact on c-Jun can be confirmed in human cancer samples. High CSN6 expression is prevalent in many types of cancer and could lead to poor cancer survival. Mouse cancer xenograft studies have demonstrated that CSN6 deficiency leads to downregulation of Cyclin E and c-Jun in tumor, thereby inhibiting tumor growth.

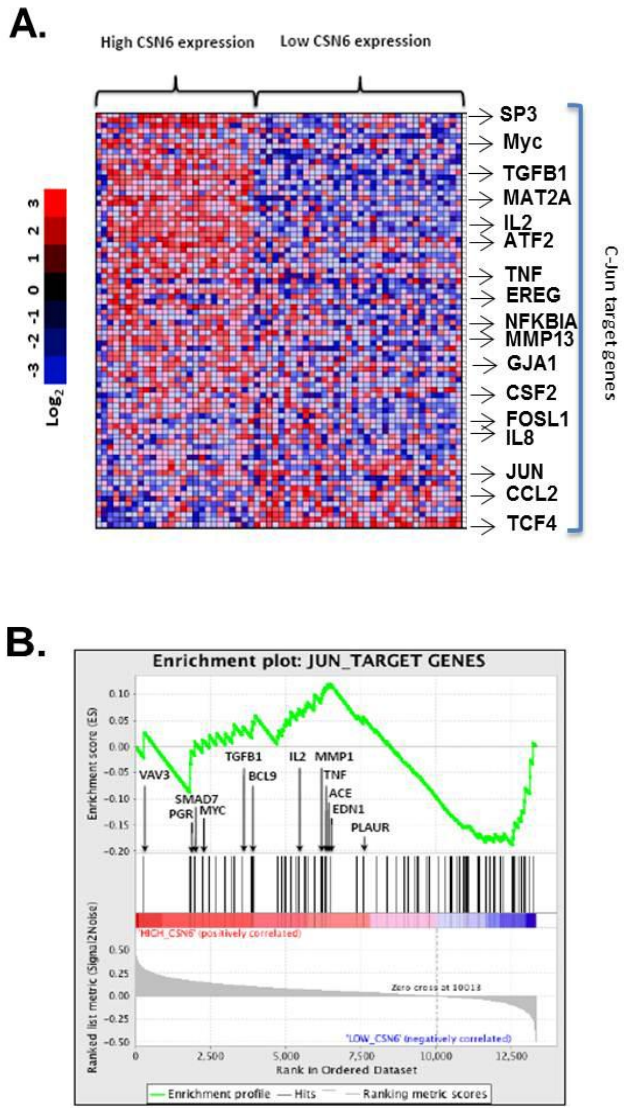

Figure 51. CSN6's role in tumorigenicity and cancer survival. (A) High CSN6 expression is associated with elevated mRNA levels of c-Jun target genes in breast cancer patients. (B) Gene set enrichment analysis indicates increased c-Jun target gene expression in high CSN6 breast cancer samples.

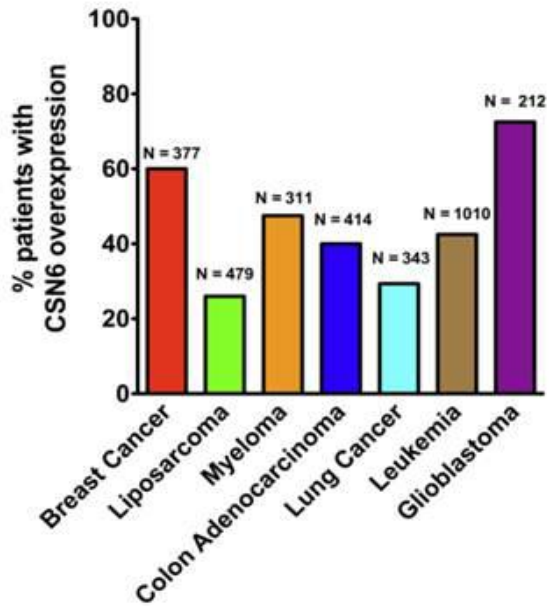

Figure 52. CSN6 is frequently overexpressed in many common types of cancer. Percentage of CSN6 overexpression was determined with use of Oncomine's database and analysis tools. N represented the total number of patients analyzed for each type of cancer. A 60% increase in CSN6 mRNA when compared with corresponding normal tissue was defined as overexpression.

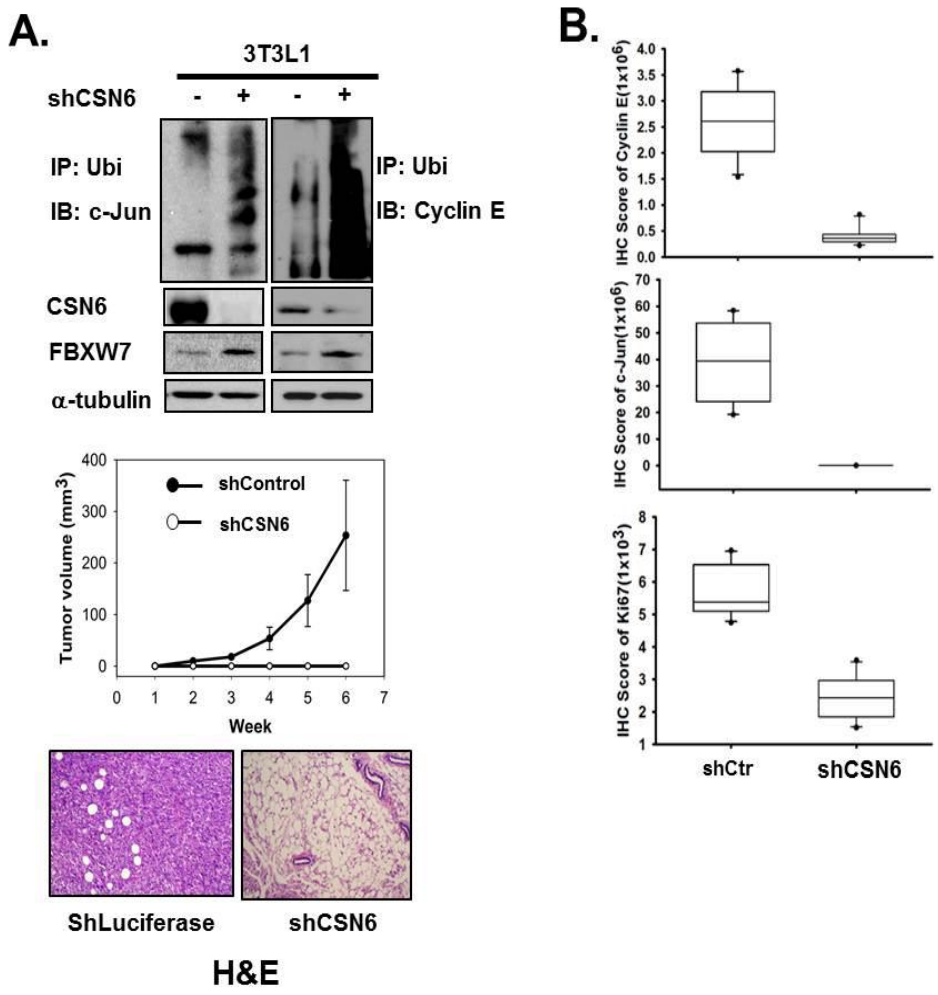

Figure 53. CSN6 - FBXW7- Cyclin E and c-Jun axis regulation induces tumorigenesis. (A) shControl and shCSN6 expressing 3T3L1 cells were harvested and s.c. injected into the flank region of nude mice. Tumor volumes were monitored for 6 weeks. Tumor volumes were measured 6-day period. Bars, SD. Tumor slides stained with hematoxylin and eosin were shown. (B) Tumor slides obtained from (A) were immunostained with Cyclin E–, c-Jun–, or Ki-67–specific antibody, and signals of proteins were quantitated on the basis of immunohistochemical staining intensity.

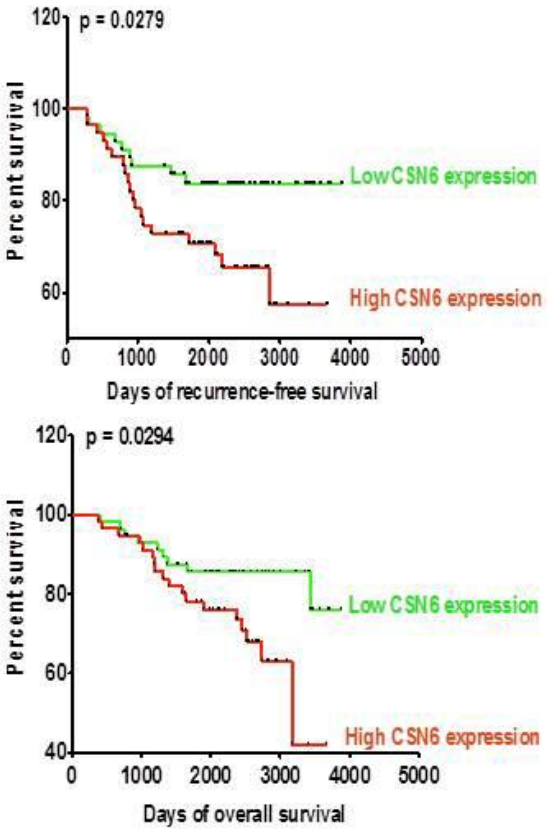

Figure 54. CSN6 reduces survival rate of breast cancer patients. Kaplan-Meier analysis showed that high expression of CSN6 was associated with poor overall survival of breast cancer patients. High CSN6 expression also shortens recurrence-free survival.

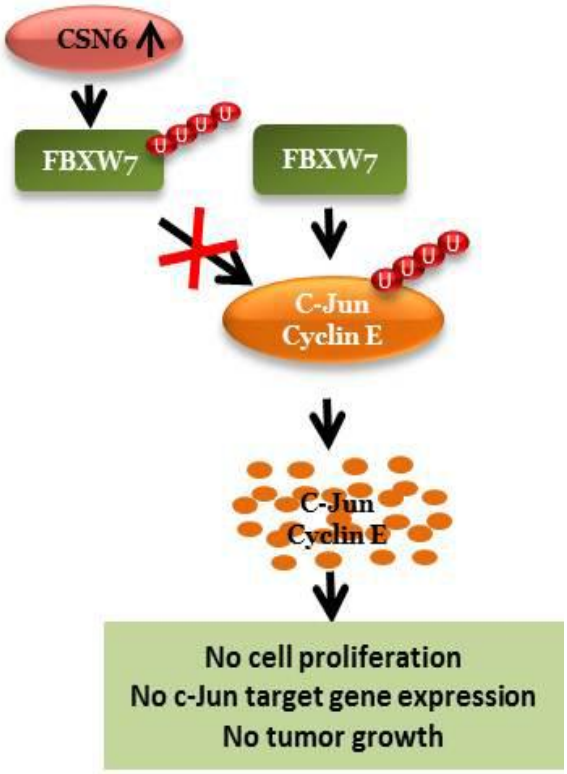

Figure 55. Model of CSN6-mediated Cyclin E and c-Jun stabilization in affecting tumor progression. Note the role of CSN6 in enhancing FBXW7 ubiquitination.

CHAPTER 5

DISCUSSION

1. COP1 ubiquitin ligase regulates FBXW7 stability in an AKT dependent manner

In chapter 3, I demonstrated that FBXW7 is phosphorylated by AKT and COP1 ubiquitinates phosphorylated FBXW7 proteins. FBXW7 is a well-defined tumor suppressor protein through recognizing and degrading several oncoproteins such as Cyclin E, c-Jun, c-Myc, and Mcl-1[10,30,95]. Therefore, abnormal expression of FBXW7 causes tumorigenesis via failing to decrease target oncoproteins [95,96]. Even though FBXW7 has a critical function in cancer regulation, only a few upstream regulators are verified to be involved in regulation of FBXW7 stability and activity [89,90,97]. In this study, the data suggests AKT and COP1 as novel upstream regulators of FBXW7.

1-1 FBXW7 is a novel substrate of AKT

It was reported that Protein Kinase C (PKC) induced mislocalization of FBXW7 increase tumorigenesis [90]. And also Polo-like Kinase (PLK)-mediated FBXW7 phosphorylation destabilized FBXW7 via regulating dimerization [89]. It suggested that FBXW7 stability affected by phosphorylation status and it could be regulated by kinase. Based on this possibility, I sought to identify a new kinase that affects FBXW7 regulation and I found an AKT consensus sequence at T226 and S227 in FBXW7 (Fig 6. A). Both *in vivo* and *in vitro* kinase assay showed that AKT have impact on FBXW7 phosphorylation (Fig 6.B,C). This result suggests that FBXW7 is a new substrate of AKT. It is known that AKT promotes tumor progression through suppressing many of the tumor suppressor substrate proteins such as BAD [77], p27 [98], GSK-3β [99], and

FOXO [100]. In this study, I found that IGF1 and EGF induced-AKT signaling activation phosphorylates tumor suppressor protein FBXW7 at T226 and S227 resulting in FBXW7 destabilization which then increased Cyclin E and c-Myc accumulation (Fig.7). However, PI3K/AKT inhibitor LY294002 suppressed kinase activity of AKT on FBXW7 and also phosphorylation site mutant form of FBXW7 rescued from AKT regulation (Fig.8 A, B). This is the first report about biological stimuli of FBXW7 signaling and also that AKT have tumorigenic function through FBXW7-mediated Cyclin E and c-Myc regulation. Steady state and turn-over rate studies of FBXW7 support negative impact of AKT (Fig.9,10).

Protein degradation is commonly regulated by either the lysosome or proteasome [101]. Lysosome mediated protein degradation is non-selective whereas proteasome mediated degradation is highly selective. Therefore, I examined which pathway AKT uses to degrade FBXW7 and I found that AKT ubiquitinates FBXW7 via the proteasomal pathway. Proteasome complex facilitate protein degradation via L48- or L63-linked polyubiquitination. L48-linkage induces proteolytic degradation but L63-linkage affects cellular localization or signaling regulation. In this study, I found that AKT degrades FBXW7 via L48-liked polyubiquitination (Fig.11, 12, 13). These results proposed that AKT-mediated phosphorylation is critical for FBXW7 ubiquitination. Therefore, I tried to verify whether other E3 ligase proteins are involved in AKT-mediated FBXW7 ubiquitination.

1-2 COP1 function as an E3 ubiquitin ligase of FBXW7

COP1 has been identified as a photo-morphogenic E3 ubiquitin ligase in plants [39]. However, recent studies report that COP1 is also involved in cancer regulation in mammals [40,53,85]. Interestingly, it has been reported that COP1 is positively regulated by the AKT induced signaling pathway [40]. In addition, most of COP1 substrates contain the COP1 binding motif 'D(E)D(E)X_nVPD(E)' [40]. In this study, I found that FBXW7 contains an evolutionary conserved COP1 binding motif at V200 and P201 (Fig.17 A). COP1 binds with FBXW7 both *in vivo* and *in vitro* (Fig.17 A). Especially, WD40 domain of COP1 which is substrate binding domain [95] associate with N-terminal of FBXW7 (Fig.15, 16). However, V200A/P201A FBXW7 fails to integrate with COP1 (Fig.17 B). This finding suggests that COP1 regulates FBXW7 stability through those binding sites. Indeed, COP1 decreased steady-state of FBXW7 whereas increased ubiquitination level both *in vivo* and *in vitro* (Fig.20-23). However, V200A/P201A mutant FBXW7 was not regulated by COP1 (Fig.21). The RING mutant of COP1 which is lost the E3 ligase function failed to induce FBXW7 poly ubiquitination (Fig.22) [48]. This data confirmed that COP1 is a major functional E3 ligase of FBXW7. Like other known regulators, a recent study reported that prolyl isomerase Pin1 also destabilizes FBXW7 via self-ubiquitination manner by destruction of dimerization [97]. However, COP1 still regulated F-box deleted FBXW7 (ΔF FBXW7) which is lost the self-ubiquitination function [95]. This data supports that FBXW7 is regulated by COP1 not through self-ubiquitination regulation but by K48 liked poly ubiquitination (Fig.24). These results suggest that COP1 recognizes FBXW7 as a substrate and causes their destruction via the proteasome pathway. However, there is no evidence as to whether

AKT-mediated FBXW7 phosphorylation is necessary to target COP1-mediated ubiquitination of FBXW7.

1-3 AKT-mediated FBXW7 phosphorylation is necessary to recognized by COP1.

E3 ubiquitin ligases commonly detect phosphorylated substrate proteins to target ubiquitination [102]. For example, FBXW7 recognizes most substrate proteins after phosphorylation by GSK3β kinase [19]. In this study, my data presented above suggests a possibility that AKT-mediated phosphorylation of FBXW7 might be critical for FBXW7 destabilization by COP1. Indeed, FBXW7 phosphorylation status is critical for binding between FBXW7 and COP1 (Fig.25, 26). In addition, phospho-mutant FBXW7 rescued from COP1 mediated ubiquitination whereas phospho-mimic FBXW7 have high levels of ubiquitination (Fig.31). *In vitro* ubiquitination assay results strongly support that catalytic activation of FBXW7 by AKT is critical for ubiquitination by COP1 (Fig.29). These results confirmed that COP1 recognizes FBXW7 in a phosphorylation dependent manner. Moreover, upon IGF1 treatment, COP1 increase FBXW7 ubiquitination followed by FBXW7 phosphorylation through AKT signaling pathway (Fig.28). However, both under AKT knock down and LY294002 treatment conditions, COP1 failed to induce FBXW7 degradation (Fig.30).

As I emphasized above, FBXW7 is a well-documented tumor suppressor protein via suppressing various oncoproteins. Loss of function FBXW7 will promote tumorigenesis. Thus, I performed further biological studies to understand whether FBXW7 phosphorylation is a key event for tumorigenesis. Indeed, high phosphorylation of FBXW7 increased cell migration, proliferation, and invasion rate of human colon

carcinoma (Fig.32). However, non-phosphorylated FBXW7 have significantly less S phase cell numbers (Fig.33). Taken together, this data supports that AKT-mediated FBXW7 phosphorylation facilitates COP1-mediated ubiquitination and increases tumorigenesis.

In conclusion, the inhibition of AKT and COP1 by therapeutic intervention can stabilize FBXW7 and this is expected to further suppress downstream cancer-promoting molecules involved in cancer growth as an effective cancer treatment.

2. CSN6 negatively regulates FBXW7's degrading activity toward Cyclin E and c-Jun

In chapter 4, I demonstrated that CSN6 destabilized the FBXW7 protein through ubiquitination and positively regulated FBXW7's oncogenic substrate proteins, Cyclin E and c-Jun. The CSN complex is involved in a wide variety of regulatory processes, including cell cycle control, signal transduction, transcriptional activation, and tumorigenesis [66,103]. CSN6 is overexpressed in many different types of cancers [66] [104]. In this study, I discovered a critical role for CSN6: controlling Cyclin E and c-Jun homeostasis by regulating their ubiquitin-proteasomal degradation mediator—FBXW7. My results provide insight into the consequences of CSN6 overexpression that can lead to elevation of Cyclin E and c-Jun expression during carcinogenesis and cancer progression.

2-1 CSN6 facilitates FBXW7 ubiquitination

Recent studies demonstrated that CSN6 is emerging as an oncoprotein [94,104]. CSN6 increases the function of oncogenic E3 ligase proteins, which in turn suppress tumor-suppressor proteins. For example, CSN6 upregulates MDM2 to facilitate p53 degradation [94]. CSN6 increases COP1 stability to suppress 14-3-3σ activity [105]. However, so far there is no evidence that CSN6 can regulate tumor-suppressive types of E3 ligase proteins. In this study, I show for the first time that CSN6 can downregulate the tumor-suppressive E3 ligase FBXW7, providing further evidence of its role as an oncogenic protein. CSN6 contains a MPN domain, which is found in the N-terminus of yeast Mpr1 and Pad1 proteins. [106,107,108]. Among 8 subunit of COP9 signalosome

complex, only CSN5 and CSN6 contain MPN domain. However, most of studies about function of MPN domain were related with CSN5 mediated- deneddylation function. CSN5 deneddylates the Cullin protein through metalloprotease containing JAMM motif in MPN domain and destabilize SCF activity. Although CSN6 consists of polar residues that resemble the active site residues of metalloproteases of CSN5, the biological function of the MPN domain of CSN6 remains to be determined [109]. In this study, my data shows that CSN6 associates with FBXW7 and facilitates the degradation of FBXW7 (Fig.35, 36, 37). Even though CSN6 involved in FBXW7 regulation, it remains to be known whether the MPN domain of CSN6 participates in FBXW7 degradation. The reason is that the most functional region of the MPN domain of CSN5 is a JAMM motif, but CSN6 does not have it. Also, although CSN6 may be directly involved in FBXW7 degradation through their structural homology with the 19S proteasome lid, it is also possible that CSN6 recruits other E3 ligases to degrade FBXW7 [56]. Although a detailed mechanism of CSN6-mediated FBXW7 ubiquitination is not clear, in this study I first found evidence of the function of CSN6 in tumor suppressor protein FBXW7 regulation. Based on this finding, I tried to demonstrate whether suppression of FBXW7 by CSN6 has impact on tumorigenesis through regulating FBXW7 substrates.

2-2 CSN6 has positive impact on FBXW7 substrates Cyclin E and c-Jun

FBXW7 is the F-box protein that functions as a substrate-recognition subunit of SCF complexes. SCF^{FBXW7} is known to regulate the degradation of several important substrates such as Notch, c-Myc, Cyclin E, aurora A, aurora B, and c-Jun [110,111] [112]. Previous I found that CSN6 negatively regulates FBXW7 stability. Therefore, I tried to verify whether CSN6-mediated FBXW7 suppression affect to FBXW7 substrate proteins.

It is noteworthy to state that cells overexpressing CSN6 bind with Cyclin E and c-Jun both *in vivo* and *in vitro* (Fig.38-40). In addition, steady-state and turn-over of both substrates of FBXW7 were regulated by CSN6 (Fig.41-43). Although CSN6 have impact on Cyclin E and c-Jun, it needs to be verified if FBXW7 is the major mediator of this regulation. Steady-state, turn-over rate, and ubiquitination assay data showed that CSN6 has no effect on the regulation of c-Jun or Cyclin E in FBXW7-null cells (Fig.44-48). This supports that FBXW7 works as a target of CSN6 during CSN6-mediated upregulation of c-Jun and Cyclin E. Although it remains to be determined whether CSN6's negative impact on FBXW7 can extend to other FBXW7 substrates and affect their upregulation, my study suggests that CSN6 overexpression or FBXW7 downregulation in cancer cells plays an important role in Cyclin E or c-Jun mediated cell proliferation and c-Jun target gene expression. Thus, I tried to characterize the axis of CSN6-FBXW7-Cyclin E/c-Jun in tumorigenesis.

2-3 CSN6 mediated FBXW7 suppression facilitate tumorigenesis via Cyclin E and c-Jun regulation

CSN6 has been identified as an oncoprotein through inducing MDM2 mediated p53 degradation and COP1 mediated 14-3-3σ ubiquitination [40,113]. In this study, I found that CSN6 also regulates FBXW7 mediated Cyclin E and c-Jun ubiquitination. It has been well known that Cyclin E and c-Jun is involved in cell cycle regulation, proliferation, and apoptosis [17,30]. Therefore, in this study I tried to demonstrate oncogenic function of CSN6 through suppressing FBXW7 mediated Cyclin E and c-Jun regulation. Previous study reported that the CSN6 gene copy number is amplified in human breast cancer and that CSN6 is overexpressed in follicular thyroid carcinoma [94].

In this study, CSN6 positively correlated with both Cyclin E and c-Jun in various human breast cancer cell lines (Fig.49). To support this finding, human clinical cancer sample analyses based on Oncomine and TCGA, showed that CSN6 is overexpressed in many types of cancer including breast cancer, liposarcoma, and glioblastoma (Fig.50). Significantly, CSN6 overexpression can lead to elevated expression of c-Jun target genes in breast cancer (Fig.51), suggesting that my biochemical studies of regulation between CSN6 and c-Jun can be confirmed by clinical sample analysis. In the same manner, cell proliferation, migration, anchorage independent colony formation, and cell cycle distribution data showed that CSN6 have oncogenic functions (Fig.52). Moreover, I demonstrated that xenografted tumor volume was significantly decreased in shCSN6-expressing tumors. Furthermore, tumor analyses obtained from this xenograft tumor model study indicated that levels of Cyclin E and c-Jun were reduced when CSN6 expression was knocked down (Fig.53A), confirming the relationship between CSN6 and Cyclin E/c-Jun *in vivo*. Cyclin E is known to be a positive regulator of the cell cycle [10], promoting cell growth; thus our CSN6-Cyclin E link provides a mechanistic explanation for the low Ki67 signals and more differentiated phenotype we observed in our shCSN6-expressing xenograft mouse tumor samples (Fig.53B). Importantly, breast cancer patients who have high CSN6 expression, compared with those expressing low levels of CSN6, showed shorter overall and recurrence-free survival rates (Fig.54).

Evidence from my study of CSN6-mediated FBXW7 degradation indicates that the CSN6-FBXW7-Cyclin E/c-Jun axis maintains equilibrium between FBXW7, Cyclin E, and c-Jun (Fig.55). FBXW7 negatively regulates Cyclin E and c-Jun, which keeps the levels of the two proteins in check and thereby prevents cancer formation. CSN6

overexpression, which tilts this delicate balance by preventing Cyclin E and c-Jun from degradation by FBXW7, may result in cancer growth. My data showed that CSN6 overexpression decreases the stability of FBXW7 and subsequently suppresses FBXW7-mediated Cyclin E and c-Jun degradation, thereby promoting cancer growth.

In conclusion, CSN6 will be an important molecular target for rational cancer therapy. Since FBXW7 expression/restoration can correct abnormal cell growth mediated by Cyclin E/c-Jun activity, targeting CSN6 will be a useful therapeutic strategy for cancer intervention.

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