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COP 9 Signalosome Subunit 6 Stabilizes COP1, which Functions as an E3 Ubiquitin Ligase for 14-3-3σ

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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 mediators 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. 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.

Keywords

14-3-3σ; CSN6; COP1; ubiquitination

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Introduction

The ubiquitin-proteasome pathway is important for controlling the abundance of proteins and plays an essential role in maintaining normal cellular function. Dysregulation of ubiquitin-mediated proteolysis results in the development of a variety of human cancers (Hoeller *et al.*, 2006). 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 (Chamovitz *et al.*, 1996; Karniol and Chamovitz, 2000; Wei and Deng, 1992). Mammalian COP9 signalosome contains eight subunits (CSN1–CSN8), which share sequence homology with subunits of the 'lid' complex of the 26S proteasome (Li and Deng, 2003). 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 (Gusmaroli *et al.*, 2007; Peng *et al.*, 2001), which will have impact on Cullin-Ring Ligases (CRL) (Cope *et al.*, 2002; Wang *et al.*, 2001). CSN5 has an MPN domain containing a JAMM (JAB1/MPN/Mov34) or MPN+ motif linked to the metalloprotease motif (EXnHXHX10D) that can deneddylate Cullins (Cope *et al.*, 2002), a process important for regulating CRL activity. By comparison, CSN6 is not recognized to have a JAMM motif and thus its function remains obscure. Recently, CSN6 was characterized as a positive regulator of MDM2 (Zhao *et al.*, 2011).

Mammalian Constitutive Photomorphogenic 1 (COP1) is an evolutionarily conserved E3 ubiquitin ligase which contains RING-finger, coiled-coil and WD40-repeat domains (Yi and Deng, 2005). 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 (Hardtke *et al.*, 2000; von Arnim and Deng, 1994). In mammalian cells, the ubiquitinated targets of COP1 include stress-responsive transcription factors p53 (Dornan *et al.*, 2004a; Su *et al.*, 2010), suggesting its role in cancer. COP1 is overexpressed in cancers (Su *et al.*, 2011), but whether COP1 targets any tumor suppressor protein for degradation during tumorigenesis remains elusive.

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 (Fu *et al.*, 2000; Laronga *et al.*, 2000; Yaffe *et al.*, 1997). Binding by 14-3-3 proteins mediates stability and/or subcellular localization of target proteins (Michaud *et al.*, 1995). 14-3-3σ was originally characterized as a human mammary epithelial-specific marker (HME1) (Prasad *et al.*, 1992), and was later found to be an essential regulator of apoptosis, cell migration, cell cycle (Hermeking *et al.*, 1997; Laronga *et al.*, 2000) and DNA damage response involving p53 and MDM2 (Lee and Lozano, 2006; Yang *et al.*, 2003; Yang *et al.*, 2007). 14-3-3σ, but not other family members, has been found to be frequently lost or decreased in various human cancers (Lee and Lozano, 2006; Li *et al.*, 2009b) 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 (Hermeking *et al.*, 1997; Lee and Lozano, 2006). 14-3-3σ also acts as a negative regulator of Akt (Yang *et al.*, 2006). 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 (Ferguson *et al.*, 2000). Posttranscriptional regulation such as ubiquitination also regulates 14-3-3σ (Horie-Inoue and Inoue, 2006). 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 demonstrated the role of the CSN6-COP1 axis in regulating 14-3-3σ

stability. Our studies indicate the applicability of employing CSN6-COP1 axis as a therapeutic intervention target in cancer.

Results

CSN6 associates with 14-3-3σ

Others have characterized proteins that associate with the CSN complex (Richardson and Zundel, 2005; Wei and Deng, 2003), and many more remain to be identified. In Fig. 3A we show that CSN6 and 14-3-3σ co-elute by gel filtration. This led us to the hypothesis that 14-3-3σ and CSN6 may associate in complex. Co-immunoprecipitation experiments indeed show their *in vivo* interaction (Fig. 1A). We then mapped the CSN6 binding region on 14-3-3σ. 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. 1B). We also mapped the 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. 1C left), and same result was observed in *In vivo* binding experiment (Fig. 1C right). These results demonstrate that the N-terminal region of CSN6 binds to the C-terminal region of 14-3-3σ.

CSN6 negatively regulates 14-3-3σ protein stability

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. 2A, left). Exogenous expression of CSN6 also down-regulated 14-3-3σ expression (Fig. 2A, right) in a dose-dependent manner (Supplemental Fig. 1A). CSN6-mediated 14-3-3σ down-regulation was suppressed by MG132, a proteasome inhibitor, suggesting the involvement of the 26S proteasome (Supplemental Fig. 1B). mRNA levels of 14-3-3σ were not affected by CSN6 overexpression or knockdown in a real-time quantitative PCR analysis (Supplemental Fig. 2A, B), suggesting that CSN6 down-regulates 14-3-3σ at the post-transcriptional level. Indeed, CSN6 knockdown can reduce the turnover rate of 14-3-3σ in the presence of the *de novo* protein synthesis inhibitor, cycloheximide (Fig. 2B). Consistently, overexpression of CSN6 increases the turnover rate of 14-3-3σ (Supplemental Fig. 1C). 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. 2C). Also, increasing amounts of CSN6 shRNA, which was cloned in the pSilencer, antagonized poly-ubiquitination of transfected 14-3-3σ in a dose-dependent manner (Fig. 2D). Same results were obtained when the in vivo ubiquitination assays wree done in denaturing conditions (Supplemental Fig. 3). Since 14-3-3σ is regulated by p53 (Hermeking *et al.*, 1997), 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. 2E). This result suggests that downregulation of 14-3-3σ by CSN6 is not dependent on p53 expression.

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

The COP9 signalosome (CSN) and COP1 are required for the dark-dependent degradation of the transcription factor HY5 (Hardtke *et al.*, 2000), a positive regulator of photomorphogenesis in plants. Although the 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. 3A 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. Fig. 3B 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. 3C). The mRNA levels of COP1 were not affected by CSN6 expression in a real-time quantitative PCR analysis (Supplemental Fig. 2C), suggesting that CSN6 up-regulates COP1 at the post-transcriptional level. Further, the turnover rate of endogenous COP1 was reduced in CSN6 overexpressing cells in a cycloheximide chase assay (Fig. 3D). We also examined the effect of CSN6 on COP1 ubiquitination. As expected, poly-ubiquitination of COP1 was reduced in CSN6 overexpressing cells compared with control cells (Fig. 3E, Supplemental Fig. 4). Taken together, these findings demonstrate that CSN6 increases COP1 stability through inhibition of ubiquitin-mediated COP1 proteasomal degradation.

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

Given that CSN6 associates with COP1 and that COP1 co-elutes with 14-3-3σ in gel filtration assays (Fig. 3A), 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. 4A). Increasing amounts of COP1 shRNA, which was cloned into pSilencer, also increased steady-state levels of 14-3-3σ (Fig. 4B). Consistently, COP1 shRNA reduced the turnover rate of 14-3-3σ in 293T cells (Fig. 4C) and COP1 shRNA reduced polyubiquitinated 14-3-3σ levels in a dose-dependent manner (Fig. 4D, Supplemental Fig. 5). COP1 shRNA also increased endogenous levels of 14-3-3σ in HCT116 cells (Supplemental Fig. 6). 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 (Supplemental Fig. 2D). It was shown that COP1 (C136S, C139S) mutant has lost its function as an E3 ligase (Su *et al.*, 2011). We thus employed this construct for studying its impact on 14-3-3σ. We transfected COP1 (Wt) and COP1 (C136S, C139S) into HCT116 cells and investigate their impact on steady-state expression or polyubiquitination of 14-3-3σ. wt COP1 can efficiently reduce steady-state expression of 14-3-3σ levels (Supplemental Fig. 7A), while COP1 (136S, C139S) mutant had no impact on 14-3-3σ levels (Supplemental Fig. 7A). Accordingly, COP1 (C136S, C139S) lost its impact on enhancing the polyubiquitination of 14-3-3σ (Supplemental Fig. 7B). Interestingly, CSN6 is unable to stabilize COP1 (C136S, C139S) mutant, while CSN6 stablizies wt COP1 (Supplemental Fig. 7C). 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. 4E). Levels of CSN6-mediated 14-3-3σ poly-ubiquitination were also compromised by increasing amounts of COP1 shRNA (Fig. 4F, Supplemental Fig. 8). As expected, COP1 also efficiently increased the ubiquitination level of 14-3-3σ in an *in vitro* ubiquitination assay (Fig. 4G). Also, CSN6 enhanced COP1-mediated ubiquitination of 14-3-3σ even further (Fig. 4G). These results suggest that COP1 is a novel E3 ligase for 14-3-3σ whose ubiquitination is also mediated by CSN6.

CSN6-14-3-3σ axis regulates Akt-mediated cell survival

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 (Chen *et al.*, 2001). Because 14-3-3σ suppresses Akt activity (Yang *et al.*, 2006), 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. 5A) as indicated by reduced phosphorylation of Akt at Serine 473. Accordingly, Akt activity in CSN6 overexpressing cells was higher than in control cells (Fig. 5B). 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. 5C). Cells were also co-transfected with constitutively active Akt (HA tagged-Akt) or treated with LY294002 (PI3K inhibitor). As expected, LY294002 had additional impact on shCSN6-mediated apoptosis in the absence of serum (Fig. 5C). In contrast, the exogenous HA tagged-Akt prevented the potentiation of CSN6 shRNA in serum starvation-mediated cell death (Fig. 5C), 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. 5D). 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.

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

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. 6A 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. 6A). On the other hand, the CSN6 knockdown cells have a slower rate of cell proliferation (Supplemental Fig. 9A) or lower number of colony formation (Supplemental Fig. 9B) compared with control cells. Additional 14-3-3σ knockdown in CSN6 knockdown cells reverses CSN6 shRNA-mediated suppression of cell proliferation and colony formation (Supplemental Fig. 9A&B).

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. 6B). 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. 6B). 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. 6C). 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.

Discussion

In mammalian cells, COP1 regulates various cellular targets, including stress-responsive transcription factors, p53 tumor suppressor (Dornan *et al.*, 2004a), c-JUN (Bianchi *et al.*,

2003; Savio *et al.*, 2008; Wertz *et al.*, 2004), acetyl-coA carboxylase (Qi *et al.*, 2006), TORC2 (Dentin *et al.*, 2007), MVP (Yi *et al.*, 2005) and nucleosome remodeling factor MTA1 (Li *et al.*, 2009a), 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 14-3-3 σ, include Cdk's (Chan *et al.*, 1999; Laronga *et al.*, 2000), p53 (Yang *et al.*, 2003) and Efp (estrogen inducible finger protein) (Urano *et al.*, 2002) 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. 6) through its binding and degradation of 14-3-3σ (Fig. 2). 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 worthwhile 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 (Dornan *et al.*, 2004b). 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 (Dornan *et al.*, 2006), while 14-3-3σ protein level is elevated by DNA damage (Hermeking *et al.*, 1997). 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 (Urano *et al.*, 2002), 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 (Horie-Inoue and Inoue, 2006; Zou and Zhang, 2006), 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 (Yang *et al.*, 2006). 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. 5) is very intriguing. This is the first discovery that links the COP9 signalosome with Akt activation. We have shown that CSN6 is involved in14-3-3σ-mediated Akt inhibition, which in turn potentiates Akt-meditated cell survival (Fig. 5). Our mechanistic studies of CSN6-mediated 14-3-3σ downregulation explains how CSN6 can activate Akt in our proposed model (Fig. 6D). 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. Indeed, we show that adenoviral gene delivery of 14-3-3σ can inhibit tumorigenicity mediated by CSN6 activity in cancer models (Fig. 6). 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.

Materials and methods

Cell lines and reagents

HCT116 $p53^{-/-}$ cells were from Dr. B. Vogelstein. 293T and U2OS cells were obtained from ATCC. Antibodies: Flag (M2, Sigma), Tubulin (Sigma), COP1 (Bethyl Lab.), CSN6 (Biomol), 14-3-3σ (RDI), Myc (9E10, Santa Cruz), HA (12CA5, Roche), pAkt (Cell Signaling Technology), Akt (Cell Signaling Technology), PARP (Cell Signaling Technology), HA (12CA5, Roche) and Actin (Sigma). His-Ubiquitin (UW 8610), E1 (UW 9410) and E2 (UW 9050) were purchased from BioMol International. pcDNA6-Myc-COP1, pcDNA6-Myc-CSN6, and deletion mutants were constructed by PCR. HA-Akt, His-ubi, DN-Akt, pCMV5-Flag-14-3-3σ, Ad-β-gal, and Ad-14-3-3σ were previously described (Yang *et al.*, 2006; Yang *et al.*, 2005; Yang *et al.*, 2003). CSN6 or COP1 shRNA were constructed using pSilencer vector (Ambion).

Immunoprecipitation, immunoblotting, *In vitro* binding assay

Total cell lysates were processed as previously described (Laronga *et al.*, 2000). Lysates were immunoprecipitated and immunoblotted with indicated antibodies. Flag-CSN6 and myc-COP1 were prepared by in vitro transcription and translation using the TNT coupled system (Promega) (Laronga *et al.*, 2000). TNT products were mixed and immunoprecipitated followed by immunoblotting as described in figure legends.

In vivo and *In vitro* ubiquitination assay

293T cells were transiently co-transfected with indicated plasmids and were treated with 5 μg/mL MG132 for 6 hr before harvesting. Ubiquitinated COP1 or 14-3-3σ was immunoprecipitated with anti-COP1 or anti-14-3-3σ, followed by immunoblotting with anti-HA. In some cases, cells were cotransfected with His-ubi, and cell were lysed in denaturing buffer (6M guanidine-HCl, 0.1M Na_2HPO_4/NaH_2PO_4, 10mM imidazole). The cell lysates were then incubated with nickel beads for 3h, washed, and immunoblotted with indicated antibodies. 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, CSN6, and ATP (2 mM) in a total volume of 50 μl for 1 h at 37°C. Reaction products were detected by immunoblotting.

Cell lysates fractionated by Gel filtration

Cell lysates were fractionated through Superose 6 column (GEHealthcare) equilibrated with lysis buffer at a flow rate of 0.3ml/min. 300-μl fractions were collected.

Quantitative PCR

Primers for real-time quantitative PCR of *CSN6*, *COP1*, *14-3-3σ*, *and GAPDH* were as referenced in Primer Bank (http://pga.mgh.harvard.edu/primerbank/). 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.

FACS Analysis for apoptosis assay

Apoptosis was determined using propidium iodide (PI) and FITC-conjugated anti-Annexin V (BD Pharmingen). Cells were stained with PI and FITC-conjugated anti-Annexin V and analyzed with a FACScalibur flow cytometer.

Generation of stable transfectants

Cells were transfected with either PCDNA6 or PCDNA6-Myc-CSN6 plasmids and were selected in 8μg/ml Blasticidin for 2 weeks. Cells were infected by lentiviral shRNA transduction particles (Sigma, NM_006833) containing either control shRNA or CSN6 shRNA. After infection, cells were selected with 2μg/ml Puromycin for two weeks.

Soft agar colony formation, Foci formation, MTT assay

The experiments were performed as previously described (Laronga *et al.*, 2000).

Xenograft experiment

Athymic (*nu/nu*) mice were housed in AAALAC-approved barrier facilities. 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. At the end of the experiment, the tumors were removed and weighed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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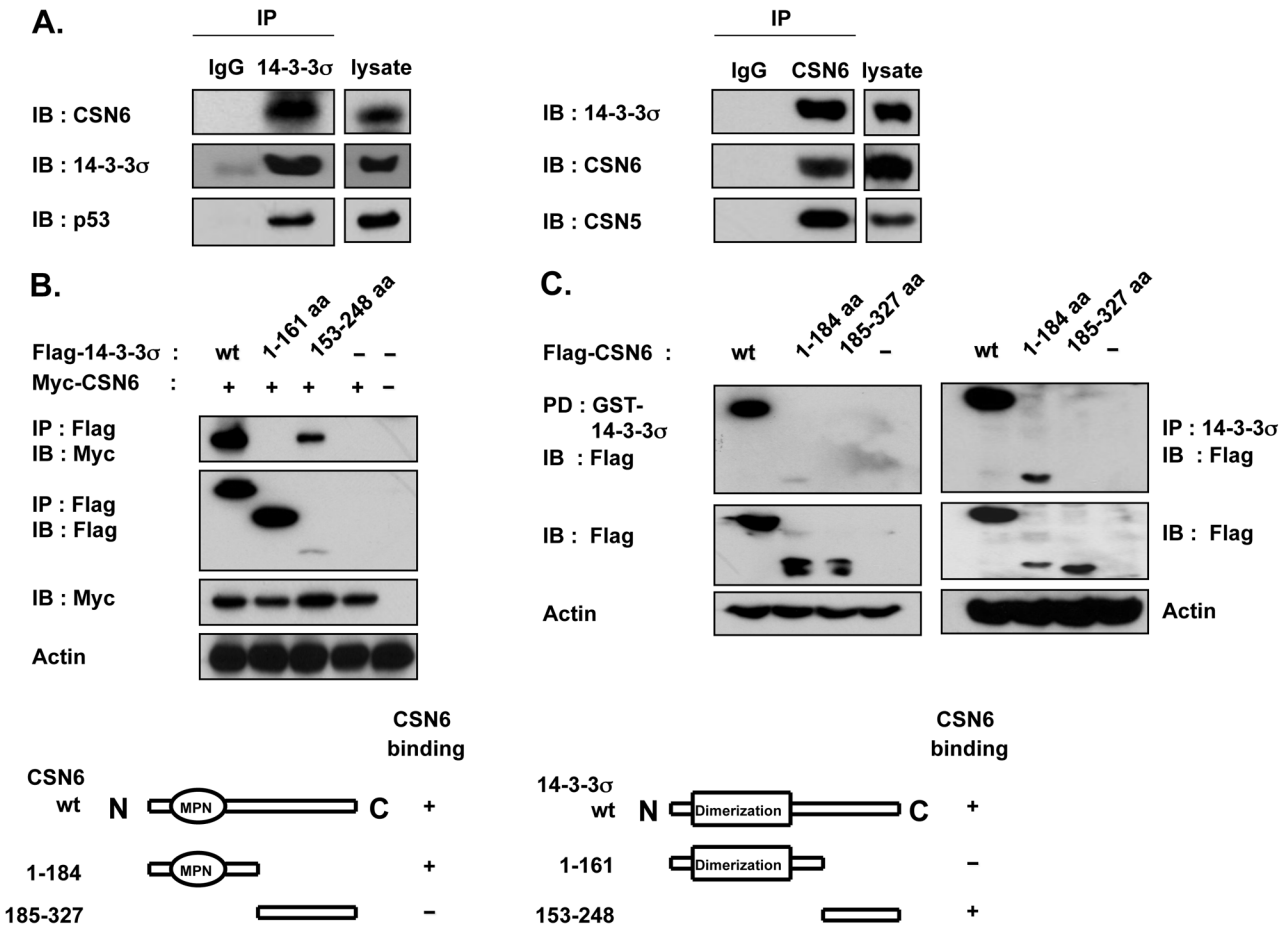

Fig. 1. CSN6 interacts with 14-3-3σ *in vivo*

A. Interaction of endogenous CSN6 with endogenous 14-3-3σ. Equal amounts of HCT116 cell lysates were immunoprecipitated with either mouse IgG or anti-14-3-3σ and immunoblotted with indicated antibodies.

B. 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 contransfected into 293T cells. Cell lysates were immunoprecipitated with anti-Flag and immunoblotted with anti-Myc.

C. 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 (left). Indicated plasmids were transfected into HCT116 cells. Cell lysates were immunoprecipitated with 14-3-3σ and immunoblotted with anti-Flag antibody (right).

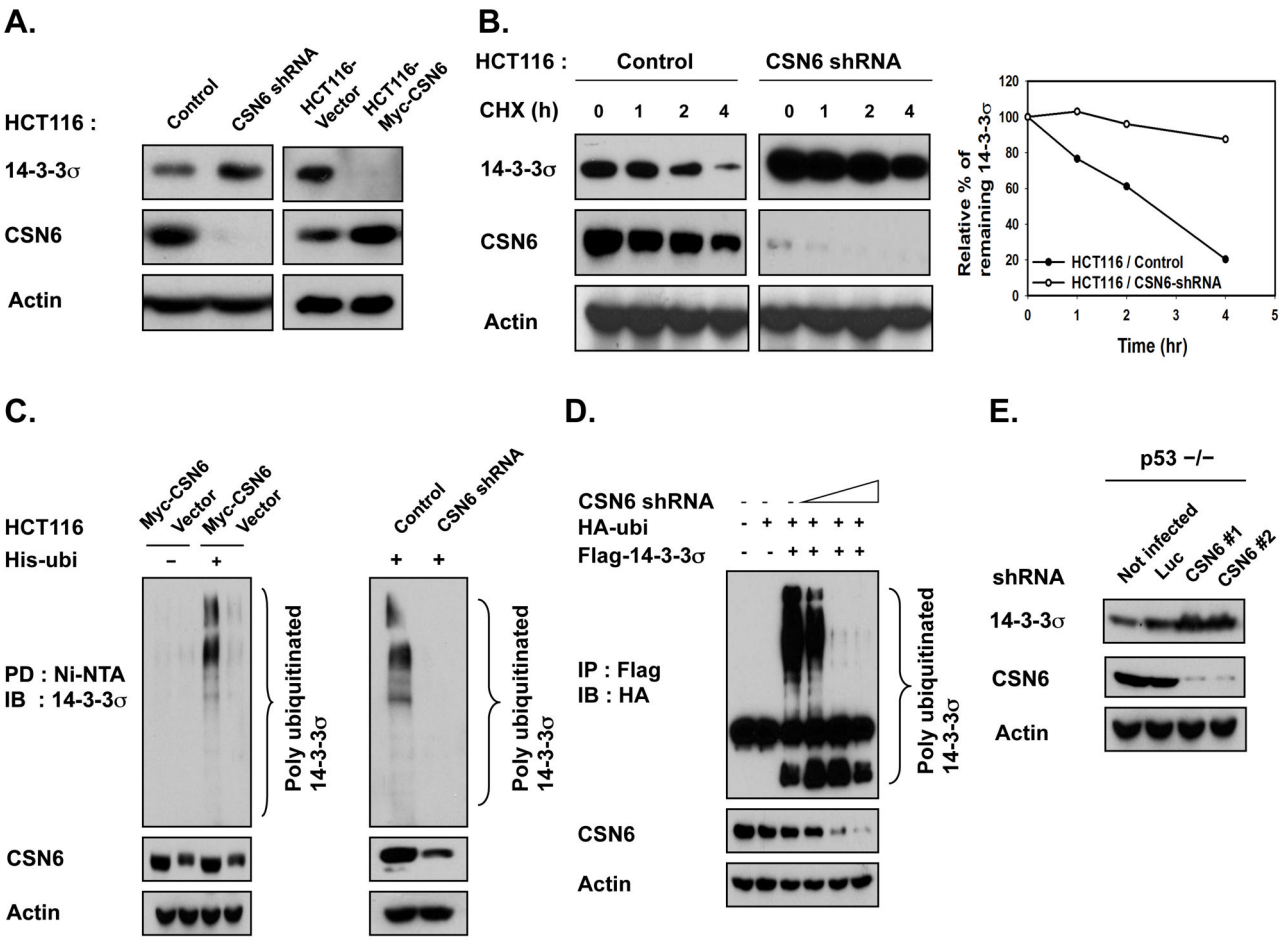

Fig. 2. CSN6 increases 14-3-3σ poly-ubiquitination

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 indicated 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 indicated antibodies.

B. 14-3-3σ turnover rate is reduced in CSN6 knockdown cells. 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 indicated 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).

C. CSN6 increases endogenous 14-3-3σ polyubiquitination. Indicated or transfected cells were treated with MG132 for six hours before harvesting. Cells were lysed in guanidine-HCl containing buffer. The cell lysates were then pulled down (PD) with nickel beads and immunoblotted with indicated antibodies. Equal amounts of cell lysates were immunoblotted with anti-CSN6 or Actin.

D. Knockdown of CSN6 reduces polyubiquitination of exogenous 14-3-3σ. 293T cells were co-transfected with indicated plasmids and increasing amounts of pSilencer expressing CSN6 shRNA. Cells were treated with MG132 for six 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 indicated antibodies.

E. 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 indicated antibodies.

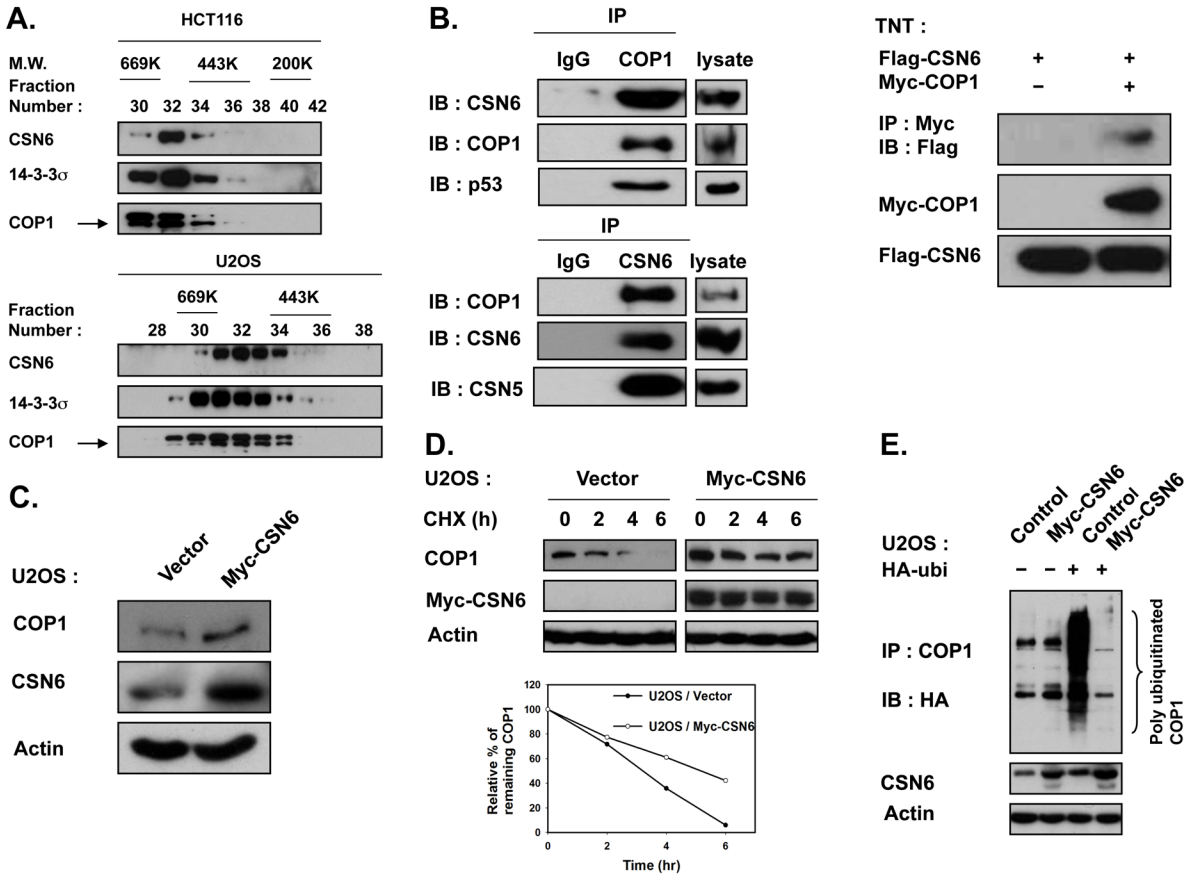

Fig. 3. CSN6 interacts with COP1 and regulates its stability

A. Gel filtration and elution profiles analysis of CSN6, COP1 and 14-3-3σ. The distributions of these proteins were analyzed by gel filtration chromatography (Superose 6). Immunoblots of the fractions for indicated proteins are shown in both HCT116 (top) and U2OS (bottom) cells. Molecular weight markers are indicated.

B. Endogenous COP1 interacts with endogenous CSN6. Equal amounts of U2OS cell lysates were immunoprecipitated with indicated antibodies followed by immunoblotting with indicated antibodies (left panels). COP1 is also shown to interact with CSN6 *in vitro* (right panel). Myc-COP1 and Flag-CSN6 cDNAs were transcribed and translated *in vitro* (TNT). COP1 and CSN6 proteins were incubated overnight and immunoprecipitated with anti-Myc followed by immunoblotting with anti-Flag.

C. Overexpression of CSN6 elevates the steady-state protein levels of COP1. Lysates of Myc-CSN6 overexpressing U2OS stable transfectants or vector control transfectants were immunoblotted with indicated antibodies.

D. 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 indicated antibodies. Integrated OD values of bands at each time point were measured using a densitometer. Levels of COP1 at time zero were set at 100%. COP1 remaining is indicated graphically.

E. CSN6 reduces polyubiquitination of COP1. Myc-CSN6 overexpressing U2OS stable transfectants or vector control transfectants were transfected with HA-Ubiquitin. Cells were treated with MG132 six 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.

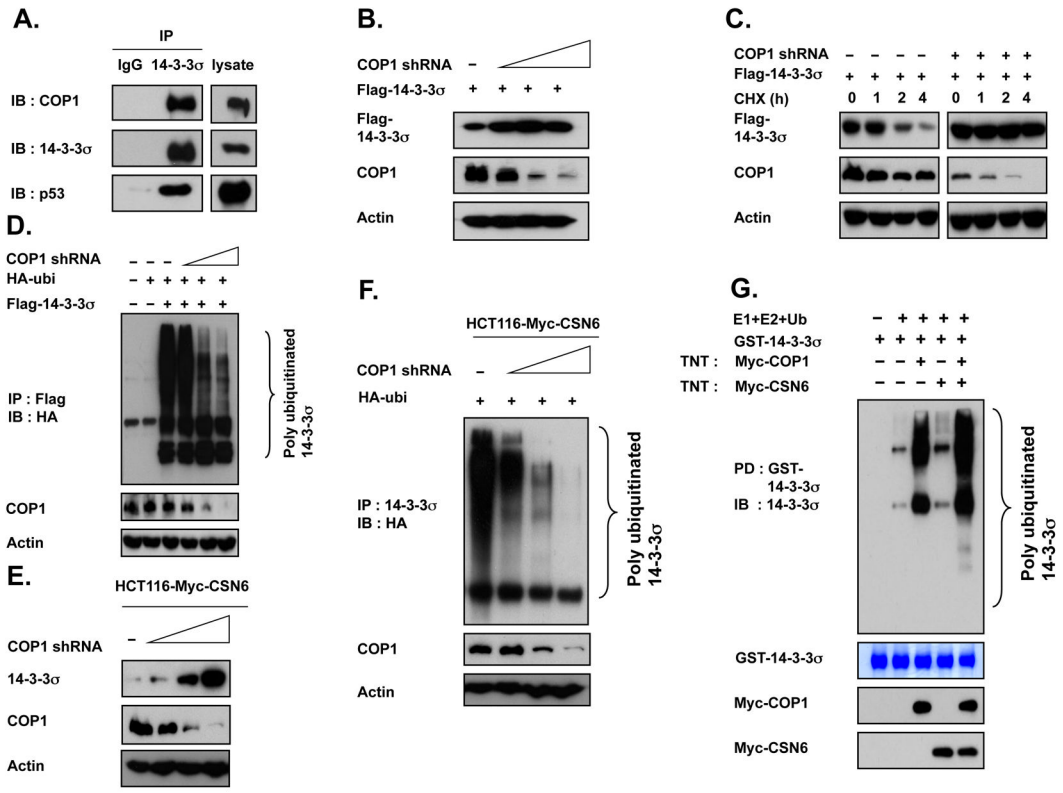

Fig. 4. COP1 is involved in CSN6-mediated 14-3-3σ ubiquitination and degradation

A. Endogenous COP1 interacts with endogenous 14-3-3σ. Equal amounts of HCT116 cell lysates were immunoprecipitated with either mouse IgG or 14-3-3σ followed by immunoblotting with indicated antibodies.

B. 14-3-3σ protein level is upregulated when endogenous COP1 expression is knocked down with shRNA. 293T cells were co-transfected with indicated plasmids and increasing amounts of pSilencer expressing COP1 shRNA. Equal amounts of cell lysates were immunoblotted with indicated antibodies.

C. 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 indicated antibodies.

D. Knockdown of COP1 expression reduces polyubiquitination of 14-3-3σ. 293T cells were co-transfected with indicated plasmids and increasing amounts of COP1 shRNA. Cells were treated with MG132 six 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 indicated antibodies.

E. 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 indicated antibodies.

F. 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 six 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 indicated antibodies.

G. COP1 induces the ubiquitination of 14-3-3σ in an *in vitro* ubiquitination assay. GST-14-3-3σ was incubated with indicated Myc-COP1 or Myc-CSN6 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-ubi antibodies.

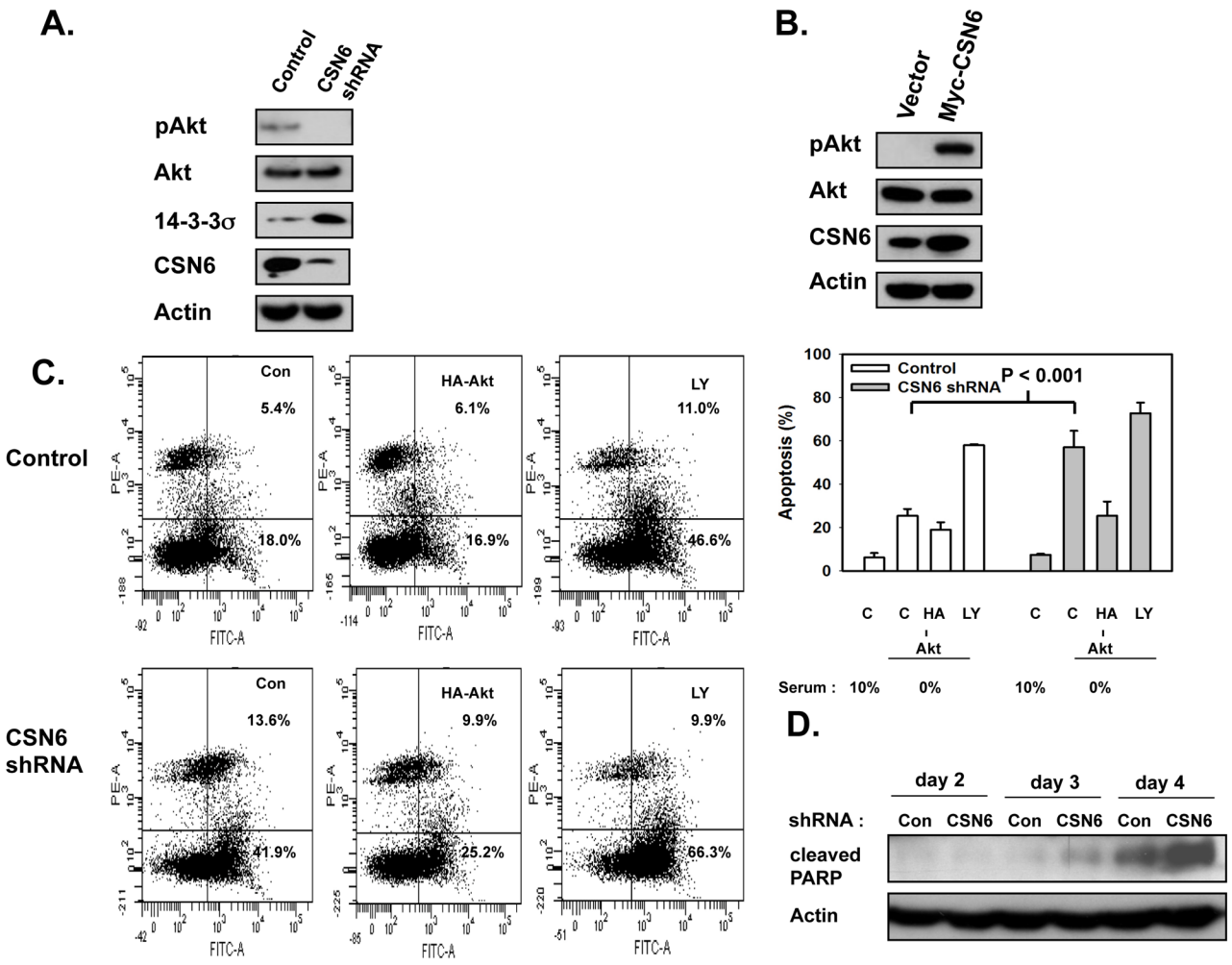

Fig. 5. CSN6 has impact on Akt-mediated survival

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. 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.

C. 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). Error bars represent 95% confidence intervals.

D. 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 indicated antibodies.

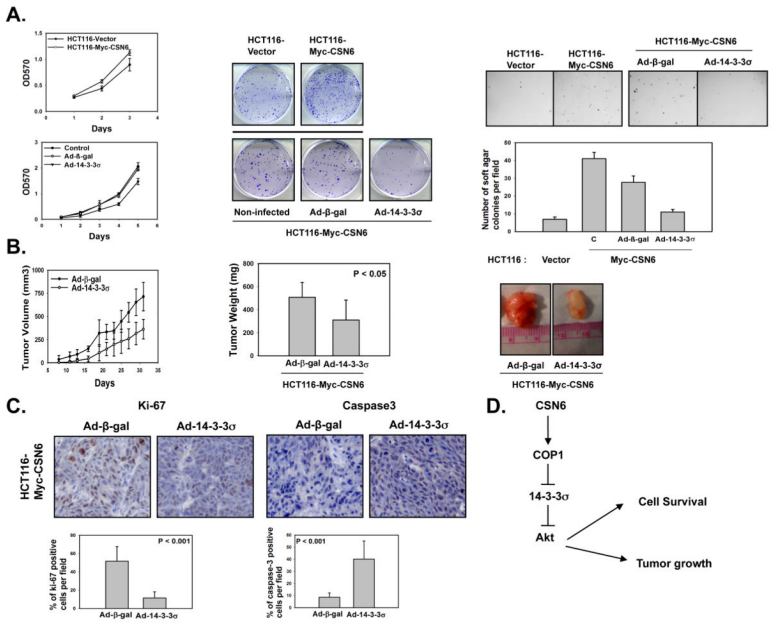

Fig. 6. 14-3-3σ inhibits CSN6-mediated cell proliferation, anchorage-independent growth, and tumorigenicity

A. 14-3-3σ inhibits CSN6-mediated cell proliferation. Myc-CSN6 overexpressing HCT116 stable transfectants and vector control transfectants were 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 (left bottom panel). Error bars represent 95% confidence intervals. 14-3-3σ antagonizes CSN6-mediated foci formation. 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 subjected to foci formation (middle, bottom). 14-3-3σ antagonizes CSN6-mediated soft agar colony formation. 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.

B. 14-3-3σ suppresses tumorigenesis of CSN6-overexpressing cells. Myc-CSN6 overexpressing HCT116 stable transfectants were infected with Ad-14-3-3σ or Ad-β-gal. Cells were harvested and s.c. injected into the flank of nude mice. Tumor volumes were monitored for 31 days. Tumor growth curves are shown (left); error bars represent 95% confidence intervals. Tumors were excised at the end of the study and tumor weights from each group measured (middle). Error bars represent 95% confidence intervals. Representative tumors from each group (right).

C. 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.

D. Model of the impact of the CSN6-COP1 axis in regulating 14-3-3σ signaling.