5-2011

Cop 9 Signalosome Subunit 6 Stabilizes Cop1, A Novel E3 Ubiquitin Ligase For 14-3-3Σ

Hyun Ho Choi

COP 9 SIGNALOSOME SUBUNIT 6 STABILIZES COP1, A NOVEL E3 UBIQUITIN LIGASE FOR 14-3-3σ

By

Hyun Ho Choi, B.S., M.S.

Approved:

_____

Mong-Hong Lee, Ph.D (Chair)

_____

Elsa Flores, Ph.D

_____

Lei Li, Ph.D

_____

Xin Lin, Ph.D

_____

Sai-Ching J. Yeung, M.D., Ph.D

Approved:

_____

Dean, The University of Texas

Health Science Center at Houston

Graduate School of Biomedical Science

COP 9 SIGNALOSOME SUBUNIT 6 STABILIZES COP1, A NOVEL E3 UBIQUITIN LIGASE FOR 14-3-3σ

A

DISSERTATION

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

and

The University of Texas

M.D. Anderson Cancer Center

Graduate School of Biomedical Sciences

In Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

by

Hyun Ho Choi, B.S., M.S.

Houston, Texas

May, 2011

ACKNOWLEDGEMENTS

It is a pleasure to thank many people who made this thesis possible. First of all, I would like to express my sincere gratitude to Dr. Mong-Hong Lee for his warm encouragement, thoughtful guidance, and giving me the confidence to explore my research interests. I also wish to express my appreciation to Dr. Sai-Ching J. Yeung who brought me a lot of thinking in science and gave me many valuable suggestions that improved the quality of this study.

I wish to thank the fellows in the laboratory: Christopher Gully, Dr. Chun-Hui Su, Guermarie Velaszquez-Torres, Ping-Chieh Chou, Chieh Tseng, Dr. Ruiying Zhao, Liem Phan, Dr. Jian Chen, Ji-Hyun Shin, and Dr. Enrique Fuentes-Mattei for sharing reagents, knowledge, laboratory experience, and most of all, providing a stimulating and fun environment. I also appreciate Dr. Tattym Shaikenov for providing valuable experimental tool.

I am grateful to my supervisory committee members: Dr. Elsa R. Flores, Dr. Lei Li, and Dr. Xin Lin for their lively discussions and constructive feedback.

I would like to thank my parents as well as my brothers Seong-Won and Jun-Ho for their continuous support and absolute confidence in me. Above all, I thank my wife, Se-Kyung who supported, encouraged, understood, and loved me constantly. My last thanks to my daughter, Emily for giving me happiness and joy.

Abstract

14-3-3σ, a gene upregulated by p53 in response to DNA damage, exists as part of a positive-feedback loop which activates p53 and is a human cancer epithelial marker downregulated in various cancer types. 14-3-3σ levels are critical for maintaining p53 activity in response to DNA damage and regulating signal mediator such as Akt. Here, we identify Mammalian Constitutive Photomorphogenic 1 (COP1) as a novel E3 ubiquitin ligase for targeting 14-3-3σ through proteasome degradation. We show for the first time that COP9 signalosome subunit 6 (CSN6) associates with COP1 and is involved in 14-3-3σ ubiquitin-mediated degradation. Mechanistic studies show that CSN6 expression leads to stabilization of COP1 through reducing COP1 self-ubiquitination and decelerating COP1's turnover rate. We also show that CSN6-mediated 14-3-3σ ubiquitination is compromised when COP1 is knocked down. Thus, CSN6 mediates 14-3-3σ ubiquitination through enhancing COP1 stability. Subsequently, we show that CSN6 causes 14-3-3σ downregulation, thereby activating Akt and promoting cell survival by suppressing FOXO, an Akt target, transcriptional activity. Also, CSN6 overexpression leads to increased cell growth, transformation and promotes tumorigenicity. Significantly, 14-3-3σ expression can correct the abnormalities mediated by CSN6 expression. These data suggest that the CSN6-COP1 axis is involved in 14-3-3σ degradation, and that deregulation of this axis will promote cell growth and tumorigenicity.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1. INTRODUCTION

1.1. COP9 signalosome 6 (CSN6), a multiprotein complex

The ubiquitin-proteasome pathway is important for controlling the abundance of proteins and plays an essential role in maintaining normal cellular functions. Dysregulation of ubiquitin-mediated proteolysis results in the development of a variety of human cancers (1). The evolutionarily conserved Constitutive Photomorphogenesis 9 Signalosome (CSN) was originally identified from plant (*Arabidopsis*) mutants that mimic light-induced seedling development when grown in the dark (2-4). Mammalian COP9 signalosome contains eight subunits (CSN1-CSN8), which share sequence homology with subunits of the 'lid' complex of the 26S proteasome (5). Because of the homology, the COP9 signalosome has been postulated to play a role in protein degradation. CSN6 and CSN5 are the only two subunits that contain an MPN (Mpr1p and Pad1p N-terminal) domain (6, 7), which will have impact on Cullin-Ring Ligases (CRL) (8, 9). CSN5 has a MPN domain containing JAMM (JAB1/MPN/Mov34) or MPN+ motif linked to the metalloprotease motif (EXnHXHX10D) that can deneddylate Cullin (9), a process important for regulating CRL activity. By comparison, CSN6 is not recognized to have JAMM motif and thus its function remain obscure.

1.2. Constitutive Photomorphogenic 1 (COP1), an E3 ubiquitin ligase

Mammalian Constitutive Photomorphogenic 1 (COP1) is an evolutionarily conserved E3 ubiquitin ligase containing RING-finger, coiled-coil, and WD40-repeat domains (10). Through association with the COP9 signalosome, COP1 has been identified as a crucial mediator to block photomorphogenesis in the dark through the ubiquitinated proteasomal

degradation of light-induced transcription factor HY5 (11)(12). In mammalian cells, the ubiquitinated targets of COP1 include stress-responsive transcription factors p53 (13) and c-JUN (14-16), as well as acetyl-coA carboxylase (17), transducer of regulated CREB activity 2 (TORC2, a glucose metabolite regulator) (18) and nucleosome remodeling factor MTA1 (19). COP1 is overexpressed in cancers, but whether COP1 targets any tumor suppressor protein for degradation during tumorigenesis remains elusive.

1.3. 14-3-3σ, a tumor suppressor gene

The 14-3-3 proteins are a family of evolutionarily conserved regulatory chaperone molecules involved in many diverse physiological functions, including signal transduction, stress response, apoptosis and cell cycle checkpoint regulation (20, 21). In mammals, the 14-3-3 family comprises seven isoforms β, ε, γ, ζ, η, σ and τ, which are widely expressed in various tissues and exert their biological functions by directly binding to phosphoproteins containing the consensus motifs RX(Y/F)XpSXP or RSXpSXP (22). Binding by 14-3-3 proteins mediates stability and/or subcellular localization of target proteins (23). 14-3-3σ (also known as Stratifin) was originally characterized as a human mammary epithelial-specific marker (HME1) (24), and was later found to be an essential regulator of apoptosis, cell migration, cell cycle (20, 25) and DNA damage response involving p53 and MDM2 (26-28). In contrast to the other 14-3-3 family members, which are able to form both homo- and heterodimers, 14-3-3σ can form only homodimers (29). This unique characteristic implies that 14-3-3σ has exclusive functions and behaviors. 14-3-3σ, but not other family members, has been found to be frequently lost or decreased in various human cancers (30)(28) and functions as a potential tumor suppressor. In response to DNA damage, 14-3-3σ is known to be a p53 downstream target and may

serve as a regulator to prevent oxidative and DNA-damage stress-induced mitotic checkpoint dysfunction (25, 28). 14-3-3σ also acts as an negative regulator of Akt (31). As for its role in cancer, a previous report showed that there is a high frequency of hypermethylation at the 14-3-3σ gene locus in breast cancer (32). Posttranscriptional regulation such as ubiquitination also regulates 14-3-3σ (33). Although 14-3-3σ may play an important role in protecting cells from DNA damage or cancer, the detailed mechanism by which 14-3-3σ is modulated remains not well characterized.

In this study, we found that CSN6, COP1 and 14-3-3σ interact and we investigated the role of the CSN6-COP1 axis in regulating 14-3-3σ stability. Additionally, we found that CSN6 induced 14-3-3σ downregulation by enhancing 14-3-3σ ubiquitination, which was abrogated when COP1 is knocked down. Further investigation of the underlying mechanism revealed that CSN6 physically interacts with COP1 and reduces COP1-mediated self-ubiquitination, thereby stabilizing COP1. This in turn reduces COP1-mediated 14-3-3σ ubiquitination. We previously showed that 14-3-3σ is a negative regulator of Akt (31). Subsequently, CSN6 potentiates Akt-mediated FOXO inactivation, which results in the transcriptional change of targets regulated by FOXO, thereby facilitating cell survival. These data provide insight into how CSN6 overexpression can lead to cell growth, transformation, and promote tumorigenicity. Taken together, our studies indicate the applicability of employing CSN6-COP1 axis as a therapeutic intervention target in cancers.

CHAPTER 2. STATEMENT OF OBJECTIVES

Aim 1 : Determine the mechanism of CSN6 mediated 14-3-3σ degradation

Our preliminary results show that CSN6 decreases levels of 14-3-3σ. Since CSN6, as part of the COP9 signalosome complex, is known to aid E3 ligases in ubiquitination of target proteins, ubiquitination of 14-3-3σ is the most likely mechanism. Therefore, I shall investigate whether CSN6 increases the ubiquitination of 14-3-3σ. To further clarify the mechanism by which CSN6 regulates 14-3-3σ, I shall identify the E3 ligase(s) involved in this process.

Aim 2 : Determine the role of CSN6 in the regulation of 14-3-3σtarget proteins

14-3-3σ has several down stream effects which include inhibition of Akt. The role of CSN6 in how 14-3-3σ regulates this effect can be determined by changing CSN6 expression in the presence or absence of 14-3-3σ.

Aim 3 : Determine the biological significance of CSN6-mediated 14-3-3 σ degradation

Our unpublished data indicate that overexpression of CSN6 increases cell proliferation and promotes tumor growth. Since CSN6 downregulates and thus antagonizes the activity of 14-3-3σ**,** I will determine the impact of 14-3-3σ on CSN6-mediated cell proliferation and tumor promotion.

CHAPTER 3. MATERIALS AND METHODS

3.1. Cell culture and reagents

HCT116 p53 –/– cells were provided by Dr. Bert Vogelstein. Human 293T were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 μg/mlstreptomycin and 0.25 μg/ml amphotericin B. HCT116 cells and U2OS cells were maintained in McCoy's 5A medium supplemented with fetal bovine serum and antimicrobials as mentioned above. For transient transfections, cells were transfected with DNA using either (Lipofectamine 2000 (Invitrogen), or FuGENE HD (Roche) reagents according to protocols of the manufacturers. Antibodies to the following epitopes and proteins were purchased from the indicated vendors: Flag (M2 monoclonal antibody, Sigma), Tubulin (Sigma), COP1 (Bethyl Laboratories), CSN6 (Biomol), 14-3-3σ (RDI), Myc (mouse monoclonal 9E10, Santa Cruz Biotechnology), pAkt (Cell Signaling Technology), Akt (Cell Signaling Technology), PARP (Cell Signaling Technology), HA (12CA5, Roche) and Actin (Sigma).

3.2. Plasmids

pcDNA6-Myc-CSN6 and pcDNA6-Myc-COP1 were constructed in our lab by PCR. pCMV5-Flag-14-3-3σ, Ad-β-gal, and Ad-14-3-3σ was previously described (26). Flag-CSN6 (wt), 1-184 aa and 185-327 aa as well as Flag-14-3-3σ (wt), 1-161 aa and 153-248 aa were generated using standard PCR cloning methods. HA-Akt and DN-Akt were previously described (31). FOXO luciferase reporter gene was used as described (47).

3.3. Immunoprecipitation and immunoblotting

Total cell lysates were solubilized in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride, 5 mM sodium orthovanadate and 1 μg/ml each of aprotinin, leupeptin, and pepstatin) and processed as previously described (20). Lysates were immunoprecipitated with indicated antibodies according to standard protocols. Proteins were resolved by SDS-PAGE gel and proteins transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked with 5% nonfat milk for 1 hr at room temperature prior to incubation with indicated primary antibodies. Subsequently, membranes were washed and incubated for 1 hr at room temperature with peroxidase-conjugated secondary antibodies (Thermo Scientific). Following several washes, chemiluminescent images of immunodetected bands on the membranes were recorded on X-ray film using the enhanced chemiluminescence (ECL) system (Roche).

3.4. *In vitro* binding assay

Flag-CSN6 and myc-COP1 were prepared by in vitro transcription and translation using the TNT coupled system as previously described (Promega)(20). TNT Proteins were mixed and immunoprecipitated with anti-Myc followed by immunoblotting with anti-Flag as described.

3.5. *In vivo* ubiquitination assay

HCT116 and U2OS cells were used to detect endogenous COP1 and 14-3-3σ ubiquitination. 293T cells were transiently co-transfected with indicated plasmids to detect exogenous 14-3-3σ ubiquitination. Forty-eight hours later, cells were treated with 5

μg/mL MG132 (Sigma) for 6 hr. Cells were harvested and lysed with lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.5% NP-40, 0.5% Triton X-100 and 5 mM NEM). Ubiquitinated COP1 was immunoprecipitated with anti-COP1 (Santa Cruz Biotechnology, Inc.) and immunoblotted with anti-HA (Roche). Ubiquitinated 14-3-3σ was immunoprecipitated with Flag (M2 monoclonal antibody, Sigma) or anti-14-3-3σ (RDI) and immunoblotted with anti-HA (Roche). Protein complexes were then resolved by 10% SDS-PAGE to allow observation of the polyubiquitinated COP1 or 14-3-3σ.

3.6. *In vitro* Ubiquitination Assay

For detection of ubiquitinated 14-3-3σ in vitro, purified 14-3-3σ proteins were incubated with different combinations of ubiquitin (200 pmol), E1 (2 pmol), E2-UbcH5a/5b (10 pmol), in vitro translated COP1 and ATP (2 mM) in a total volume of 50 ìl for 1 h at 37^{o}C. Reaction products were resolved with 8% SDS-polyacrylamide gels and probed with anti-14-3-3σ or anti-GST. His-Ubiquitin (UW 8610), E1 (UW 9410) and E2 (UW 9050) were purchased from BioMol International.

3.7. Cell lysates fractionated by Gel filtration

HCT116 and U2OS cell lysates were fractionated through Superose 6 column (GEHealthcare) equilibrated with lysis buffer at a flow rate of 0.3ml/min. Fractions of 300μl each were collected and subjected to immunoblot.

3.8. Quantitative PCR

Primers for real-time quantitative PCR of *CSN6* (5'-GCACAGACAAGTTCAAGA; 5'-GTGATGGTGCCGAGGTAG), *COP1* (5'- CTGCAACGGGCTCATCAACT; 5'-GGCCACATTTTGTCATGTATGCT), *14-3-3σ* (5'-CTCTCCTGCGAAGAGCGAAAC; 5'-

CCTCGTTGCTTTTCTGCTCAA) 14-3-3σ target genes— *BTG* (5'-CGACAGCTGCAGACCTTCAG; 5'-GGTTGATGCGAATACAACGGTA), *FOXO1* (5'-GTCAAGAGCGTGCCCTACTTC; 5'-CTTGCCACCCTCTGGATTGAG), *BCL6* (5'-AGCCCATAAAACGGTCCTCAT; 5'-GAGGATGCAGAATCCCTCAG), *BCLxL* (5'-AAGCGGTCCCGTGGATAGA; 5'-TCCGGTATTCGCAGAAGTCC) *and GAPDH* (5'-AAGGTGAAGGTCGGAGTCAAC; 5'-GAGTTAAAAGCAGCCCTGGTG) were as referenced in Primer Bank (http://pga.mgh.harvard.edu/primerbank/). Quantitative PCR amplification of *GAPDH* was used for normalization. Total RNAs were extracted from cells using Trizol (Invitrogen); 1 μg RNA was used for producing cDNA by iScript cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR analyses were performed using iQ SYBR Green Super mix (Bio-Rad, 170-8882) and the iCycler iQ Real-time PCR detection system.

3.9. Luciferase assay

A FOXO luciferase reporter gene containing a FOXO transcription factor binding site was co-transfected with the pCMV-Myc-CSN6, pCMV-Flag-14-3-3σ or DN-Akt expressing vectors into 293T or HCT116 cells. Luciferase activity was assayed with the dual luciferase assay system (Promega) according to the manufacturer's instructions.

3.10. FACS Analysis for apoptosis assay

Apoptosis was determined by two-color analysis using propidium iodide (PI) and FITC-conjugated anti-Annexin V (BD Pharmingen, USA) according to the manufacturer's instructions. Cells were harvested and washed three times with PBS then cells were stained with PI and FITC-conjugated anti-Annexin V and analyzed with a FACScalibur flow cytometer.

3.11. Generation of stable transfectants

For generation of Myc-CSN6 overexpression stable transfectants, HCT116 and U2OS cells were transfected with either PCDNA6 or PCDNA6-Myc-CSN6 plasmids by electroporation (Amaxa). Forty-eight hours later, cells were selected in 8μg/ml Blasticidin containing culture medium for 2 weeks. For generation of CSN6 knock-down stable cell lines, HCT116 and U2OS cells were infected by lentiviral shRNA transduction particles (Sigma, NM_006833 COPS6 MISSION shRNA lentiviral transduction particles) containing either control shRNA or CSN6 shRNA. After infection, cells were selected with 2μg/ml Puromycin for two weeks.

3.12. Soft agar colony formation

10,000 cells were mixed in 0.35% agarose/complete media were plated on 0.7% agarose/complete media (bottom) layer and grown for 8 weeks. Colonies were stained with 0.5 mg/ml p-iodonitrotetrazolium violet (Sigma) and were counted under a light microscope (Olympus IX70). Each experiment was done at least two times in triplicate wells.

3.13. Foci formation

1000, 2000 or 3000 cells were plated in 6-well plates and then incubated for 7–10 days. Cells were stained with 0.005% crystal violet to visualize the colonies growing in each well.

3.14. MTT assay

Vector control or CSN6-expressing cells were infected with Ad-β-gal (MOI = 100) or Ad-

HA-14-3-3σ (MOI =100). Cells were plated at 3000 cells/well in 96-well plates and grew in the continued presence of selective antibiotics. Cells were counted every day using a colorimetric 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 3 hrs. Following MTT incubation, cells were lysed in 200 μL of 100% DMSO (Fisher). Samples were then analyzed using a spectrophotomer to measure the optical density at 570 nm.

3.15. Xenograft experiment

Four to six week-old female athymic (*nu/nu*) mice (Experimental Radiation Oncology, M. D. Anderson Cancer Center, Houston, TX, USA) were housed in AAALAC-approved barrier facilities with food and water ad libitum and maintained in the animal facility at The University of Texas M. D. Anderson Cancer Center. Mice were divided into different groups. CSN6-expressing cells infected with Ad-β-gal (MOI = 100) or Ad-14-3-3σ (MOI =100) were harvested and injected into the flank of each mouse. Tumor volumes were measured and recorded from day 8 after injection. At the end of the experiment, the mice were euthanized with CO_2 and the tumors were removed and weighed.

CHAPTER 4. RESULTS

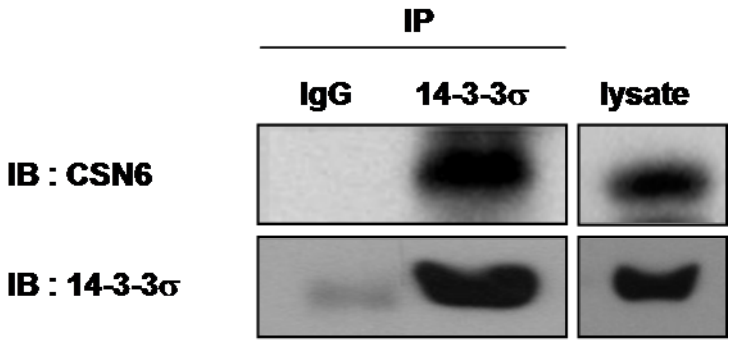
4.1. CSN6 associates with 14-3-3σ

Others have characterized proteins that associate with the CSN complex (34)(35), and many more remain to be identified. In Fig. 3A we show that CSN6 and 14-3-3σ coelute by gel filtration. This led us to the hypothesis that 14-3-3σ and CSN6 may associate in complex. Coimmunoprecipitation experiments. indeed shows their *in vivo* interaction (Fig. 1). We then mapped CSN6 binding region on14-3-3σ. The results showed that CSN6 binds to the C-terminus of 14-3-3σ (aa 153-248), but not the N-terminus (aa 1-161 containing dimerization domain) (Fig. 2). We also mapped 14-3-3σ binding region on CSN6 *in vitro*. A GST-pull-down assay suggests that the N-terminus of CSN6 was responsible for binding 14-3-3σ. (Fig. 3). These results demonstrate that the N-terminal region of CSN6 binds to the C-terminal region of 14-3-3σ.

Figure 1. CSN6 interacts with 14-3-3σ *in vivo*.

Interaction of endogenous CSN6 with endogenous 14-3-3σ. Lysates of HCT116 cells were prepared and equal amounts of cell lysates were immunoprecipitated with indicated antibodies followed by immunoblotting with indicated antbibodies.

Figure 1


IP
IgG
14-3-3σ
lysate
IB : CSN6
IB : 14-3-3σ

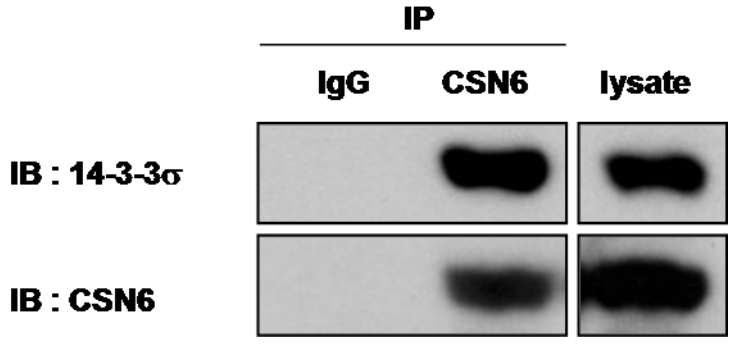
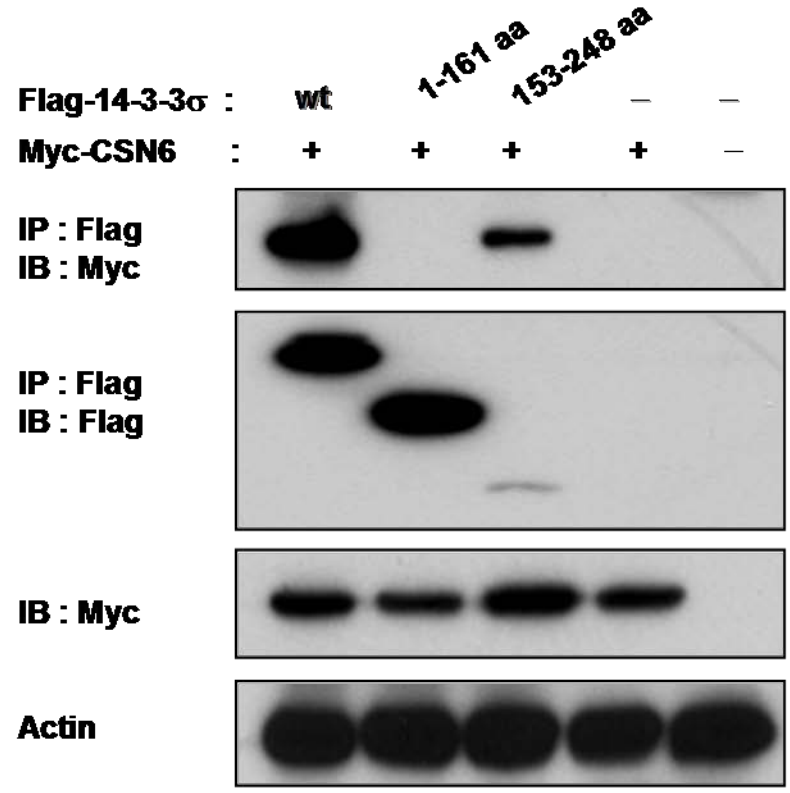

IP
IgG
CSN6
lysate
IB : 14-3-3σ
IB : CSN6

Figure 2. Mapping of 14-3-3σ domain interaction with CSN6.

Mapping of CSN6 binding domains on 14-3-3σ. Myc-CSN6 and Flag-14-3-3σ (aa 1-248), N-terminus (aa 1-161) or C-terminus (aa 153-248) was co-transfected into 293T cells. Cell lysates were immunoprecipitated with anti-Flag and immunoblotted with anti-Myc.

Figure 2


Flag-14-3-3σ :
wt
1-161 aa
153-248 aa
–
–
Myc-CSN6 :
+
+
+
+
–
IP : Flag
IB : Myc
IP : Flag
IB : Flag
IB : Myc
Actin

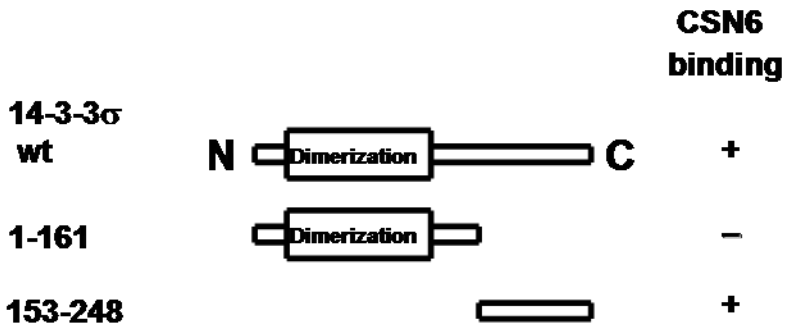
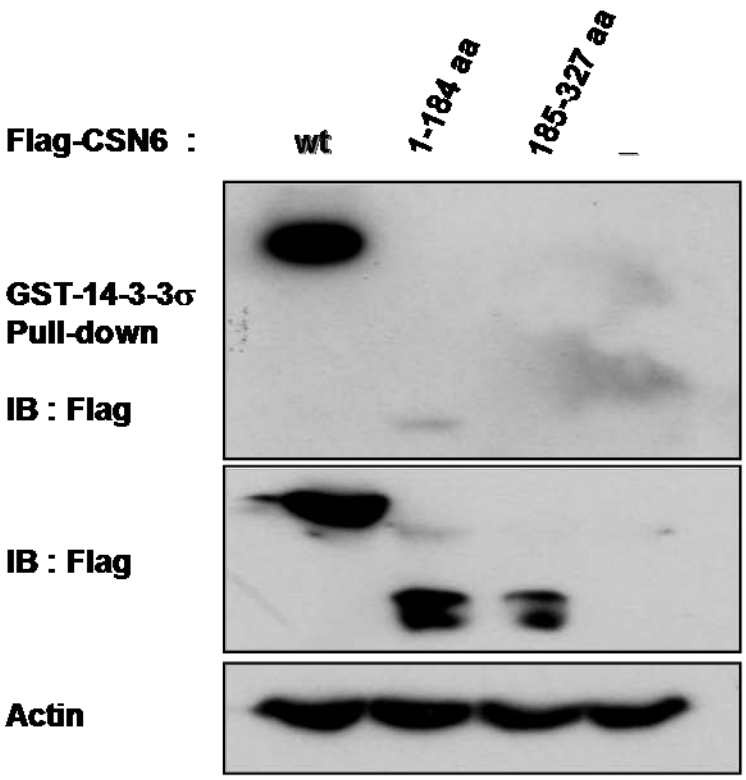
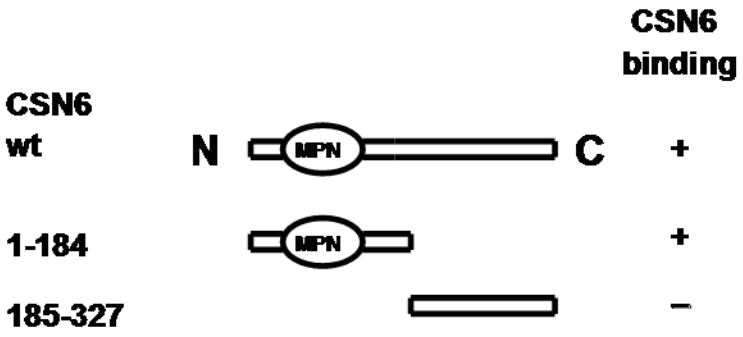

CSN6
binding
14-3-3σ
wt
N
Dimerization
C
+
1-161
Dimerization
–
153-248
+

Figure 3. Mapping of the 14-3-3σ binding region within CSN6.

Mapping of 14-3-3σ binding domains on CSN6. Flag-CSN6 (aa 1-327), N-terminal (aa 1-184) or C-terminal (aa 185-327) were transfected into 293T cells. Cell lysates were subjected to GST-14-3-3σ pull-down (PD) and immunoblotted with anti-Flag antibody.

Figure 3


Flag-CSN6 :
wt
1-184 aa
185-327 aa
_
GST-14-3-3σ
Pull-down
IB : Flag
IB : Flag
Actin


CSN6
binding
CSN6
wt
N
MPN
C
+
1-184
MPN
+
185-327
–

4.2. CSN6 negatively regulates 14-3-3σ protein stability

4.2.1 CSN6 down-regulates 14-3-3σ at the post-transcriptional level

Because CSN6 interacts with 14-3-3σ, we reasoned that CSN6 has some biological impact on 14-3-3σ. The 14-3-3σ levels were elevated when cells were infected with CSN6-shRNA virus to perform CSN6 knockdown (Fig. 4A, left). Exogenous expression of CSN6 also downregulated 14-3-3 σ expression (Fig. 4A, right) in a dose-dependent manner (Fig. 4B). CSN6-mediated 14-3-3σ downregulation was suppressed by MG132, a proteasome inhibitor, suggesting the involvement of 26S proteasome. (Fig. 5). The mRNA levels of 14-3-3σ were not affected by CSN6 overexpression or knockdown in a real-time quantitative PCR analysis (Fig. 6), suggesting that CSN6 downregulates 14-3-3σ at the post-transcriptional level.

4.2.2 CSN6 increases 14-3-3σ polyubiquitination

To investigate if CSN6 can destabilize 14-3-3σ at the protein level we performed a turnover rate experiment. Indeed, CSN6 knockdown can reduce the turnover rate of 14-3-3σ in the presence of the *de novo* protein synthesis inhibitor, cycloheximide (Fig. 7A). Consistently, overexpression of CSN6 increases turnover rate of 14-3-3σ (Fig. 7B). Further, we found that CSN6 increased the endogenous ubiquitination level of 14-3-3σ (while CSN6 knockdown reduced the endogenous ubiquitination level of 14-3-3σ (Fig. 8 top). Also, increasing amounts of CSN6 shRNA antagonized poly-ubiquitination of transfected 14-3-3σ in a dose-dependent manner (Fig. 8 bottom).

4.2.3 Downregulation of 14-3-3σ by CSN6 is not dependent on p53 expression

Since 14-3-3σ is regulated by p53 (25), at issue is whether CSN6-mediated 14-3-3σ downregulation involves p53. We compared 14-3-3σ protein levels in HCT116 p53 -/- cells infected with CSN6 shRNA virus or control shRNA virus and found that 14-3-3σ protein levels were still higher in HCT116 p53 -/- cells knocked down for CSN6 versus control virus (Fig. 9). This result suggests that downregulation of 14-3-3σ by CSN6 is not dependent on p53 expression.

Figure 4. CSN6 reduces the steady-state expression levels of 14-3-3σ.

A. 14-3-3σ protein level is upregulated when endogenous CSN6 expression is inhibited with shRNA (left). Lysates of HCT116 cells infected with either CSN6 shRNA or control shRNA were immunoblotted with anti-14-3-3σ, CSN6 or Actin antibodies. 14-3-3σ protein level was downregulated in CSN6 overexpressing cells (right). Lysates of Myc-CSN6 overexpressing HCT116 stable transfectants and vector control transfectants were immunoblotted with anti-14-3-3σ, CSN6, or Actin antibodies.

B. Ectopic expression of CSN6 reduces 14-3-3σ expression. 293T cells were co-transfected with the indicated expression vectors. Equal amounts of protein from cell lysates were immunoblotted with anti-Flag, Myc, or Actin antibodies.

Figure 4

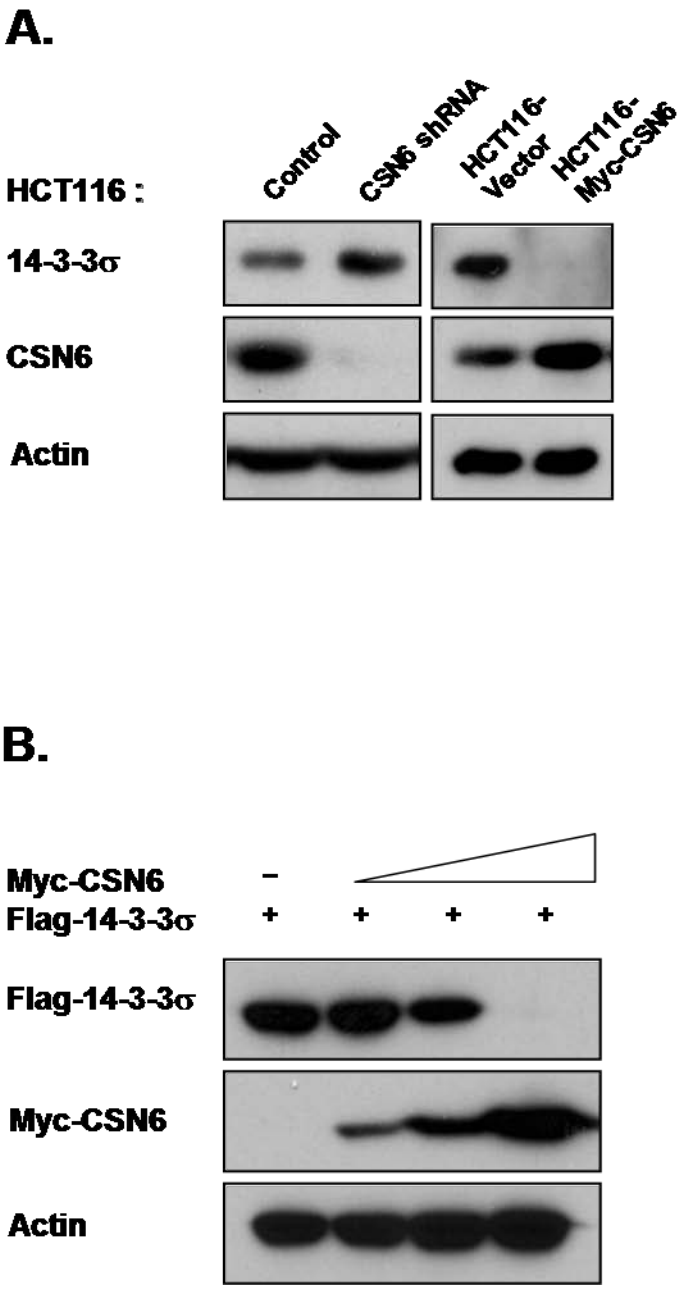

A.
HCT116 :
Control
CSN6 shRNA
HCT116-Vector
HCT116-Myc-CSN6
14-3-3σ
CSN6
Actin
B.
Myc-CSN6
Flag-14-3-3σ
– + + +
Flag-14-3-3σ
Myc-CSN6
Actin

Figure 5. CSN6 down-regulates 14-3-3σ through 26S proteasome pathway.

293T cells were co-transfected with the indicated plasmids. Cells were treated with or without proteasome inhibitor MG132 before collecting lysates. Lysates were immunoblotted with anti-Flag or Tubulin antibodies.

Figure 5

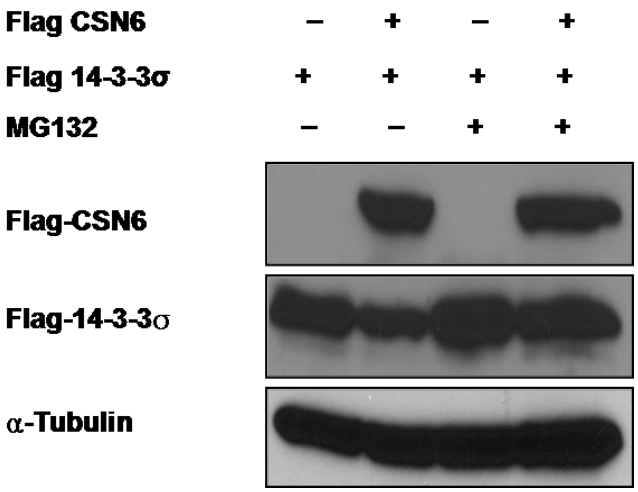


Figure 6. CSN6 down-regulates 14-3-3σ at the post-transcriptional level.

A. mRNA levels of 14-3-3σ are not affected by overexpression of CSN6. Real-time quantitative PCR analysis of 14-3-3σ in 293T cells transfected with or without Myc-CSN6 showed no obvious difference in 14-3-3σ mRNA levels.

B. mRNA levels of 14-3-3σ are not affected by knockdown of CSN6 expression. Real-time quantitative PCR analysis of 14-3-3σ in HCT116 cells infected with CSN6 shRNA or control shRNA showed no obvious difference in 14-3-3σ mRNA levels.

Figure 6

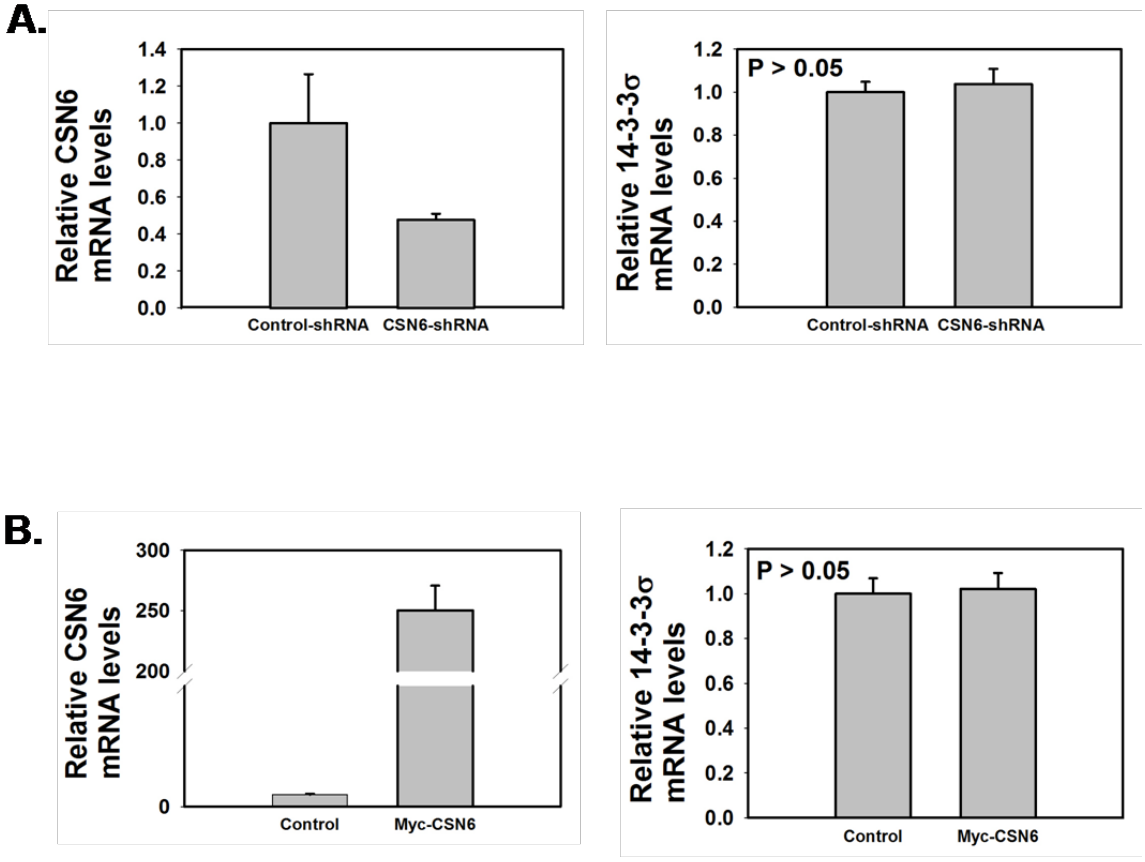
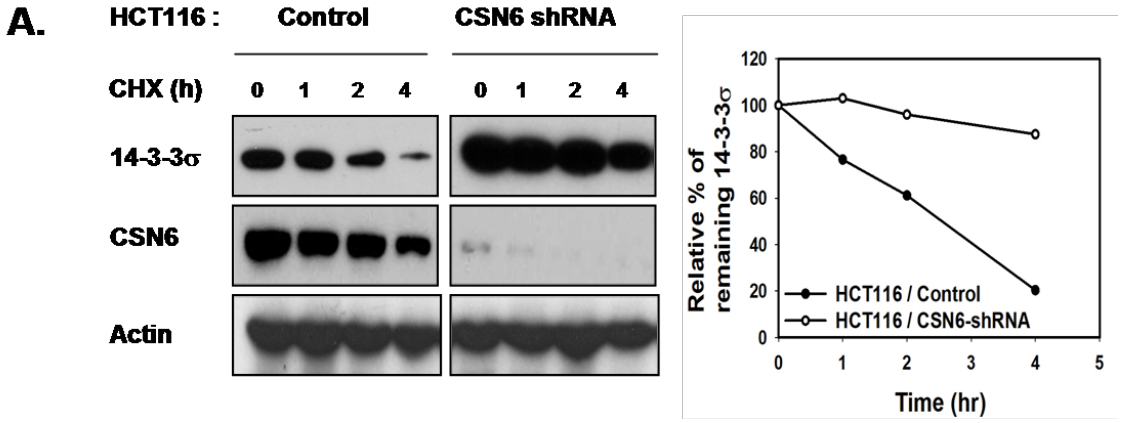

A.
Relative CSN6 mRNA levels
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control-shRNA
CSN6-shRNA
Relative 14-3-3σ mRNA levels
P > 0.05
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control-shRNA
CSN6-shRNA
B.
Relative CSN6 mRNA levels
300
250
200
0
Control
Myc-CSN6
Relative 14-3-3σ mRNA levels
P > 0.05
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Myc-CSN6

Figure 7. CSN6 increases 14-3-3σ turnover rate.

A. HCT116 cells infected with either lentiviral CSN6 shRNA or control shRNA were treated with cycloheximide (CHX) (100 μg/ml) for the indicated times. Cell lysates were immunoblotted with anti-14-3-3σ, CSN6, or Actin antibodies. Integrated OD values of bands at each time point were measured using a densitometer. Levels of 14-3-3σ at time zero were set at 100%. Remaining 14-3-3σ is indicated graphically (right).

B. 293T cells were co-transfected with the indicated expression vectors. Forty-eight hours after transfection, the cells were treated with cycloheximide (CHX) (100 μg/ml) for the indicated times. Cell lysates were immunoblotted with anti-Flag, Myc, or Actin antibodies. Integrated OD values of bands at each time point were measured using a densitometer. Levels of 14-3-3σ at time zero were set at 100%. 14-3-3σ remaining is indicated graphically.

Figure 7


A.
HCT116 :
Control
CSN6 shRNA
CHX (h)
0 1 2 4 0 1 2 4
14-3-3σ
CSN6
Actin
Relative % of remaining 14-3-3σ
120
100
80
60
40
20
0
0 1 2 3 4 5
HCT116 / Control
HCT116 / CSN6-shRNA
Time (hr)

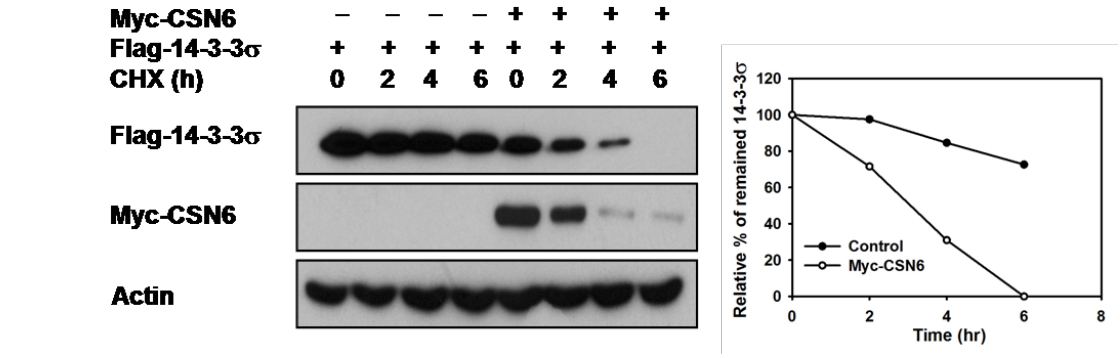

B.
Myc-CSN6 – – – – + + + +
Flag-14-3-3σ + + + + + + + +
CHX (h) 0 2 4 6 0 2 4 6
Flag-14-3-3σ
Myc-CSN6
Actin
Relative % of remained 14-3-3σ
120
100
80
60
40
20
0
0 2 4 6 8
Control
Myc-CSN6
Time (hr)

Figure 8. CSN6 increases 14-3-3σ poly-ubiquitination.

CSN6 increases endogenous 14-3-3σ polyubiquitination (top)**.** Indicated or transfected cells were treated with MG132 for four hours before harvesting. Polyubiquitinated 14-3-3σ was immunoprecipitated with anti-14-3-3σ and immunoblotted with anti-HA. Equal amounts of cell lysates were immunoblotted with anti-CSN6 or Actin.

Knockdown of CSN6 reduces poly-ubiquitination of exogenous 14-3-3σ (bottom). 293T cells were co-transfected with indicated plasmids and increasing amounts of CSN6 shRNA. Cells were treated with MG132 for four hours before harvesting. Polyubiquitinated 14-3-3σ was immunoprecipitated with anti-Flag and immunoblotted with anti-HA. Equal amounts of cell lysates were immunoblotted with anti-CSN6 or Actin antibodies.

Figure 8

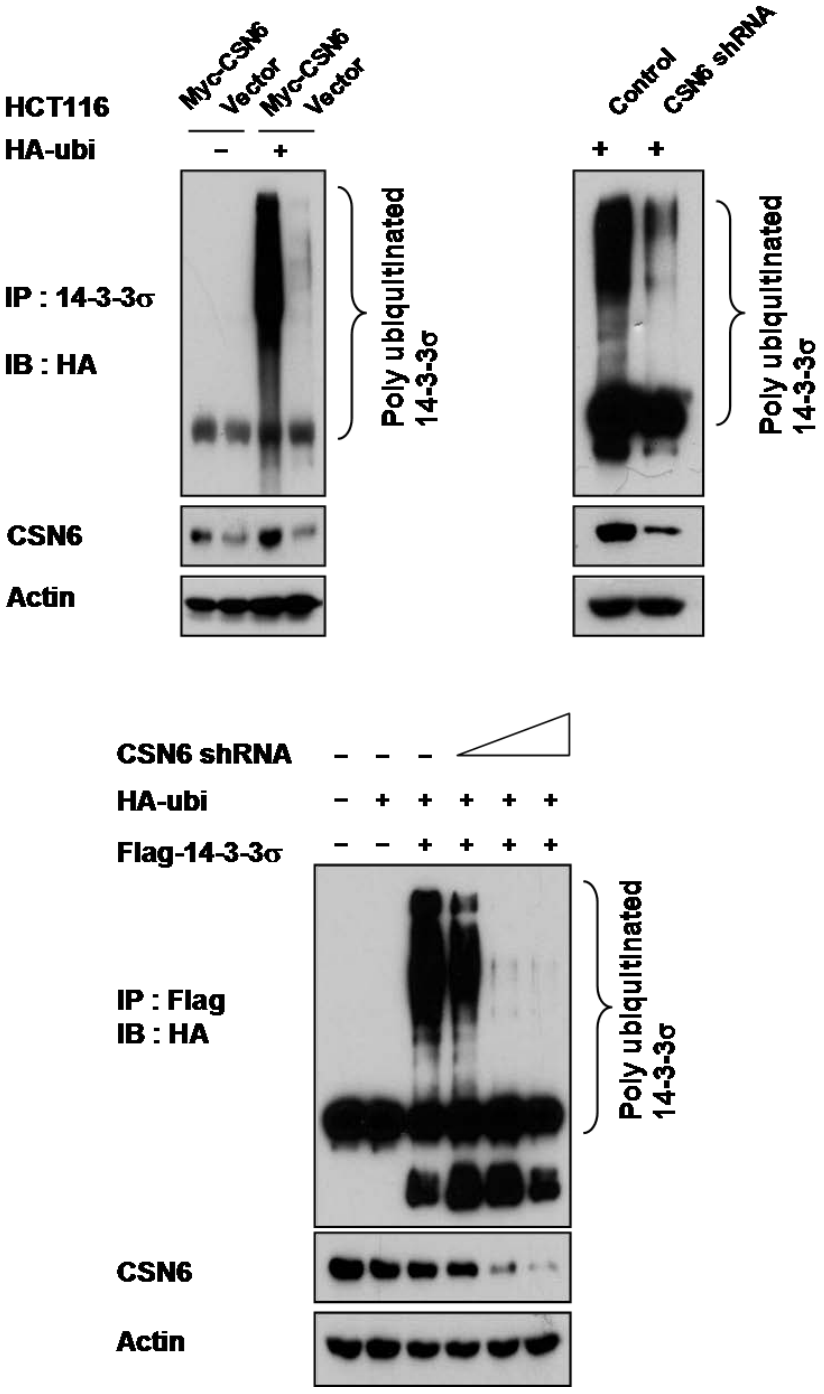
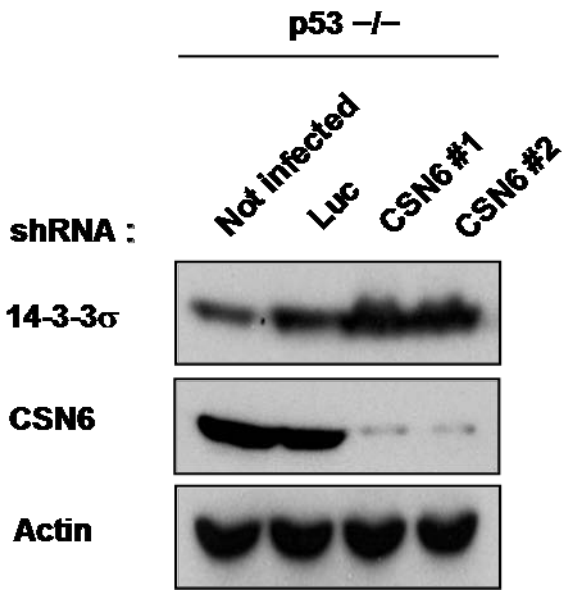

HCT116
HA-ubi
Myc-CSN6
Vector
Myc-CSN6
Vector
– +
IP : 14-3-3σ
IB : HA
Poly ubiquitinated 14-3-3σ
CSN6
Actin
Control
CSN6 shRNA
+ +
Poly ubiquitinated 14-3-3σ
CSN6 shRNA – – –
HA-ubi – + + + + +
Flag-14-3-3σ – – + + + +
IP : Flag
IB : HA
Poly ubiquitinated 14-3-3σ
CSN6
Actin

Figure 9. Downregulation of 14-3-3σ by CSN6 is not dependent on p53 expression.

HCT116 p53 -/- cells were infected with either CSN6 shRNA or luciferase shRNA. Equal amounts of cell lysates were immunoblotted with anti-14-3-3σ, CSN6, or Actin antibodies.

Figure 9



4.3. CSN6 regulates 14-3-3σ protein stability through E3 ubiquitin ligase COP1

4.3.1 Gel filtration and elution profiles analysis of CSN6, COP1 and 14-3-3σ

The COP9 signalosome (CSN) and COP1 are required for the dark-dependent degradation of the transcription factor HY5 (12), a positive regulator of photomorphogenesis in plants. Although CSN is required for proper functioning of COP1, a direct interaction between CSN and COP1 has not been characterized. To investigate this possible direct interaction, we analyzed HCT116 and U2OS cell lysates separated with gel filtration columns. Lysate fractions were analyzed by SDS-PAGE and immunoblotting. Fig. 10 shows that COP1, CSN6, and 14-3-3σ are present in the fractions corresponding to the molecular size of the COP9 holocomplex (between 400-693 kDa), suggesting the interaction of COP1 and 14-3-3σ with the COP9 signalosome. These observations led us to investigate the functional relevance of the interaction of CSN6 and COP1.

4.3.2 CSN6 interacts with COP1 and elevates the steady-state protein levels of COP1 at the post-transcriptional level

Fig. 11 shows that CSN6 associates with COP1 endogenously as assayed by co-ip and an *In vitro* binding assay confirms that CSN6 directly binds to COP1. To address the significance of the CSN6 and COP1 interaction, we examined the impact of CSN6 on COP1 levels and noted that the steady-state level of COP1 increased when CSN6 was overexpressed (Fig. 12A). The mRNA levels of COP1 were not affected by CSN6 expression in a real-time quantitative PCR analysis (Fig. 12B), suggesting that CSN6 up-regulates COP1 at the post-transcriptional level.

4.3.3 CSN6 reduces poly-ubiquitination of COP1

Further, the turnover rate of endogenous COP1 was reduced in CSN6 overexpressing cells in a cycloheximide chase assay (Fig. 13). We also examined the effect of CSN6 on COP1 ubiquitination. As expected, polyubiquitinated COP1 was reduced in CSN6 overexpressing cells compared with control cells (Fig. 14). Taken together, these findings demonstrate that CSN6 increases COP1 stability through inhibition of ubiquitin-mediated COP1 proteasomal degradation.

Figure 10. Gel filtration and elution profiles analysis of CSN6, COP1 and 14-3-3σ.

The distributions of CSN6, 14-3-3σ, and COP1 were analyzed by gel filtration chromatography. Immunoblots of the fractions for indicated proteins are shown in both HCT116 (A) and U2OS (B) cells. Molecular weight markers are indicated.

Figure 10

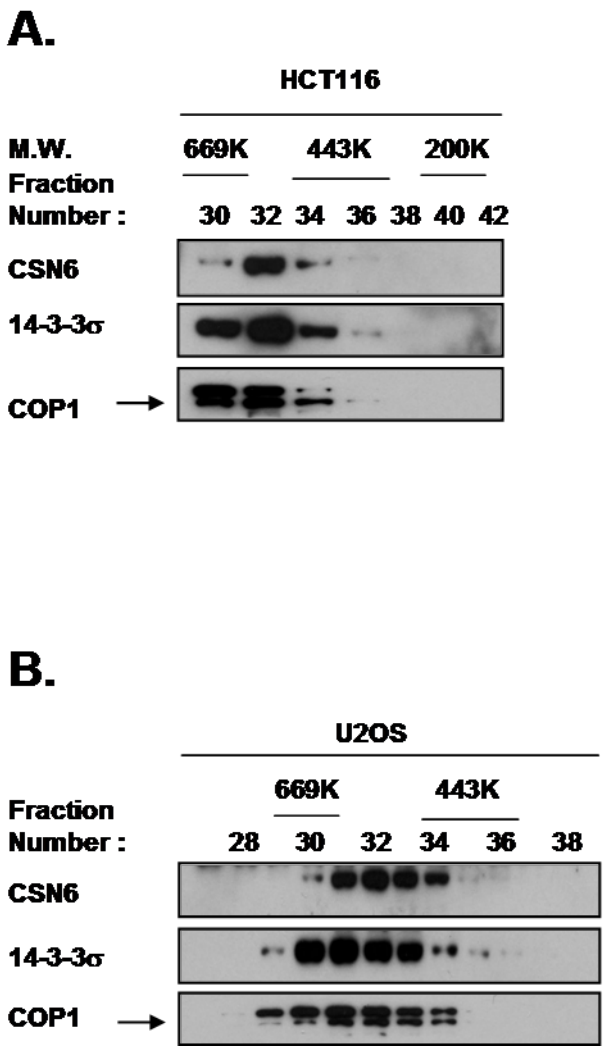
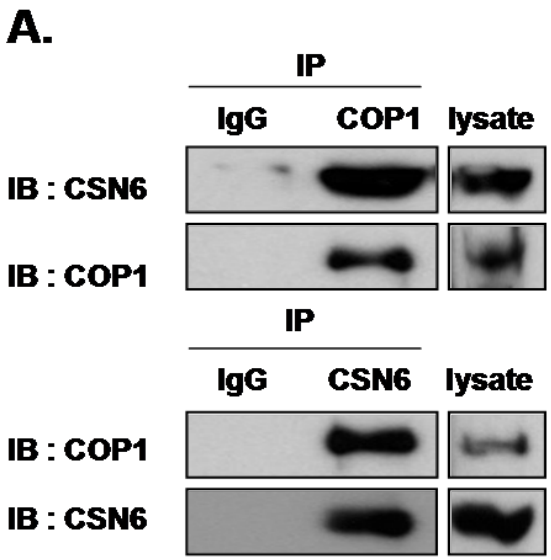

A.
HCT116
M.W.
669K
443K
200K
Fraction
Number :
30 32 34 36 38 40 42
CSN6
14-3-3σ
COP1
B.
U2OS
669K
443K
Fraction
Number :
28 30 32 34 36 38
CSN6
14-3-3σ
COP1

Figure 11. CSN6 interacts with COP1.

A. Endogenous COP1 interacts with endogenous CSN6. Lysates of U2OS cells were prepared and equal amounts of cell lysates immunoprecipitated with either rabbit IgG or anti-COP1 antibodies followed by immunoblotting with anti-CSN6 antibody (left panel).

B. COP1 is shown to interact with CSN6 *in vitro* (right panels). Myc-COP1 and Flag-CSN6 cDNAs were transcribed and translated *in vitro*. COP1 and CSN6 proteins were incubated overnight and immunoprecipitated with anti-Myc followed by immunoblotting with anti-Flag.

Figure 11


A.
IP
IgG
COP1
lysate
IB : CSN6
IB : COP1
IP
IgG
CSN6
lysate
IB : COP1
IB : CSN6

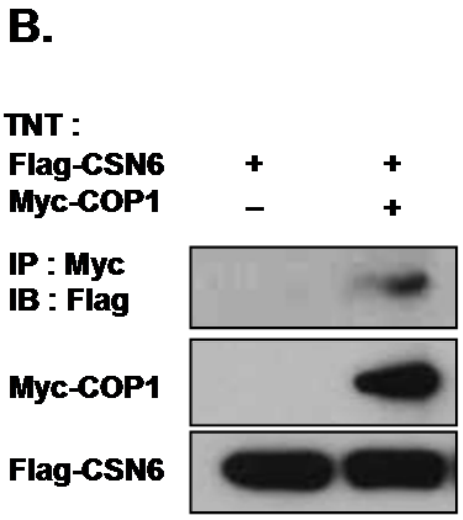
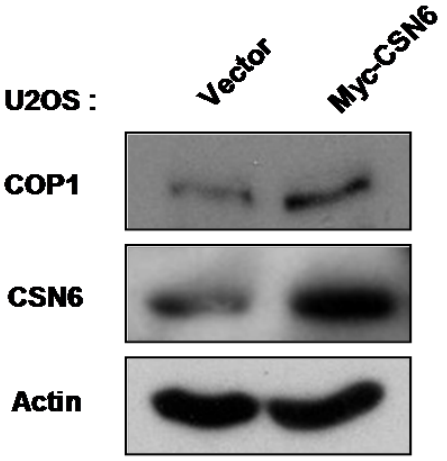

B.
TNT :
Flag-CSN6 + +
Myc-COP1 – +
IP : Myc
IB : Flag
Myc-COP1
Flag-CSN6

Figure 12. Overexpression of CSN6 elevates the steady-state protein levels of COP1 at the post-transcriptional level.

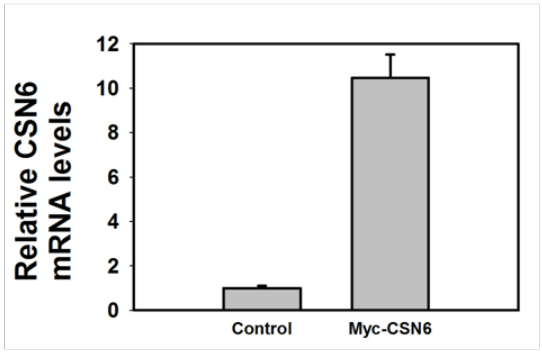
A. Lysates of Myc-CSN6 overexpressing U2OS stable transfectants or vector control transfectants were immunoblotted with anti-COP1, CSN6, or Actin antibodies.

B. mRNA levels of COP1 are not affected by overexpression of CSN6. Real-time quantitative PCR analysis of COP1 in Myc-CSN6 overexpressing U2OS stable transfectants or vector control transfectants showed no obvious difference in COP1 mRNA levels.

Figure 12


U2OS :
Vector
Myc-CSN6
COP1
CSN6
Actin

B.


Relative CSN6 mRNA levels
12
10
8
6
4
2
0
Control
Myc-CSN6

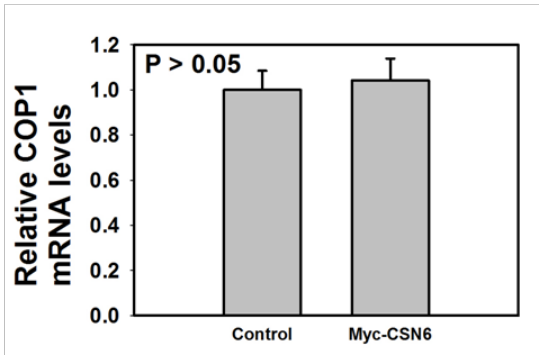
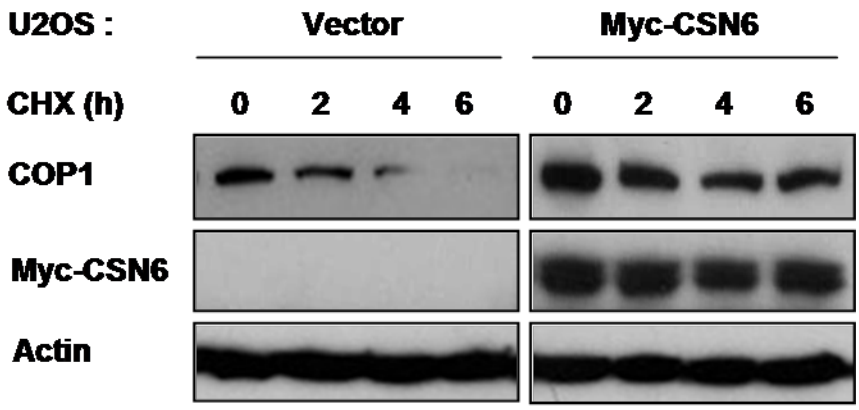

Relative COP1 mRNA levels
1.2
1.0
0.8
0.6
0.4
0.2
0.0
P > 0.05
Control
Myc-CSN6

Figure 13. CSN6 decreases COP1 turnover rate.

Myc-CSN6 overexpressing U2OS stable transfectants and vector control transfectants were treated with cycloheximide (CHX) (100 μg/ml) for indicated times. Cell lysates were immunoblotted with anti-COP1, Myc, or Actin antibodies. Integrated OD values of bands at each time point were measured using a densitometer. Levels of COP1 at time zero wwere set at 100%. COP1 remaining is indicated graphically.

Figure 13


U2OS :
Vector
Myc-CSN6
CHX (h)
0 2 4 6 0 2 4 6
COP1
Myc-CSN6
Actin

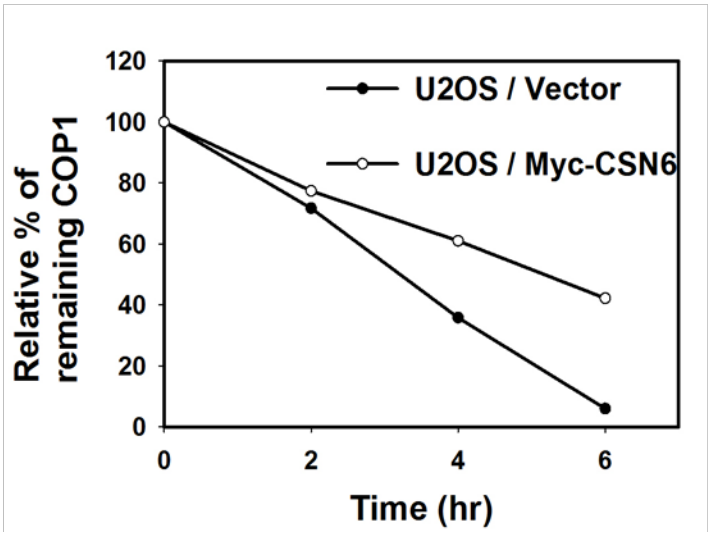
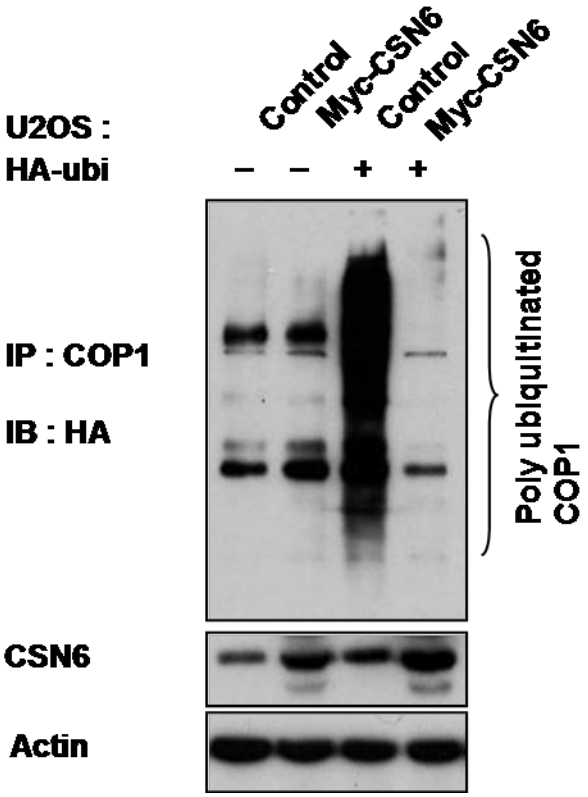

Relative % of remaining COP1
120
100
80
60
40
20
0
0
2
4
6
Time (hr)
U2OS / Vector
U2OS / Myc-CSN6

Figure 14. CSN6 reduces poly-ubiquitination of COP1.

Myc-CSN6 overexpressing U2OS stable transfectants or vector control transfectants were transfected with HA-Ubiquitin. Cells were treated with MG132 four hours before harvesting. Polyubiquitinated COP1 was immunoprecipitated with anti-COP1 and immunoblotted with anti-HA antibodies. Equal amounts of cell lysates were immunoblotted with anti-CSN6 or Actin antibodies.

Figure 14


Control
Myc-CSN6
Control
Myc-CSN6
U2OS :
HA-ubi
– – + +
IP : COP1
IB : HA
Poly ubiquitinated COP1
CSN6
Actin

4.4. COP1 is required for CSN6-mediated 14-3-3σ degradation

4.4.1 COP1 interacts with 14-3-3σ and Knockdown of COP1 expression reduces poly-ubiquitination of 14-3-3σ

Given that CSN6 associates with COP1 and that COP1 coelutes with 14-3-3σ in gel filtration assays (Fig. 10), we reasoned that COP1 could be involved in CSN6-mediated 14-3-3σ ubiquitination. Indeed, 14-3-3σ was able to associate with COP1 endogenously as assayed by co-ip (Fig. 15). Increasing amounts of COP1 shRNA also increased steady-state levels of 14-3-3σ (Fig. 16A). Consistently, COP1 shRNA reduced the turnover rate of 14-3-3σ in 293T cells (Fig. 17) and COP1 shRNA reduced polyubiquitinated 14-3-3σ levels in a dose-dependent manner (Fig. 18).

4.4.2 Knockdown of COP1 expression in CSN6 overexpressing cells increases 14-3-3σ expression through reducing CSN6-mediated poly-ubiquitination of 14-3-3σ

COP1 shRNA also increased endogenous levels of 14-3-3σ in HCT116 cells (Fig. 16B**)** and the mRNA levels of 14-3-3σ were not significantly affected by COP1 shRNA, suggesting that COP1 shRNA upregulates 14-3-3σ at the post-transcriptional level (Fig. 16C). In light of the observation that CSN6 stabilized COP1 and negatively regulated 14-3-3σ, we further evaluated the impact of COP1 on CSN6-mediated 14-3-3σ poly-ubiquitination. We found that increasing amounts of COP1 shRNA in HCT116 cells overexpressing CSN6 led to increased 14-3-3σ steady-state expression (Fig. 19A). Levels of CSN6-mediated 14-3-3σ poly-ubiquitination were also compromised by increasing amounts of COP1 shRNA (Fig. 19B).

4.4.3 COP1 induces the ubiquitination of 14-3-3σ in an *in vitro* ubiquitination assay

As expected, COP1 also efficiently increased the ubiquitination level of 14-3-3σ in an *in vitro* ubiquitination assay (Fig. 20). These results suggested that COP1 is a novel E3 ligase for 14-3-3σ whose ubiquitination is also mediated by CSN6.

Figure 15. COP1 interacts with 14-3-3σ *in vivo*.

Endogenous COP1 interacts with endogenous 14-3-3σ. Lysates of HCT116 cells were prepared and equal amounts of cell lysates immunoprecipitated with either mouse IgG or 14-3-3σ followed by immunoblotting with anti-COP1 antibody.

Figure 15

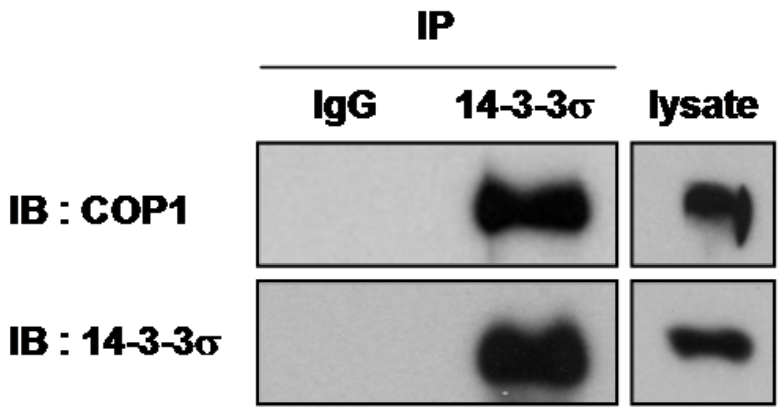


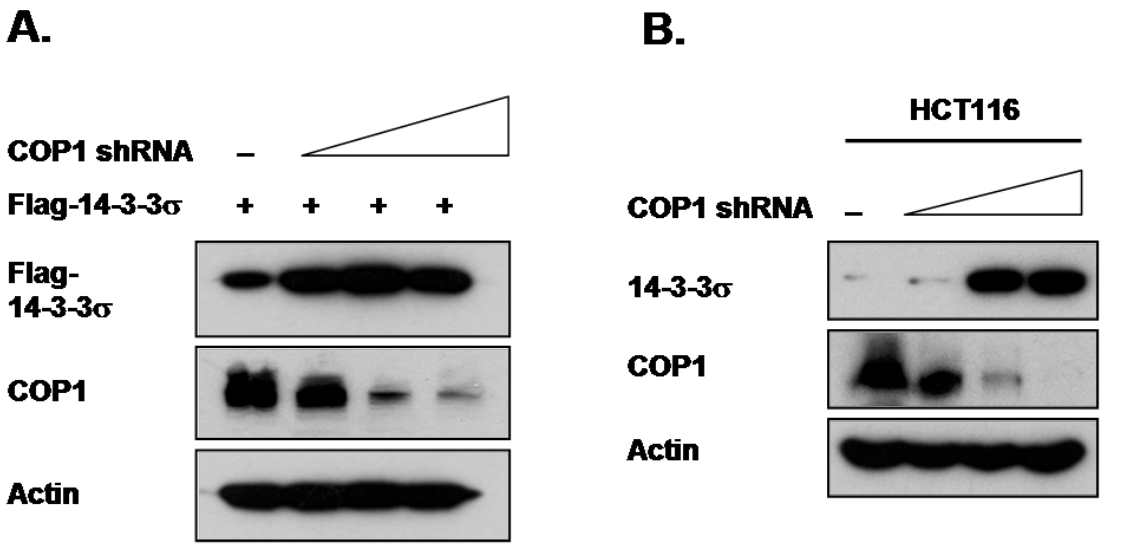
Figure 16. Knockdown of COP1 expression elevates the steady-state levels of 14-3-3σ at the post-transcriptional level.

A. 14-3-3σ protein level is upregulated when endogenous COP1 expression is inhibited with shRNA. 293T cells were co-transfected with indicated plasmids and increasing amounts of COP1 shRNA. Equal amounts of cell lysates were immunoblotted with anti-Flag, COP1, or Actin antibodies.

B. 14-3-3σ protein level was upregulated when endogenous CSN6 expression was inhibited by shRNA. Lysates of HCT116 cells transfected with increasing amounts of CSN6 shRNA were immunoblotted with anti-14-3-3σ, COP1, or Actin antibodies.

C. mRNA levels of 14-3-3σ are not significantly affected by knockdown of COP1 expression. Real-time quantitative PCR analysis of 14-3-3σ in HCT116 cells transfected with COP1 shRNA or control shRNA showed no significant difference in 14-3-3σ mRNA levels.

Figure 16


A.
COP1 shRNA –
Flag-14-3-3σ + + + +
Flag-14-3-3σ
COP1
Actin
B.
HCT116
COP1 shRNA –
14-3-3σ
COP1
Actin

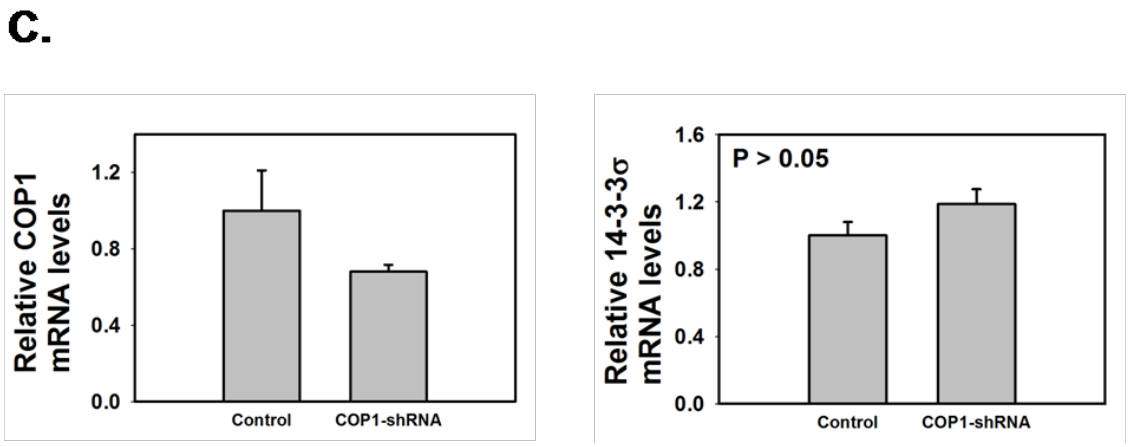
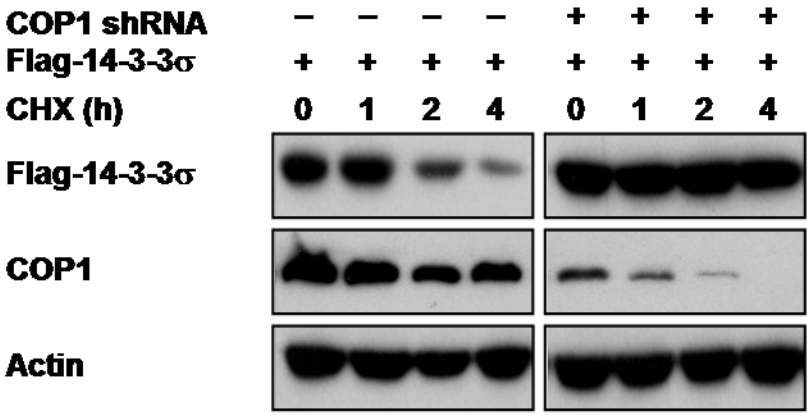

C.
Relative COP1 mRNA levels
1.2
0.8
0.4
0.0
Control
COP1-shRNA
Relative 14-3-3σ mRNA levels
1.6
1.2
0.8
0.4
0.0
P > 0.05
Control
COP1-shRNA

Figure 17. Knockdown of COP1 expression reduces 14-3-3σ turnover rate.

293T cells were transfected with the indicated expression vectors. Forty-eight hours after transfection, the cells were treated with cycloheximide (CHX) (100 μg/ml) for the indicated times. Cell lysates were immunoblotted with anti-Flag, COP1, or Actin antibodies. Integrated OD values of bands at each time point were measured using a densitometer. The level of 14-3-3σ at time zero was set at 100%. 14-3-3σ remaining is indicated graphically.

Figure 17


COP1 shRNA – – – – + + + +
Flag-14-3-3σ + + + + + + + +
CHX (h) 0 1 2 4 0 1 2 4
Flag-14-3-3σ
COP1
Actin

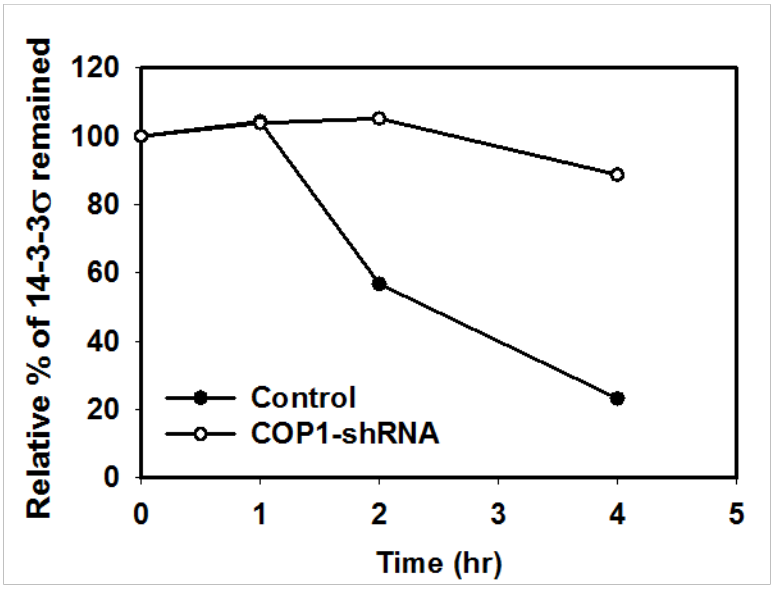

Relative % of 14-3-3σ remained
120
100
80
60
40
20
0
0 1 2 3 4 5
Time (hr)
Control
COP1-shRNA

Figure 18. Knockdown of COP1 expression reduces poly-ubiquitination of 14-3-3σ.

293T cells were co-transfected with indicated plasmids and increasing amounts of COP1 shRNA. Cells were treated with MG132 four hours before harvest, and polyubiquitinated 14-3-3σ immunoprecipitated with anti-Flag followed by immunoblotting with anti-HA. Equal amounts of cell lysates were immunoblotted with anti-COP1 or Actin antibodies.

Figure 18

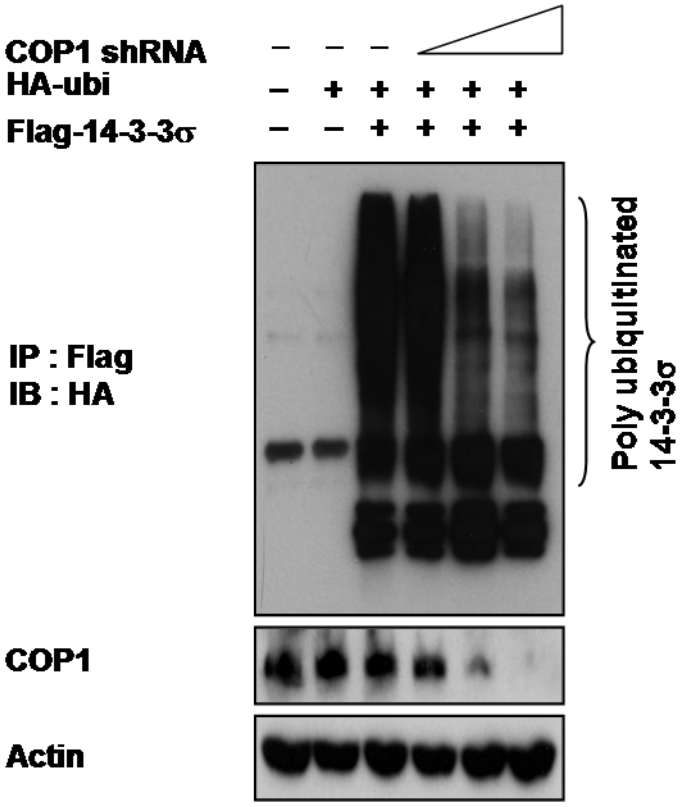

COP1 shRNA
HA-ubi
Flag-14-3-3σ
IP : Flag
IB : HA
Poly ubiquitinated
14-3-3σ
COP1
Actin

Figure 19. Knockdown of COP1 expression in CSN6 overexpressing cells increases 14-3-3σ expression through reducing CSN6-mediated poly-ubiquitination of 14-3-3σ.

A. Knockdown of COP1 expression in CSN6 overexpressing cells increases the steady-state expression of 14-3-3σ. Lysates of Myc-CSN6 overexpressing HCT116 cells transfected with increasing amounts of COP1 shRNA were immunoblotted with anti-14-3-3σ, COP1, or Actin antibodies.

B. Knockdown of COP1 expression in CSN6 overexpressing cells reduced the poly-ubiquitination of 14-3-3σ. Myc-CSN6 overexpressing HCT116 cells were co-transfected with indicated plasmids and increasing amounts of COP1 shRNA. Cells were treated with MG132 four hours before harvest. Polyubiquitinated 14-3-3σ was immunoprecipitated with anti-14-3-3σ and immunoblotted with anti-HA antibodies. Equal amounts of cell lysates were immunoblotted with anti-COP1 or Actin antibodies.

Figure 19

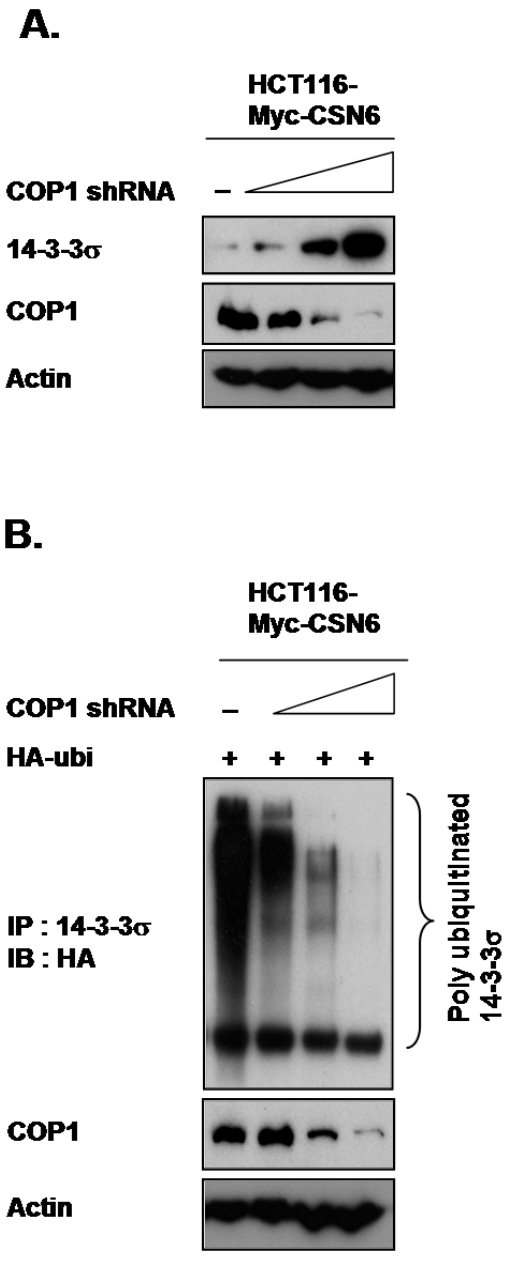
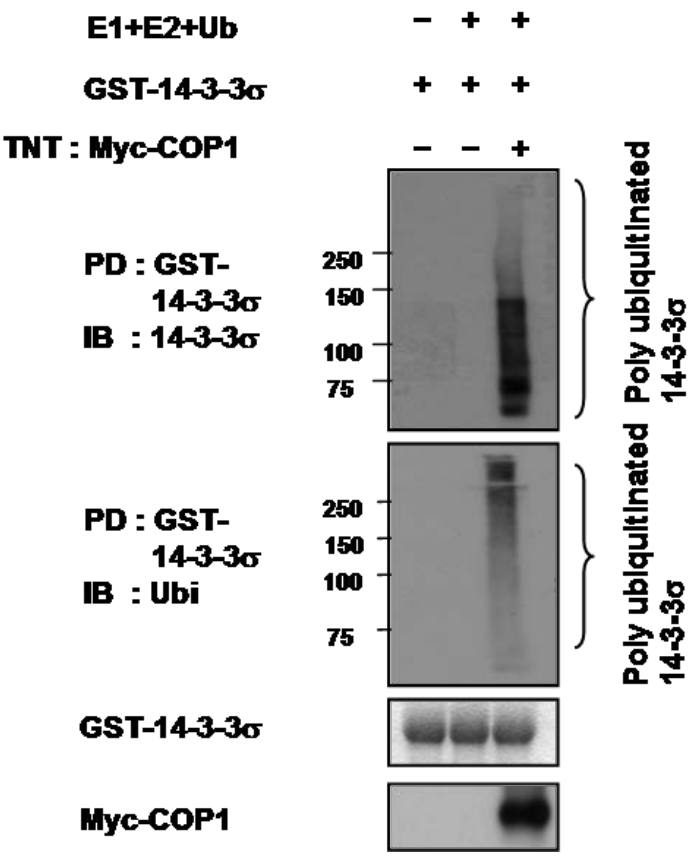

A.
HCT116-
Myc-CSN6
COP1 shRNA
–
14-3-3σ
COP1
Actin
B.
HCT116-
Myc-CSN6
COP1 shRNA
–
HA-ubi
+ + + +
IP : 14-3-3σ
IB : HA
Poly ubiquitinated
14-3-3σ
COP1
Actin

Figure 20. COP1 induces the ubiquitination of 14-3-3σ in an *in vitro* ubiquitination assay.

GST-14-3-3σ was incubated with or without Myc-COP1 which was prepared with *in vitro* translation (TNT) in the presence of E1, E2, His-Ubiquitin and ATP as indicated. Ubiquitinated 14-3-3σ was detected by immunoblotting with anti-14-3-3σ or anti-GST antibodies.

Figure 20


E1+E2+Ub
GST-14-3-3σ
TNT : Myc-COP1
PD : GST-14-3-3σ
IB : 14-3-3σ
250
150
100
75
Poly ubiquitinated 14-3-3σ
PD : GST-14-3-3σ
IB : Ubi
250
150
100
75
Poly ubiquitinated 14-3-3σ
GST-14-3-3σ
Myc-COP1

4.5. CSN6-14-3-3σ axis regulates Akt-mediated cell survival and FOXO transcriptional activity

4.5.1 CSN6 has impact on Akt activity

Akt has been implicated in the control of cell survival. For example, mice with targeted disruption of the *akt1* gene are more sensitive to apoptosis-inducing stimuli (36). Because 14-3-3σ suppresses Akt activity (31), we first examined whether knockdown of CSN6 could suppress Akt activity and inhibit Akt-mediated cell survival. To address this, we used U2OS cells infected with CSN6 shRNA virus or control virus and found that Akt activity was suppressed when CSN6 was knocked down versus control (Fig. 21A) as indicated by reduced phosphorylation of Akt at Serine 473. In order to determine whether lower Akt activity in CSN6 knockdown cells leads to apoptosis, cells were analyzed for Annexin V staining at day 4 after serum starvation. Knockdown of CSN6 demonstrated significant increases in both early apoptotic (Annexin V staining 41.9%, at right bottom quadrant) and late apoptotic cells (Annexin V staining 13.6% at right upper quadrant) compared to control cells (18.0% and 5.4%, respectively) (Fig. 21B). Cells were also co-transfected with constitutively active Akt (HA tagged-Akt) or treated with LY294002 (PI3K inhibitor). As expected, LY294002 have added the impact on shCSN6-mediated apoptosis in the absence of serum (Fig. 21B). In contrast, the exogenous HA tagged-Akt prevented the potentiation of CSN6 shRNA in serum starvation-mediated cell death (Fig. 21B), suggesting that the CSN6-Akt axis is involved in cell survival. Also, cells were serum starved and analyzed for the presence of the cleaved form of PARP, an apoptosis marker. CSN6 knockdown cells showed more PARP cleavage compared with control cells (Fig. 21C). Taken together, these results suggest that knockdown of CSN6 resulted

in enhanced apoptotic cell death when the cells were cultured in serum free medium and that loss of cell viability was likely to be due to the suppression of Akt activity.

4.5.2 CSN6 suppresses FOXO target gene expression

Akt has been reported to suppress FOXO transcriptional activity through phosphorylation (37, 38). Since CSN6 downregulates 14-3-3σ expression and thus enhances Akt activity, we next examined the impact of CSN6 on the Akt-mediated suppression of FOXO transcriptional activity. As expected, Akt activity in CSN6 overexpressing HCT116 cells was higher than in control cells (Fig. 22A). We then examined the target gene expression of FOXO transcription factors under the condition of CSN6 overexpression. In Fig. 22B we show that FOXO transcriptional targets, such as BTG, FOXO1 and BCL6, were decreased in HCT116 CSN6-overexpressing cells (Fig. 22B). Consistently, BCL-xL, which is repressed by BCL6, was upregulated in CSN6 overexpressing HCT116 cells reflecting the impact of CSN6-mediated suppression of BCL6 (Fig. 22B).

4.5.3 14-3-3σ and DN-Akt antagonizes CSN6-mediated FOXO transcriptional activity

To demonstrate the link between CSN6, 14-3-3σ, Akt and FOXO, we analyzed the functional link by employing a FOXO-responsive luciferase reporter gene assay. We found that CSN6 expression impaired FOXO transcriptional activity (Fig. 23), and this effect was reversed by increased expression of Flag-14-3-3σ or DN-Akt (Fig. 23). Taken together, overexpression of CSN6 leads to downregulation of 14-3-3σ, which in turn activates Akt kinase, resulting in the suppression of FOXO transcriptional activity.

Figure 21. CSN6 has impact on Akt activity.

A. Knockdown of CSN6 expression suppresses Akt activity. Lysates of U2OS cells infected with either CSN6 shRNA or control shRNA were immunoblotted with anti-phospho-Akt, Akt, 14-3-3σ, CSN6, or Actin antibodies.

B. Knockdown of CSN6 accelerates apoptosis induced by serum starvation. U2OS cells infected with either CSN6 shRNA or control shRNA were transfected with either empty vector or HA-Akt. Cells were cultured in 0% FBS containing either DMSO or 10 μM of LY294002 (LY) for four days. Binding of Annexin V and uptake of propidium iodide were analyzed by flow cytometry. Left panel, representative analysis of apoptotic cells. The lower left quadrant contains the viable population of cells, the lower right quadrant contains early apoptotic cells, the upper left quadrant contains necrotic cells and the upper right quadrant contains late apoptotic cells. The mean of three data sets was taken and the values shown from the corresponding quadrant (right panel).

C. Knockdown of CSN6 expression leads to PARP cleavage. U2OS cells infected with either CSN6 shRNA or control shRNA were cultured in 0% FBS for indicated times. Cell lysates were immunoblotted with anti-PARP or anti-Actin antibodies.

Figure 21

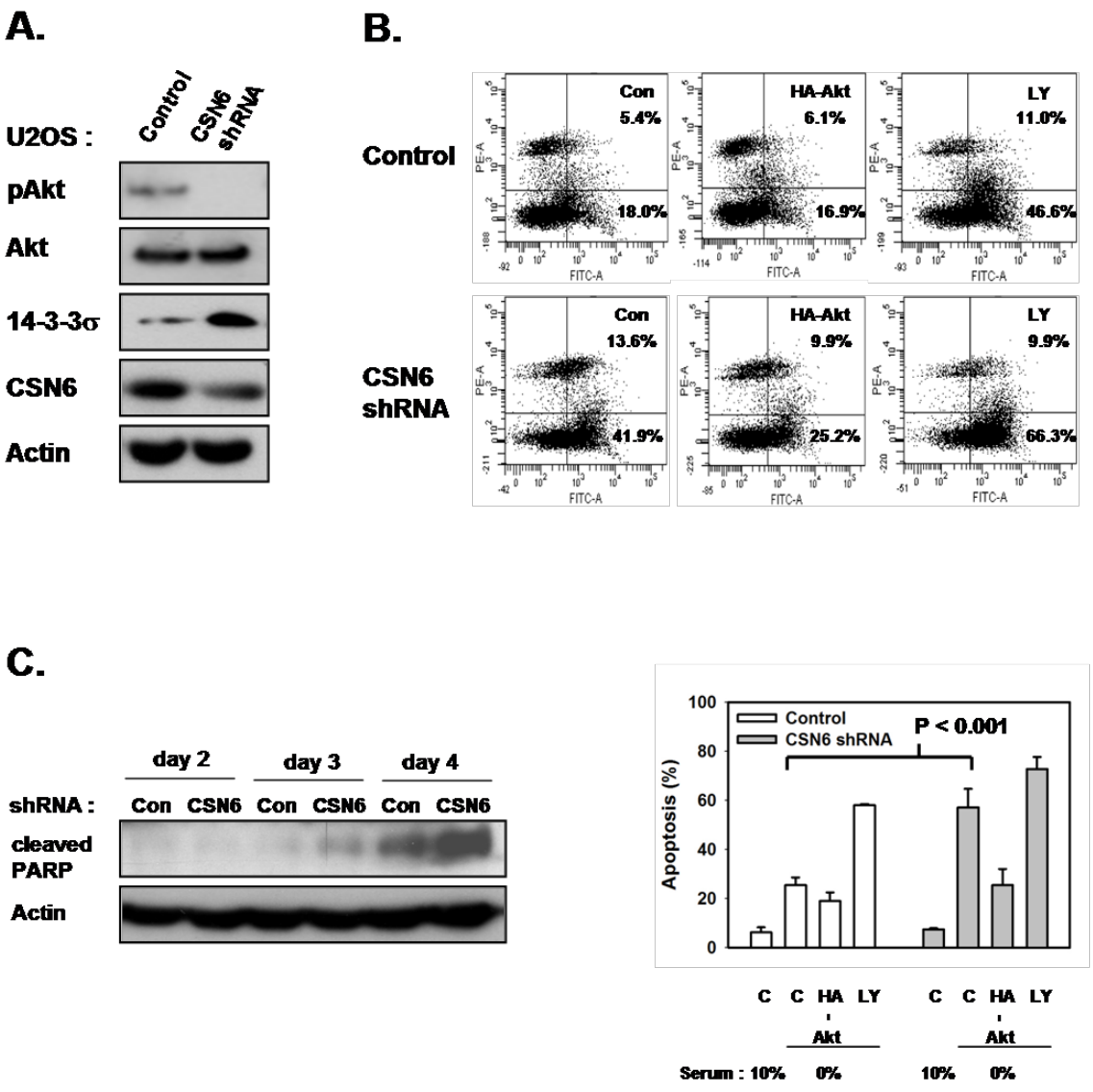
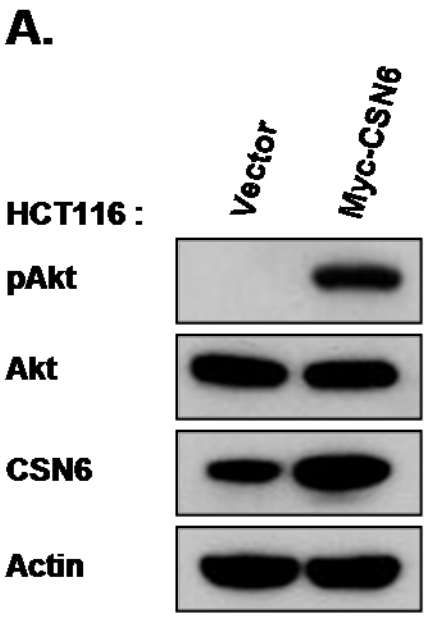

A.
U2OS :
Control
CSN6 shRNA
pAkt
Akt
14-3-3σ
CSN6
Actin
B.
Control
CSN6 shRNA
Con
5.4%
18.0%
HA-Akt
6.1%
16.9%
LY
11.0%
46.6%
Con
13.6%
41.9%
HA-Akt
9.9%
25.2%
LY
9.9%
66.3%
PE-A
FITC-A
C.
day 2
day 3
day 4
shRNA :
Con CSN6 Con CSN6 Con CSN6
cleaved PARP
Actin
Control
CSN6 shRNA
P < 0.001
Apoptosis (%)
100
80
60
40
20
0
C C HA LY C C HA LY
Akt
Akt
Serum : 10% 0% 10% 0%

Figure 22. CSN6 suppresses FOXO target gene expression.

A. Overexpression of CSN6 enhances Akt activity. Lysates of Myc-CSN6 overexpressing HCT116 stable transfectants or vector control transfectants were immunoblotted with anti-phospho-Akt, Akt, CSN6, or Actin antibodies.

B. CSN6 suppresses FOXO target gene expression. mRNA levels of the indicated FOXO target genes were determined by quantitative RT-PCR in HCT116 cells stably expressing either Myc-CSN6 or vector control.

Figure 22


A.
HCT116 :
Vector
Myc-CSN6
pAkt
Akt
CSN6
Actin

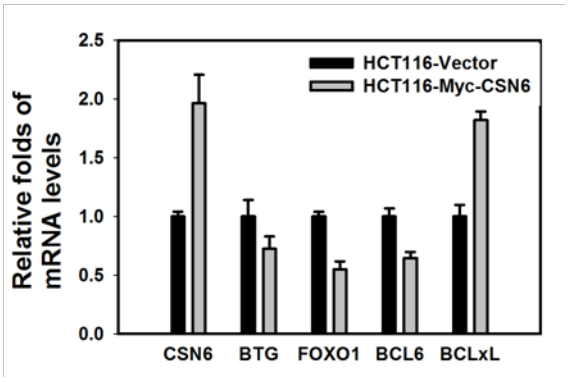
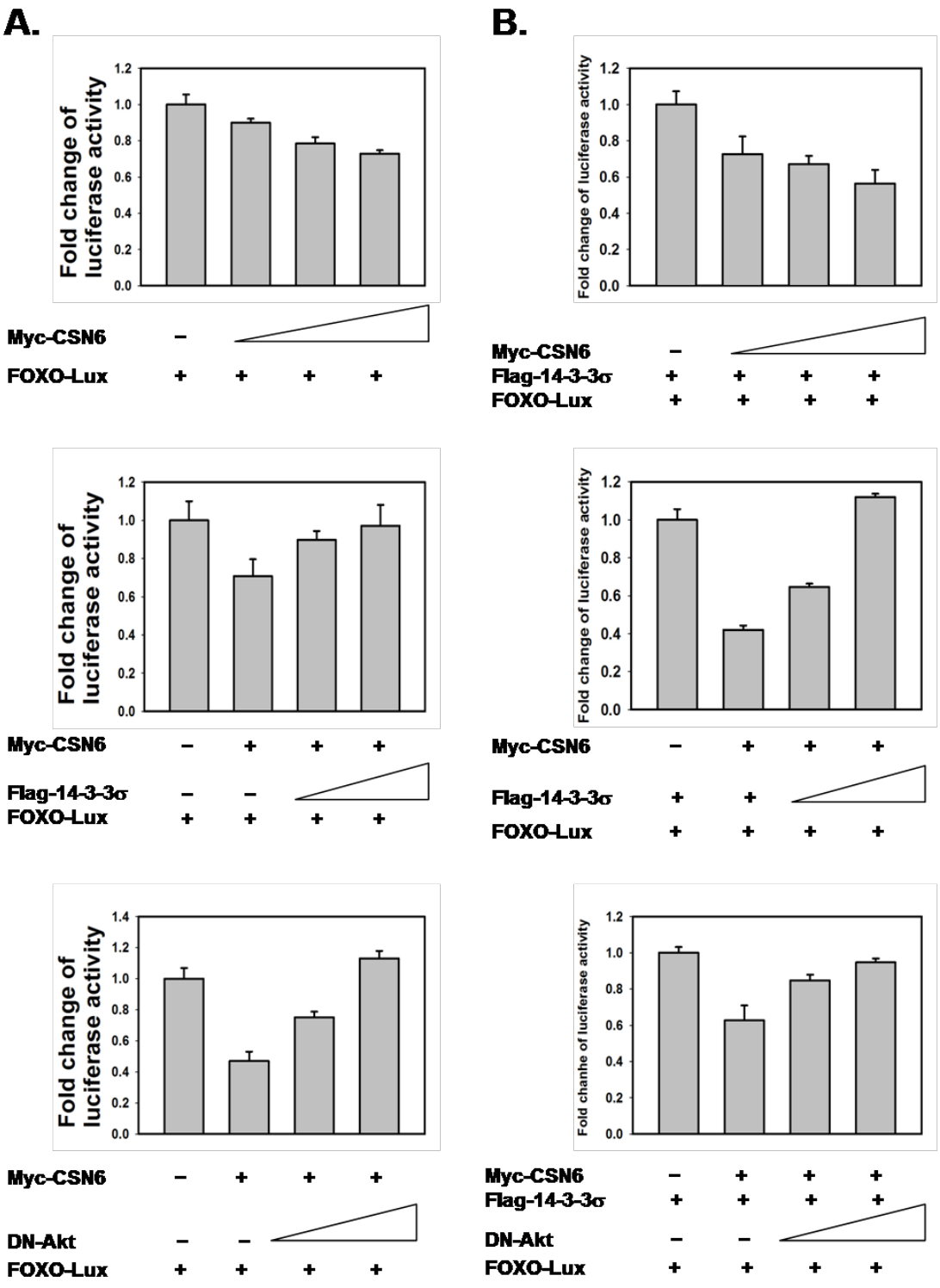

B.
Relative folds of mRNA levels
2.5
2.0
1.5
1.0
0.5
0.0
HCT116-Vector
HCT116-Myc-CSN6
CSN6
BTG
FOXO1
BCL6
BCLxL

Figure 23. 14-3-3σ and DN-Akt antagonizes CSN6-mediated FOXO transcriptional repression.

HCT116 (A) and 293T (B) cells were co-transfected with a FOXO-responsive luciferase reporter and the indicated expression vectors. Relative luciferase activity is shown as a bar graph.

Figure 23



4.6. CSN6-14-3-3σ axis regulates cell growth and tumorigenicity.

4.6.1 14-3-3σ inhibits CSN6-mediated cell proliferation

Since CSN6 downregulates and thus antagonizes the activity of 14-3-3σ, we reasoned that CSN6 would have a role in cell proliferation and anchorage-independent growth. In Fig. 24 we show that indeed CSN6 overexpression facilitates cell growth. Since CSN6 can mediate 14-3-3σ inhibition, we sought to examine the growth effect of expressing 14-3-3σ in terms of cell proliferation, foci formation, and anchorage-independence in CSN6-overexpressing cells. We found that CSN6 overexpressing HCT116 cells infected with Ad-14-3-3σ showed inhibition of cell proliferation, foci formation, and anchorage-independent growth when compared with the Ad-β-gal control (Fig. 24).

4.6.2 14-3-3σ suppresses tumorigenesis of CSN6-overexpressing cells

Our unpublished data indicate that overexpression of CSN6 promotes tumor growth (39). Because 14-3-3σ suppresses CSN6-mediated cell proliferation and anchorage-independent growth, we next examined the impact of 14-3-3σ on CSN6-mediated tumor promotion. We observed that xenografted tumor volume was significantly decreased in Ad-14-3-3σ-treated mice compared with Ad-β-gal-treated mice (Fig. 25A). Further, the average excised tumor weight per mouse in the Ad-β-gal-treated group (507 mg) was higher than in the Ad-14-3-3σ-treated group (310 mg) (Fig. 25B). Tumors obtained from this study were fixed, embedded in paraffin, and sections were examined. Immunohistochemistry staining indicated that cell proliferation marker, Ki67, was reduced in tumors from Ad-14-3-3σ-treated mice when compared with the control group, while the signal intensity of apoptotic marker, cleaved Caspase 3, was increased in the Ad-14-3-3σ-treated group when compared with the Ad-β-gal-treated group (Fig. 25D). Together, these data illustrate that the COP9 signalosome subunit 6-14-3-3σ axis is deregulated when CSN6 is overexpressed, and this promotes cell growth and tumorigenicity.

Significantly, 14-3-3σ expression can correct the abnormalities mediated by CSN6 expression.

Figure 24. 14-3-3σ inhibits CSN6-mediated cell proliferation.

A. Myc-CSN6 overexpressing HCT116 stable transfectants and vector control transfectants were plated in 96-well plates and the number of live cells estimated by MTT assay every day for a total of 3 days (left, top panel). Results are expressed as OD570. Myc-CSN6 overexpressing HCT116 cells were infected with Ad-β-gal or Ad-HA-14-3-3σ (leftbottom panel). Error bars represent 95% confidence intervals. 14-3-3σ antagonizes CSN6-mediated foci formation.

B. Myc-CSN6 overexpressing HCT116 stable transfectants and vector control transfectants were analyzed for foci formation (Middle, top). Myc-CSN6 overexpressing HCT116 cells infected with Ad-β-gal or Ad-HA-14-3-3σ were plated at low density and allowed to form foci (middle, bottom).

C. Myc-CSN6 overexpressing HCT116 stable transfectants, vector control transfectants or Myc-CSN6 overexpressing HCT116 cells infected with Ad-β-gal or Ad-HA-14-3-3σ were analyzed for soft agar colony formation (right, top). Average numbers of colonies per field were scored. Error bars represent 95% confidence intervals.

Figure 24

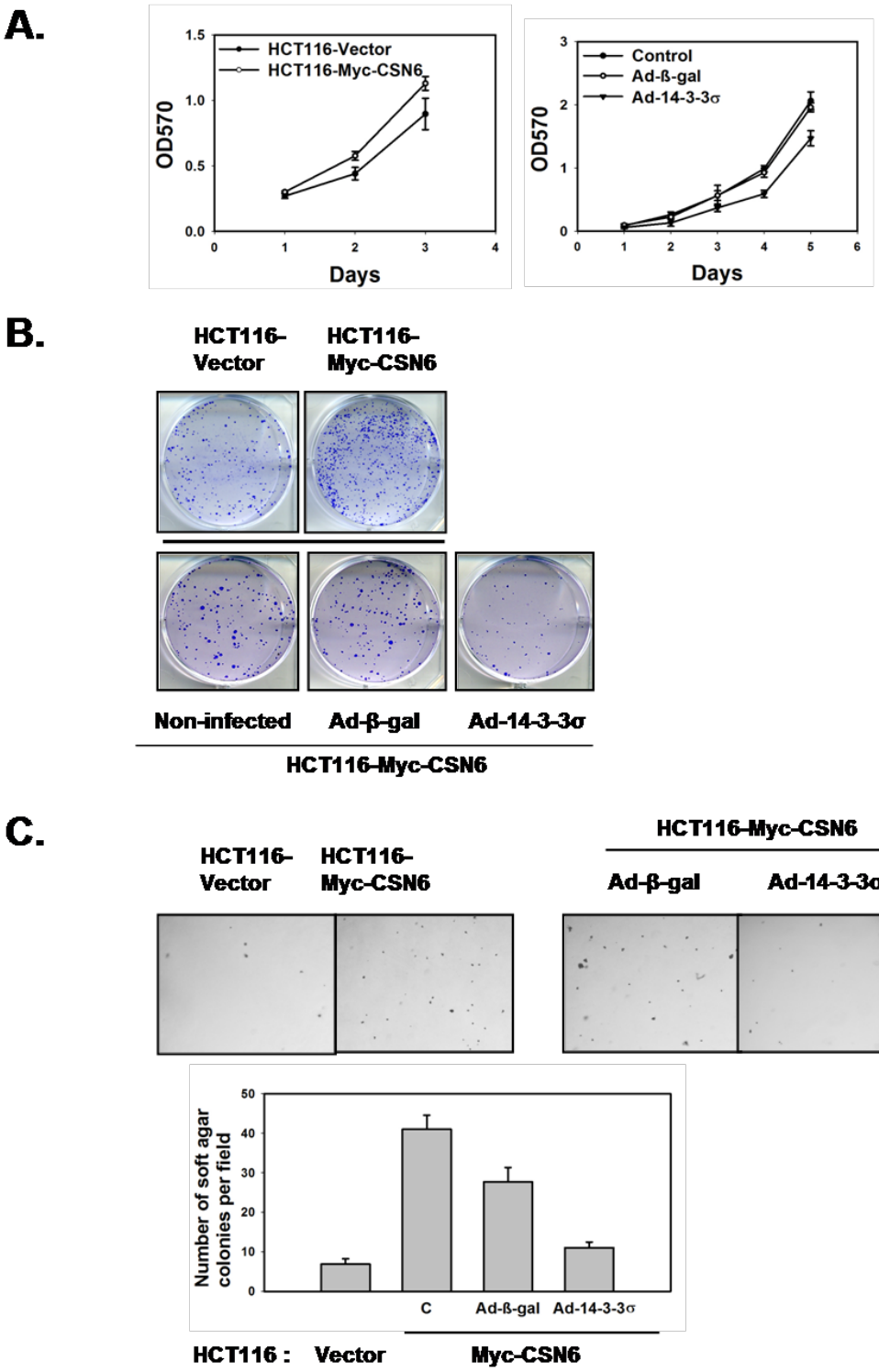


Figure 25. 14-3-3σ suppresses tumorigenesis of CSN6-overexpressing cells.

A. Myc-CSN6 overexpressing HCT116 stable transfectants were infected with Ad-14-3-3σ or Ad-β-gal. Cells were harvested and subcutaneously injected into the flank of female nude mice. Tumor volumes were monitored for 31 days. Tumor growth curves are shown (left); error bars represent 95% confidence intervals.

B. C. Tumors were exised at the end of the study and tumor weights from each group measured (B). Error bars represent 95% confidence intervals. Representative tumors from each group (C).

D. Tumor sections were stained with anti-Ki67 and anti-cleaved Caspase 3. Percentage positive signal is plotted as a bar graph. Error bars represent 95% confidence intervals.

Figure 25

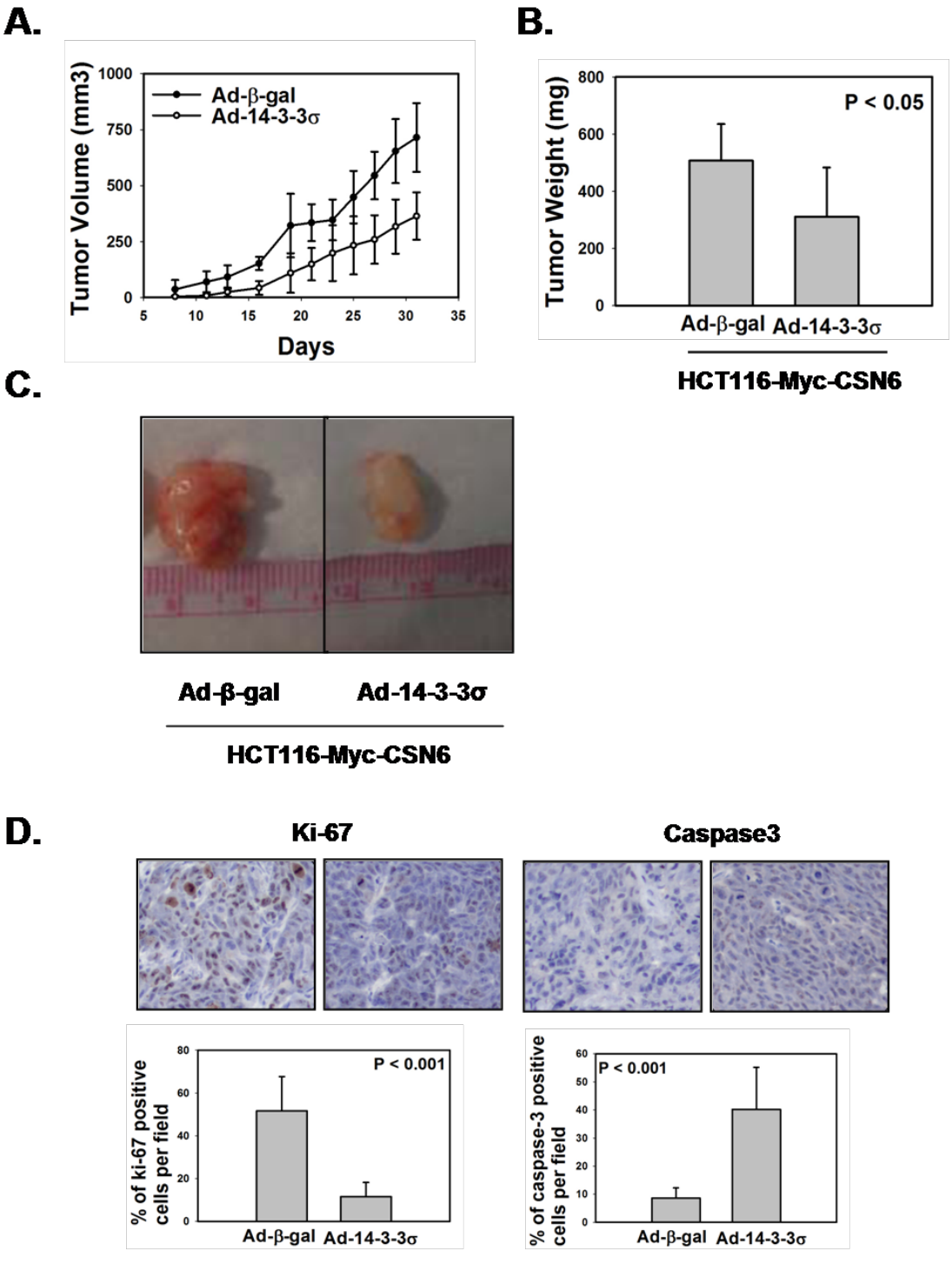
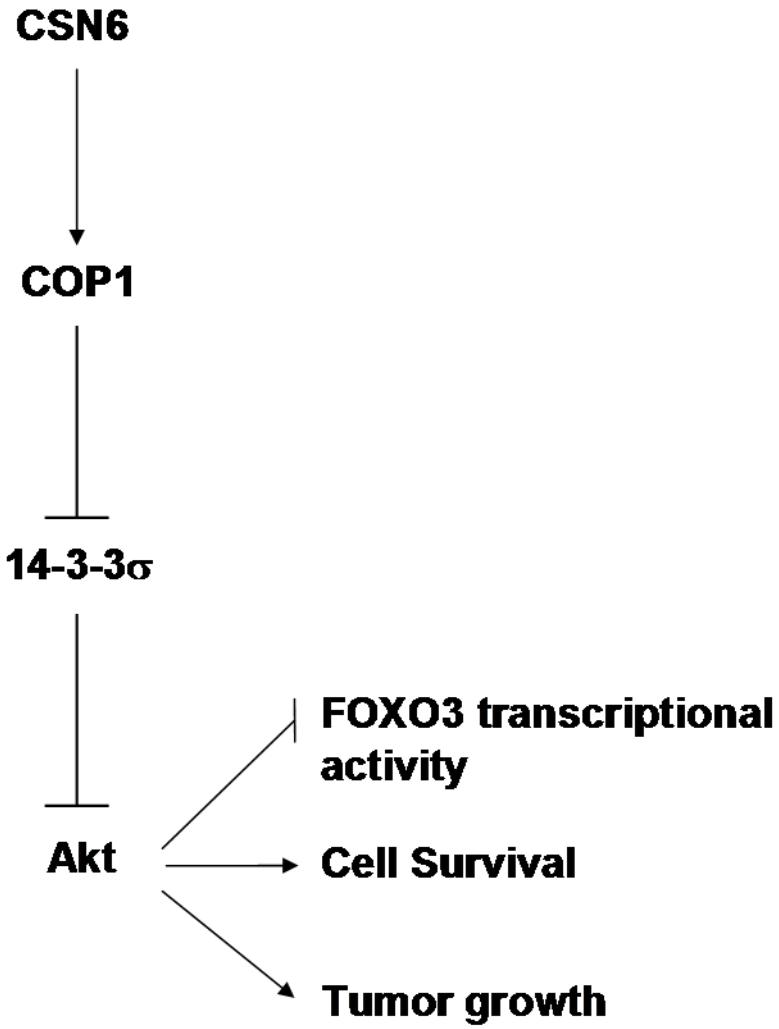


Figure 26. Model of the impact of the CSN6-COP1 axis in regulating 14-3-3σ

signaling.

Figure 26


CSN6
COP1
14-3-3σ
Akt
FOXO3 transcriptional activity
Cell Survival
Tumor growth

In mammalian cells, COP1 regulates various cellular targets, including stress-responsive transcription factors, p53 tumor suppressor (13), c-JUN (14-16), acetyl-coA carboxylase (17), TORC2 (18), MVP (40) and nucleosome remodeling factor MTA1 (19), suggesting its versatile functions. Many COP1 associated proteins remain to be characterized. In this study, our results indicate that COP1 binds and is a novel E3 ligase for 14-3-3σ.

The known proteins interacting with 14-3-3σ, include Cdk's (20, 41), p53 (26) and Efp (estrogen inducible finger protein) (42), and all play important roles in tumorigenesis. We find here that CSN6 is a new 14-3-3σ-associating protein (Fig. 1) and is important in promoting cancer growth (Fig. 25) through its binding and degradation of 14-3-3σ (Fig. 8). We further determined that CSN6 regulates 14-3-3σ posttransccriptionally by enhancing 14-3-3σ ubiquitination. CSN6 does not have the conserved RING or HECT domains found in well-characterized E3 ligases. Therefore, at issue is how CSN6 causes 14-3-3σ downregulation. Thus we searched for potential ligases that could associate with the COP9 signalosome and found that the COP1 E3 ligase is a potential candidate. It is wothwhile to point out that as an E3 ligase for p53, COP1 drives the ubiquitination and proteasomal degradation of p53, thereby maintaining low steady-state levels of p53 in unstressed cells (43). In this way, COP1 may antagonize the activity of positive p53 regulators such as 14-3-3σ, which can stabilize p53 by reducing p53 ubiquitination level. Also, COP1 is downregulated in response to DNA damage (44), while 14-3-3σ protein level is elevated by DNA damage (25). Together, it is conceivable that COP1 and 14-3-3σ may have a functional relationship. Indeed, we observed for the first time that CSN6 recruits RING containing COP1 to degrade 14-3-3σ. This is based on the observation that

CSN6-mediated 14-3-3σ degradation is compromised when COP1 is knocked down with shRNA. Importantly, CSN6 not only associates with COP1 but also prevents COP1's self-ubiquitination, adding yet another layer of regulation.

Although Efp, another RING containing protein, was previously characterized as an E3 ligase for 14-3-3σ (42), it remains to be studied whether CSN6 also recruits Efp to degrade 14-3-3σ. Interestingly, Efp has another activity and seems to serve as an E3 ligase to add ISG15 (15 Kd protein encoded by an interferon stimulated gene) for 14-3-3σ-ISGylation (33, 45), but the role of this type of modification in terms of 14-3-3σ protein degradation remains unclear. We previously showed that 14-3-3σ is a negative regulator of Akt (31). Consistent with this, we found that CSN6 can downregulate the expression of 14-3-3σ and lead to Akt activation. The observation that CSN6 increases the activity of Akt (Fig. 21) is very intriguing. This is the first discovery that links the COP9 signalosome with Akt activation. We have shown that CSN6 is involved in 14-3-3σ-mediated Akt inhibition, which in turn antagonizes FOXO-meditated transcriptional activity for pro-apoptotic genes (Fig. 22, 23). Our mechanistic studies of CSN6-mediated 14-3-3σ downregulation explains how CSN6 can activate Akt in our proposed model (Fig. 26). This study shows that CSN6 positively regulates Akt and promotes Akt-mediated cell survival to prevent apoptosis. Clearly, the CSN6-Akt link will be an important molecular target for rational cancer therapy. Our primary breast cancer studies showed that CSN6 is amplified in 75% of primary breast cancers (39). Also, COP1 and 14-3-3σ inversely correlate in primary pancreatic and breast cancer (46). Together, it demonstrates that the CSN6-COP1-14-3-3σ-axis is deregulated in cancer. Here, we show that adenoviral gene delivery of 14-3-3σ can inhibit tumorigenicity mediated by CSN6 activity in cancer models (Fig. 25). Also, similar results were observed in that 14-3-3σ can compromise

tumorigenicity mediated by COP1 activity in xenograft cancer models (46). In conclusion, since both CSN6 and COP1 are involved in degrading 14-3-3σ, promoting cell survival and increasing tumorigenicity, targeting the CSN6-COP1 axis may be a useful therapeutic strategy for cancer intervention.

CHAPTER 6. BIBLIOGRAPHY

1. Hoeller D, Hecker CM, & Dikic I (2006) Ubiquitin and ubiquitin-like proteins in cancer pathogenesis. *Nat Rev Cancer* 6(10):776-788.

2. Chamovitz DA, Wei N, Osterlund MT, von Arnim AG, Staub JM, Matsui M, Deng XW (1996) The COP9 complex, a novel multisubunit nuclear regulator involved in light control of a plant developmental switch. (Translated from eng) *Cell* 86(1):115-121 (in eng).

3. Wei N & Deng XW (1992) COP9: a new genetic locus involved in light-regulated development and gene expression in arabidopsis. *Plant Cell* 4(12):1507-1518.

4. Karniol B & Chamovitz DA (2000) The COP9 signalosome: from light signaling to general developmental regulation and back. *Curr Opin Plant Biol* 3(5):387-393.

5. Li L & Deng XW (2003) The COP9 signalosome: an alternative lid for the 26S proteasome? *Trends Cell Biol* 13(10):507-509.

6. Peng Z, Serino G, & Deng XW (2001) Molecular characterization of subunit 6 of the COP9 signalosome and its role in multifaceted developmental processes in Arabidopsis. *Plant Cell* 13(11):2393-2407.

7. Gusmaroli G, Figueroa P, Serino G, & Deng XW (2007) Role of the MPN subunits in COP9 signalosome assembly and activity, and their regulatory interaction with Arabidopsis Cullin3-based E3 ligases. *Plant Cell* 19(2):564-581.

8. Wang H, Ma LG, Li JM, Zhao HY, & Deng XW (2001) Direct interaction of Arabidopsis cryptochromes with COP1 in light control development. (Translated from eng) *Science (New York, N.Y* 294(5540):154-158 (in eng).

9. Cope GA, Suh GS, Aravind L, Schwarz SE, Zipursky SL, Koonin EV, Deshaies RJ (2002) Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. *Science (New York, N.Y* 298(5593):608-611. Epub 2002 Aug 2015.

10. Yi C & Deng XW (2005) COP1 - from plant photomorphogenesis to mammalian

tumorigenesis. (Translated from eng) *Trends Cell Biol* 15(11):618-625 (in eng).

11. von Arnim AG & Deng XW (1994) Light inactivation of Arabidopsis photomorphogenic repressor COP1 involves a cell-specific regulation of its nucleocytoplasmic partitioning. *Cell* 79(6):1035-1045.

12. Hardtke CS, Gohda K, Osterlund MT, Oyama T, Okada K, Deng XW (2000) HY5 stability and activity in arabidopsis is regulated by phosphorylation in its COP1 binding domain. *Embo J* 19(18):4997-5006.

13. Dornan D, Bheddah S, Newton K, Ince W, Frantz GD, Dowd P, Koeppen H, Dixit VM, French DM (2004) COP1, the negative regulator of p53, is overexpressed in breast and ovarian adenocarcinomas. *Cancer Res* 64(20):7226-7230.

14. Wertz IE, O'Rourke KM, Zhang Z, Dornan D, Arnott D, Deshaies RJ, Dixit VM (2004) Human De-etiolated-1 regulates c-Jun by assembling a CUL4A ubiquitin ligase. *Science (New York, N.Y* 303(5662):1371-1374.

15. Savio MG, Rotondo G, Maglie S, Rossetti G, Bender JR, Pardi R (2008) COP1D, an alternatively spliced constitutive photomorphogenic-1 (COP1) product, stabilizes UV stress-induced c-Jun through inhibition of full-length COP1. *Oncogene* 27(17):2401-2411.

16. Bianchi E, Denti S, Catena R, Rossetti G, Polo S, Gasparian S, Putignano S, Rogge L, Pardi R (2003) Characterization of human constitutive photomorphogenesis protein 1, a RING finger ubiquitin ligase that interacts with Jun transcription factors and modulates their transcriptional activity. *J Biol Chem* 278(22):19682-19690.

17. Qi L, Heredia JE, Altarejos JY, Screaton R, Goebel N, Niessen S, Macleod IX, Liew CW, Kulkarni RN, Bain J, Newgard C, Nelson M, Evans RM, Yates J, Montminy M (2006) TRB3 links the E3 ubiquitin ligase COP1 to lipid metabolism. (Translated from eng) *Science (New York, N.Y* 312(5781):1763-1766 (in eng).

18. Dentin R, Liu Y, Koo SH, Hedrick S, Vargas T, Heredia J, Yates J 3rd, Montminy M (2007)

Insulin modulates gluconeogenesis by inhibition of the coactivator TORC2. *Nature* 449(7160):366-369.

19. Li DQ, Ohshiro K, Reddy SD, Pakala SB, Lee MH, Zhang Y, Rayala SK, Kumar R (2009) E3 ubiquitin ligase COP1 regulates the stability and functions of MTA1. *Proc Natl Acad Sci U S A* 106(41):17493-17498.

20. Laronga C, Yang HY, Neal C, & Lee MH (2000) Association of the cyclin-dependent kinases and 14-3-3 sigma negatively regulates cell cycle progression. *J Biol Chem* 275(30):23106-23112.

21. Fu H, Subramanian RR, & Masters SC (2000) 14-3-3 proteins: structure, function, and regulation. *Annu Rev Pharmacol Toxicol* 40:617-647.

22. Yaffe MB, Rittinger K, Volinia S, Caron PR, Aitken A, Leffers H, Gamblin SJ, Smerdon SJ, Cantley LC (1997) The structural basis for 14-3-3:phosphopeptide binding specificity. *Cell* 91(7):961-971.

23. Michaud NR, Fabian JR, Mathes KD, & Morrison DK (1995) 14-3-3 is not essential for Raf-1 function: identification of Raf-1 proteins that are biologically activated in a 14-3-3- and Ras-independent manner. *Mol Cell Biol* 15(6):3390-3397.

24. Prasad GL, Valverius EM, McDuffie E, & Cooper HL (1992) Complementary DNA cloning of a novel epithelial cell marker protein, HME1, that may be down-regulated in neoplastic mammary cells. *Cell Growth Differ* 3(8):507-513.

25. Hermeking H, Lengauer C, Polyak K, He TC, Zhang L, Thiagalingam S, Kinzler KW, Vogelstein B (1997) 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Mol Cell* 1(1):3-11.

26. Yang HY, Wen YY, Chen CH, Lozano G, & Lee MH (2003) 14-3-3 sigma positively regulates p53 and suppresses tumor growth. *Mol Cell Biol* 23(20):7096-7107.

27. Yang HY, Wen YY, Lin YI, Pham L, Su CH, Yang H, Chen J, Lee MH (2007) Roles for

negative cell regulator 14-3-3sigma in control of MDM2 activities. *Oncogene* 26(52):7355-7362.

28. Lee MH & Lozano G (2006) Regulation of the p53-MDM2 pathway by 14-3-3 sigma and other proteins. *Semin Cancer Biol* 16(3):225-234.

29. Verdoodt B, Benzinger A, Popowicz GM, Holak TA, & Hermeking H (2006) Characterization of 14-3-3sigma dimerization determinants: requirement of homodimerization for inhibition of cell proliferation. *Cell Cycle* 5(24):2920-2926.

30. Li Z, Liu JY, & Zhang JT (2009) 14-3-3sigma, the double-edged sword of human cancers. *Am J Transl Res* 1(4):326-340.

31. Yang H, Wen YY, Zhao R, Lin YL, Fournier K, Yang HY, Qiu Y, Diaz J, Laronga C, Lee MH (2006) DNA damage-induced protein 14-3-3 sigma inhibits protein kinase B/Akt activation and suppresses Akt-activated cancer. *Cancer Res* 66(6):3096-3105.

32. Ferguson AT, Evron E, Umbricht CB, Pandita TK, Chan TA, Hermeking H, Marks JR, Lambers AR, Futreal PA, Stampfer MR, Sukumar S (2000) High frequency of hypermethylation at the 14-3-3 sigma locus leads to gene silencing in breast cancer. (Translated from eng) *Proc Natl Acad Sci U S A* 97(11):6049-6054 (in eng).

33. Horie-Inoue K & Inoue S (2006) Epigenetic and proteolytic inactivation of 14-3-3sigma in breast and prostate cancers. (Translated from eng) *Semin Cancer Biol* 16(3):235-239 (in eng).

34. Richardson KS & Zundel W (2005) The emerging role of the COP9 signalosome in cancer. (Translated from eng) *Mol Cancer Res* 3(12):645-653 (in eng).

35. Wei N & Deng XW (2003) The COP9 signalosome. (Translated from eng) *Annu Rev Cell Dev Biol* 19:261-286 (in eng).

36. Chen WS, Xu PZ, Gottlob K, Chen ML, Sokol K, Shiyanova T, Roninson I, Weng W, Suzuki R, Tobe K, Kadowaki T, Hay N (2001) Growth retardation and increased apoptosis

in mice with homozygous disruption of the Akt1 gene. (Translated from eng) *Genes Dev* 15(17):2203-2208 (in eng).

37. Kops GJ, de Ruiter ND, De Vries-Smits AM, Powell DR, Bos JL, Burgering BM (1999) Direct control of the Forkhead transcription factor AFX by protein kinase B. (Translated from eng) *Nature* 398(6728):630-634 (in eng).

38. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. (Translated from eng) *Cell* 96(6):857-868 (in eng).

39. Zhao R, J Yeung SC, Chen J, Iwakuma T, Su CH, Chen B, Qu C, Zhang F, Chen YT, Lin YL, Lee DF, Jin F, Zhu R, Shaikenov T, Sarbassov D, Sahin A, Wang H, Wang H, Lai CC, Tsai FJ, Lozano G, Lee MH (2010) Impact of COP9 signalosome on MDM2-p53 axis in DNA damage-mediated apoptosis and tumorigenesis. *Journal of Clinical Investigation* in revision.

40. Yi C, Li S, Chen X, Wiemer EA, Wang J, Wei N, Deng XW (2005) Major vault protein, in concert with constitutively photomorphogenic 1, negatively regulates c-Jun-mediated activator protein 1 transcription in mammalian cells. (Translated from eng) *Cancer Res* 65(13):5835-5840 (in eng).

41. Chan TA, Hermeking H, Lengauer C, Kinzler KW, & Vogelstein B (1999) 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. *Nature* 401(6753):616-620.

42. Urano T, Saito T, Tsukui T, Fujita M, Hosoi T, Muramatsu M, Ouchi Y, Inoue S (2002) Efp targets 14-3-3 sigma for proteolysis and promotes breast tumour growth. (Translated from eng) *Nature* 417(6891):871-875 (in eng).

43. Dornan D, Wertz I, Shimizu H, Arnott D, Frantz GD, Dowd P, O'Rourke K, Koeppen H, Dixit VM (2004) The ubiquitin ligase COP1 is a critical negative regulator of p53. *Nature*

429(6987):86-92.

44. Dornan D, Shimizu H, Mah A, Dudhela T, Eby M, O'rourke K, Seshagiri S, Dixit VM (2006) ATM engages autodegradation of the E3 ubiquitin ligase COP1 after DNA damage. *Science (New York, N.Y* 313(5790):1122-1126.

45. Zou W & Zhang DE (2006) The interferon-inducible ubiquitin-protein isopeptide ligase (E3) EFP also functions as an ISG15 E3 ligase. (Translated from eng) *J Biol Chem* 281(7):3989-3994 (in eng).

46. Su CH, Zhao R, Zhang F, Qu C, Chen B, Feng YH, Phan L, Chen J, Wang H, Wang H, Yeung SC, Lee MH (2010) 14-3-3σ has tumor suppressing activity via regulating COP1 stability *Cancer Research* submitted.

47. Yang H, Zhao R, Yang HY, & Lee MH (2005) Constitutively active FOXO4 inhibits Akt activity, regulates p27 Kip1 stability, and suppresses HER2-mediated tumorigenicity. *Oncogene* 24(11):1924-1935.

CHAPTER 7. VITA

PERSONAL DATA

Name : Hyun Ho Choi

Date of Birth: Feb. 28, 1973

Citizenship: Korea

Marital Status: Married

Mailing Address : 2250 Holly Hall St. Apt. # 269

Houston, TX 77054

e-mail : hhchoi0228@yahoo.com

hyun.ho.choi@uth.tmc.edu

EDUCATION

Year	Degree	Major	Institution
1995-1997	Master	Food Technology	Korea University
1991-1995	Bachelor	Food Engineering	Korea University

RESEARCH AND PROFESSIONAL EXPERIENCE

2005 – Present : Graduate Student. Cancer Biology. Univ. of Texas Health Science Center, Houston, TX

1998 - 2002 : Researcher. Cancer Research Institute, Seoul National University College of Medicine, Seoul, Korea

1996 – 1998 : Research Institute of Crown Confectionery Corp., Seoul, Korea

1996: Teaching Assistant : Department of Food Technology, Korea Univ.

Master's Thesis : Studies on the blood anticoagulant protein- bound polysaccharide isolated from *Gyrophora-esculenta*

RESEARCH PUBLICATIONS

12. **Hyun Ho Choi**, Christopher Gully, Chun-Hui Su, Guermarie Velaszquez-Torres, Ping-Chieh Chou, Chieh Tseng, Ruiying Zhao, Liem Phan, Tattym Shaikenov, Jian Chen, Sai-Ching Yeung, Mong-Hong Lee. COP 9 Signalosome Subunit 6 Stabilizes COP1, which functions as an E3 Ubiquitin Ligase for 14-3-3σ. *Oncogene, in press*

11. Choi S, **Choi HH**, Lee SH, Ko SH, You HJ, Ye SK, Chung MH. Anti-inflammatory effects of 8-hydroxy-2'-deoxyguanosine on lipopolysaccharide-induced inflammation via Rac suppression in Balb/c mice. *Free Radic Biol Med.* 2007 Dec 15;43(12):1594-603.

10. Hsu EL, Yoon D, **Choi HH**, Wang F, Taylor RT, Chen N, Zhang R, Hankinson O. A proposed mechanism for the protective effect of dioxin against breast cancer. *Toxicol Sci.* 2007 Aug;98(2):436-44.

9. Choi S, **Choi HH**, Choi J, Hyun J, Yoon B, Yeh S, Yoon S, Kim J, Yoo J, Chung M. Inhibitory effect of 8-oxo-7,8-dihydro-2'-deoxyguanosine on the growth of KG-1 myelosarcoma in Balb/c nude mice. *Leuk Res*. 2006 Nov;30(11):1425-36.

8. Yim HW, Jong HS, Kim TY, **Choi HH**, Kim SG, Song SH, Kim J, Ko SG, Lee JW, Kim TY, Bang YJ. Cyclooxygenase-2 inhibits novel ginseng metabolite-mediated apoptosis. *Cancer Res.* 2005 Mar 1;65(5):1952-60.

7. Rivera SP, **Choi HH**, Chapman B, Whitekus MJ, Terao M, Garattini E, Hankinson O. Identification of aldehyde oxidase 1 and aldehyde oxidase homologue 1 as dioxin-inducible genes. *Toxicology*. 2005 Feb 28;207(3):401-9

6. **Choi HH**, Jong HS, Park JH, Choi S, Lee JW, Kim TY, Otsuki T, Namba M, Bang YJ. A novel ginseng saponin metabolite induces apoptosis and down-regulates fibroblast growth factor receptor 3 in myeloma cells. *Int J Oncol*. 2003 Oct;23(4):1087-93.

5. **Choi HH**, Jong HS, Hyun Song S, You Kim T, Kyeong Kim N, and Bang YJ. p130 mediates TGF-beta-induced cell-cycle arrest in Rb mutant HT-3 cells. *Gynecol Oncol*. 2002 Aug;86(2):184-9.

4. Song SH, Jong HS, **Choi HH**, Inoue H, Tanabe T, Kim NK, and Bang YJ. Transcriptional silencing of Cyclooxygenase-2 by hyper-methylation of the 5' CpG island in human gastric carcinoma cells. *Cancer Res*. 2001 Jun 1;61(11):4628-35

3. Song SH, Jong HS, **Choi HH**, Kang SH, Ryu MH, Kim NK, Kim WH, and Bang YJ. Methylation of specific CpG sites in the promoter region could significantly down-regulate p16(INK4a) expression in gastric adenocarcinoma. *Int J Cancer*. 2000 Jul 15;87(2):236-40.

2. Kang SH, **Choi HH**, Kim SG, Jong HS, Kim NK, Kim SJ, and Bang YJ. Transcriptional inactivation of the tissue inhibitor of metalloproteinase-3 gene by dna hypermethylation of the 5'-CpG island in human gastric cancer cell lines. *Int J Cancer* 2000 Jun 1;86(5):632-5

1. Kweon M.-H., **Choi H.-H**., and Yang H.-C. An anticoagulant polysaccharide isolated from the alkali extracts of Gyrophora esculenta. J. Indus. & Tech. Sci. 1: 81-93 (1998)

RESEARCH PRESENTATION

1. **Choi HH**. Role of COP9 Signalosome Subunit (CSN6) in the Regulation of Tumor Suppressor 14-3-3σ. UT GSBS Cancer Biology Program retreat. Feb. 19-20, 2010.

2. **Choi HH**, Jong HS, Song SH, and Bang YJ. p130 Mediates TGF-beta induced Cell-Cycle Arrest in Cervical Carcinoma Cells. Korean Cancer Association (Oral Presentation). June, 07, 2001.

3. **Choi HH**, Jong HS, Song SH, and Bang YJ. TGF-beta mediated cell cycle arrest is induced by hypophosphorylation of p130, in consistent with inactivation of CDK2:Cyc E in Rb mutant HT-3 cells. American Association for Cancer Research (# 902. Poster Session). March 25, 2001

4. **Choi HH**, Kwon MH, and Yang HC. Studies on the blood anticoagulant protein-bound polysaccharide isolated from *Gyrophora esculenta.* The Korean Society of Agricultural Chemical and Biotech (Poster presentation). October, 1996.

5. **Choi HH**, Kwon MH, and Yang HC. Purification of the blood anticoagulant from *Gyrophora esculenta.* The Korean Society of Agricultural Chemical and Biotech (Poster presentation). May, 1996.