Journal of Cellular Biochemistry 103:1219–1230 (2008)

Roles for CSN5 in Control of p53/MDM2 Activities

Xiao-Chun Zhang,[1] Jian Chen,[1] Chun-Hui Su,[1,2] Heng-Yin Yang,[1] and Mong-Hong Lee[1,2,3]*

[1]Department of Molecular and Cellular Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

[2]The Programs in Genes & Development, The University of Texas Graduate School of Biomedical Sciences at Houston, Houston, Texas

[3]The Programs in Cancer Biology, The University of Texas Graduate School of Biomedical Sciences at Houston, Houston, Texas

Abstract The 5th subunit of COP9 signalosome (CSN5, also known as Jab1 or COPS5) is implicated in regulating p53 activity and is overexpressed in various tumors. However, the precise roles of CSN5 in p53 network and tumorigenesis are not well characterized. Here we show that CSN5 is a critical regulator of both p53 and MDM2. We show that curcumin, an important inhibitor of CSN-associated kinases, can downregulate not only CSN5 but also MDM2, which results in p53 stabilization. Importantly, CSN5 interacts with p53. CSN5 expression leads to p53 degradation, facilitating MDM2-mediated p53 ubiquitination, and promoting p53 nuclear export. Additionally, CSN5 expression results in stabilization of MDM2 through reducing MDM2 self-ubiquitination and decelerating turnover rate of MDM2. Significantly, we further show that CSN5 antagonizes the transcriptional activity of p53. These results demonstrate that CSN5 is a pivotal regulator for both p53 and MDM2. Our studies may pave the way for targeting CSN5 for anti-cancer drug development. J. Cell. Biochem. 103: 1219–1230, 2008.

Key words: MDM2; CSN5; ubiquitination; p53; curcumin

The COP9 signalosome (CSN) is an evolutionarily conserved multiprotein complex that has been found in plants and animals. It is a protein complex, which consists of eight subunits (CSN1–CSN8), first characterized as a repressor of plant photomorphogenesis. While the exact biochemical function of the CSN in animal cells remains to be characterized, the complex or its individual subunits have been implicated in a wide variety of regulatory processes, including cell cycle control, signal transduction, transcriptional activation, and possibly tumorigenesis (reviewed in Wei and Deng [2003]). Notably, the eight subunits of the CSN are each paralogous to one of the eight subunits that form the lid complex (19S) of the 26S proteasome [Karniol and Chamovitz, 2000; Li and Deng, 2003]. The lid complex is thought to recognize ubiquitinated substrates and then funnel them into the proteolytic core complex for degradation. Because of the homology between the CSN and the 19S lid complex, the CSN has been postulated to play a role in protein degradation. Indeed, it is shown that CSN is involved in degrading proteins, such as p27 [Tomoda et al., 1999], c-Jun [Naumann et al., 1999], and p53 [Bech-Otschir et al., 2001], via the ubiquitin-proteasome pathway. In the p53 degradation study, it has been demonstrated that the CSN can coordinate with the ubiquitin ligase activity of MDM2 in regulating the stability of p53 [Bech-Otschir et al., 2001]. This study implicates that CSN plays an important role in p53 stability; however, the detailed mechanism remains to be characterized.

In addition, three kinases, including inositol 1,3,4-trisphosphate 5/6-kinase (5/6-kinase) [Sun et al., 2002], protein kinase D (PKD) and CK2 [Uhle et al., 2003], have been copurified with the CSN and are implicated in

Grant sponsor: NCI; Grant number: RO1CA89266; Grant sponsor: NIHRO1CA; Grant number: 089266; Grant sponsor: Cancer Center Core; Grant number: CA16672.

*Correspondence to: Mong-Hong Lee, Box 79, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030.
E-mail: mhlee@mdanderson.org

Received 24 February 2007; Accepted 28 June 2007
DOI 10.1002/jcb.21504

phosphorylating p53 and subsequent p53 degradation. Interestingly, the protein kinase activity of these CSN-associated kinases can be inhibited by curcumin [Bech-Otschir et al., 2001], a naturally occurring yellow pigment from the rhizomes of the plant *Curcuma longa.* Furthermore, p53 protein levels were elevated in the presence of curcumin. These data suggest that curcumin inhibits CSN-associated kinases, thereby affecting the stability of p53. Nonetheless, the mechanism by which curcumin antagonizes CSN activities to regulate p53 stability remains to be elucidated. Recently, gene disruption of CSN5 leads to embryonic lethality [Tomoda et al., 2004]. Importantly, p53 protein levels are elevated in CSN5−/− embryos, suggesting that CSN5 is involved in regulating p53 stability. CSN5 has an important feature that is different from other subunits. It is a NEDD8 (an ubiquitin-like protein) isopeptidase that impacts the effect of the CSN on the activity of associated E3 ubiquitin ligases SCF (SKP1-Cullin-F-box protein) [Cope et al., 2002]. To enhance the ligase activity of SCF, cullin protein of SCF needs to be covalently modified by NEDD8 [Kawakami et al., 2001]. CSN5 can cleave NEDD8−Cul1 conjugates, thereby regulating SCF activity and subsequent degradation of targeted proteins [Cope et al., 2002]. MDM2, an E3 ubiquitin ligase for tumor suppressor p53, can decrease the stability and half-life of p53 through the ubiquitin-proteasome pathway [Iwakuma and Lozano, 2003]. Although CSN5 is implicated in degrading p53, it is not clear whether MDM2, a p53 ubiquitin ligase, is regulated by CSN5 during the process.

It is clear that the tumor suppressor gene p53 is the most commonly mutated gene in human cancers. Its function is to protect normal cells from undergoing transformation in response to stress. Stabilization of p53 is essential for the maintenance of its tumor suppressor function. It is expected that when regulators involved in p53 protein stability are deregulated, they will have an impact on human tumorigenesis. For example, human MDM2 is amplified in greater than 30% of sarcomas [Lozano and Montes de Oca Luna, 1998]. Recently, it has been shown that CSN5 is overexpressed in a high percentage of invasive breast cancers [Esteva et al., 2003; Kouvaraki et al., 2003]. CSN5 overexpression also associates with poor prognosis for ovarian cancer patients [Sui et al., 2001]. Additionally, CSN5 can enhance the invasive capabilities of cancer cells [Adler et al., 2006]. These observations suggest that CSN5 protein plays a role in tumorigenesis, which may be traced to a defect in its role in p53 network control. However, the detailed role or regulation of CSN5 is not clear. Here, we show that CSN5 and MDM2 are important targets downregulated by curcumin, which contributes to p53 stabilization. We then assessed the roles of CSN5 in regulating p53 stability and function. We found that CSN5 interacts with and decreases the stability of p53. CSN5 can facilitate MDM2-mediated p53 degradation. We discovered that CSN5 reduces MDM2-mediated self-ubiquitination, thus stabilizing MDM2 protein, which in turn promotes p53 ubiquitination. Also, live-cell images indicated that CSN5 induces the translocation of p53 from the nucleus to the cytoplasm; therefore, CSN5-mediated MDM2 stabilization also contributes to cytoplasmic localization of p53. We show that CSN5 expression antagonizes p53-mediated transcriptional activity. Thus, our studies indicate that CSN5 is an important negative regulator of p53, exerting a stimulatory effect toward MDM2-mediated p53 ubiquitination. Hence, as a positive regulator of MDM2, inhibition of CSN5-mediated regulation of MDM2 may be a useful therapeutic strategy for treating cancers with activated MDM2.

RESULTS

Impact of Curcumin on CSN5 and p53 Expression

Curcumin is known to possess anti-tumor activity. Also, it is known to inhibit CSN-associated kinase [Bech-Otschir et al., 2001], thereby preventing p53 phosphorylation and subsequent degradation. However, it remains unclear how curcumin can regulate the p53-MDM2 network. Here, we showed that curcumin has impact on the expression of p53 and CSN5 in cells. R1B/L17 cells were treated with curcumin. As expected, curcumin treatment leads to increased amounts of p53 (Fig. 1A). Consistently, expression of p21, a target gene of p53, was related with p53 upregulation. Importantly, decreasing amounts of CSN5 were detected from cells harvested after 24 h of treatment. Furthermore, increased p53 correlates with the downregulation of CSN5. We obtained similar results when using Rat1 and Rat1-akt cells for studies (Fig. 1B), thus excluding the possibility that this phenomenon is cell

type specific. To further confirm this observation, we used curcumin to treat H1299 (p53 null) cells, which were cotransfected with CSN5 and p53, and found that again curcumin can cause downregulation of CSN5 and upregulate p53 (Fig. 1C). Hence, these data highlight the critical role of curcumin in affecting the expression of p53 via affecting the expression of CSN5.

This inverse relationship between p53 and CSN5 prompted us to determine whether CSN5 interacts with p53. We showed that CSN5 interacts with p53 in vivo as characterized by coimmunoprecipitation experiments (Fig. 1D). 293T cells were transfected with Flag-tagged CSN5-expressing plasmid in the presence or absence of p53-expressing plasmid. Importantly, p53 was detected in the anti-Flag immunoprecipitation (IP) complex (Fig. 1D). Reciprocal IP also indicated that Flag-CSN5 is detected in the anti-p53 IP complex. In addition, we tested the specificity of the interaction between CSN5 and p53 using the GST pull-down assay. GST-tagged p53 was able to bind to CSN5 (Fig. 1E). To further confirm the binding between endogenous proteins, we performed the coimmunoprecipitation experiments in A549 cells. Importantly, CSN5 was detected in the anti-p53 IP complex (Fig. 1F). Thus, compelling evidence indicates that the two proteins are associated in the cells.

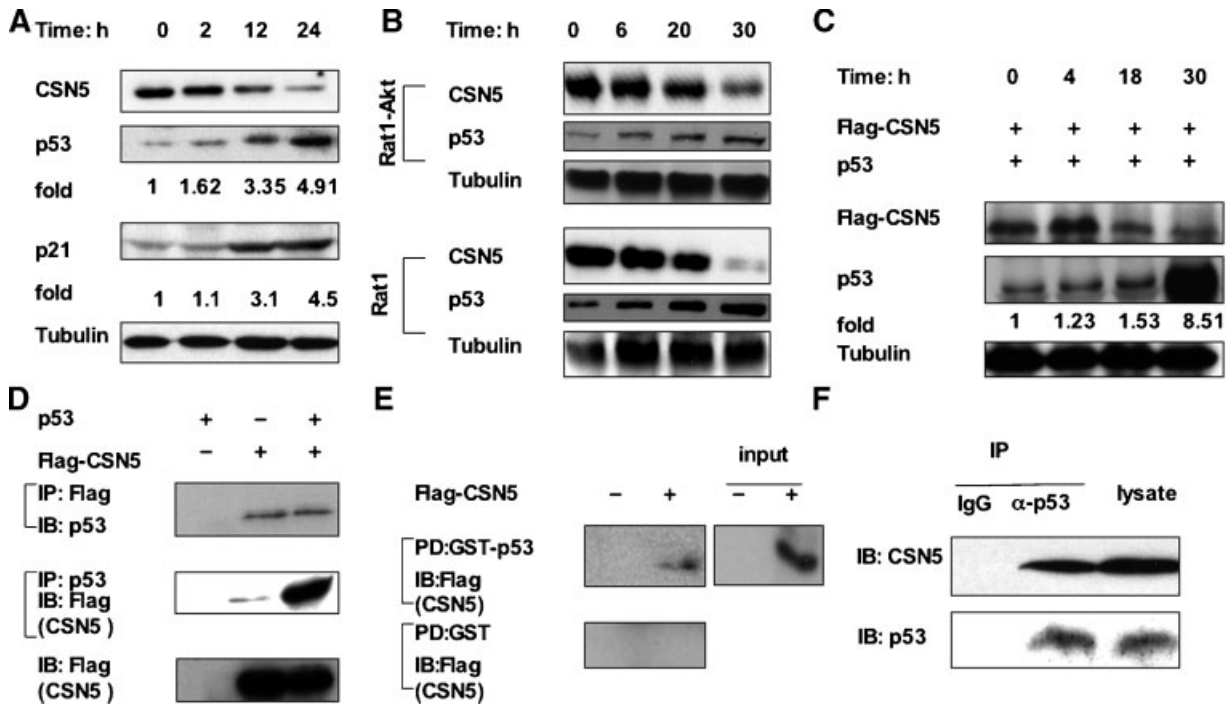

Fig. 1. Curcumin causes downregulation of CSN5 and stabilization of p53. **A**: Curcumin downregulates CSN5 to stabilize p53. R1B/l17 cells were treated with curcumin (10 μM) for the indicated hours. Equal amounts of protein from cell lysates were IB with anti-CSN5, anti-p53, and anti-p21. Tubulin served as a loading control. The quantitative analysis of expression of p53 was preformed with a NIH imaging program. The increased fold was expressed as comparison with the value at time 0. **B**: Downregulation of CSN5 in Rat1 and Rat1-akt cells treated with curcumin (10 μM). Rat1 and Rat1-akt cells were treated with curcumin for the indicated hours. Equal amounts of protein from cell lysates were IB with anti-CSN5, anti-p53, and anti-tubulin. **C**: Curcumin causes downregulation of CSN5 to stabilize exogenous p53 in H1299 cells. H1299 cells were transfected with indicated Flag-tagged CSN5 and the equal amount of p53 expression vector, followed by the treatment of curcumin (10 μM) for the indicated hours. Equal amounts of protein from cell lysates were IB with anti-p53, anti-Flag, and anti-tubulin. Tubulin served as a loading control. The expression level of p53 or p21 was quantitated with a NIH imaging program. The increased fold was expressed as comparison with the value at time 0. **D**: CSN5 associates with p53. 293T cells were transfected with the indicated Flag-tagged CSN5 construct and p53 expression vectors. Cell lysates were immunoprecipitated (IP) with anti-Flag antibody (M2), and the IP were immunoblotted (IB) with anti-p53. Cell lysates were also IP with anti-p53 antibody, and then IB with anti-Flag antibody (M2). The cell lysates were IB with anti-Flag antibody (M2, Sigma) to demonstrate the amount of CSN5 expressed. **E**: p53 specifically interacts with CSN5. 293T cells were transfected with indicated Flag-CSN5 expression plasmid. Cell lysates were subjected to GST or GST-p53 pull-down (PD) followed by IB with anti-Flag antibody. The **right panel** shows 10% of the inputs. **F**: Endogenous interaction. Cells lysates of A549 cells were IP with control IgG or anti-p53 antibody, followed by IB with anti-CSN5 or anti-p53. Cells were treated with MG132 for 8 h prior to collection.

Role of CSN5 in p53 Degradation

It was shown that CSN can regulate p53 degradation [Bech-Otschir et al., 2001]. How CSN exerts its negative impact toward p53 remains to be elucidated. We have found that CSN5 downregulation correlates with p53 upregulation (Fig. 1). Thus, CSN5 may mediate p53 degradation. Structurally, CSN5 contains a Mpr1p and Pad1p N-terminal (MPN) domain and leucine-rich part (Fig. 2A). However, the biological function of these domains in CSN5 is not clear. The MPN domain is found in the N-terminus of yeast Mpr1and Pad1 proteins, which are yeast proteasome subunits, suggesting that MPN domain may participate in protein degradation [Rinaldi et al., 1995; Penney et al., 1998; Wei et al., 1998]. The MPN domain can be further divided into six conserved regions (I–VI) [Asano et al., 1997]. To determine the contribution of domains of CSN5 in affecting p53 stability, we performed a p53 expression assay in the presence of various CSN5 expression constructs (Fig. 2A,B). We examined the role of different CSN5 constructs in regulating the protein level of p53 by transfection, using H1299 lung carcinoma cells (p53 null). Cells were cotransfected with equal amount of p53 and increasing amounts of CSN5 expression constructs. Significantly, the expression level of p53 was decreased when high level of wtCSN5 was present (Fig. 2B), suggesting that CSN5 had a negative impact on p53 stability. CSN5 (1–222aa) is a protein with MPN I–VI domain, while other CSN5 mutants contain deletion in either MPN I–III or MPN IV–VI domain. We found that only wt CSN5 and CSN5 (1–222aa) were able to downregulate the expression of p53, suggesting that MPN I–VI domain is involved in affecting the expression of p53 (Fig. 2B).

p53 is a protein with short half-life; therefore, it is not very stable. The stability of p53 is

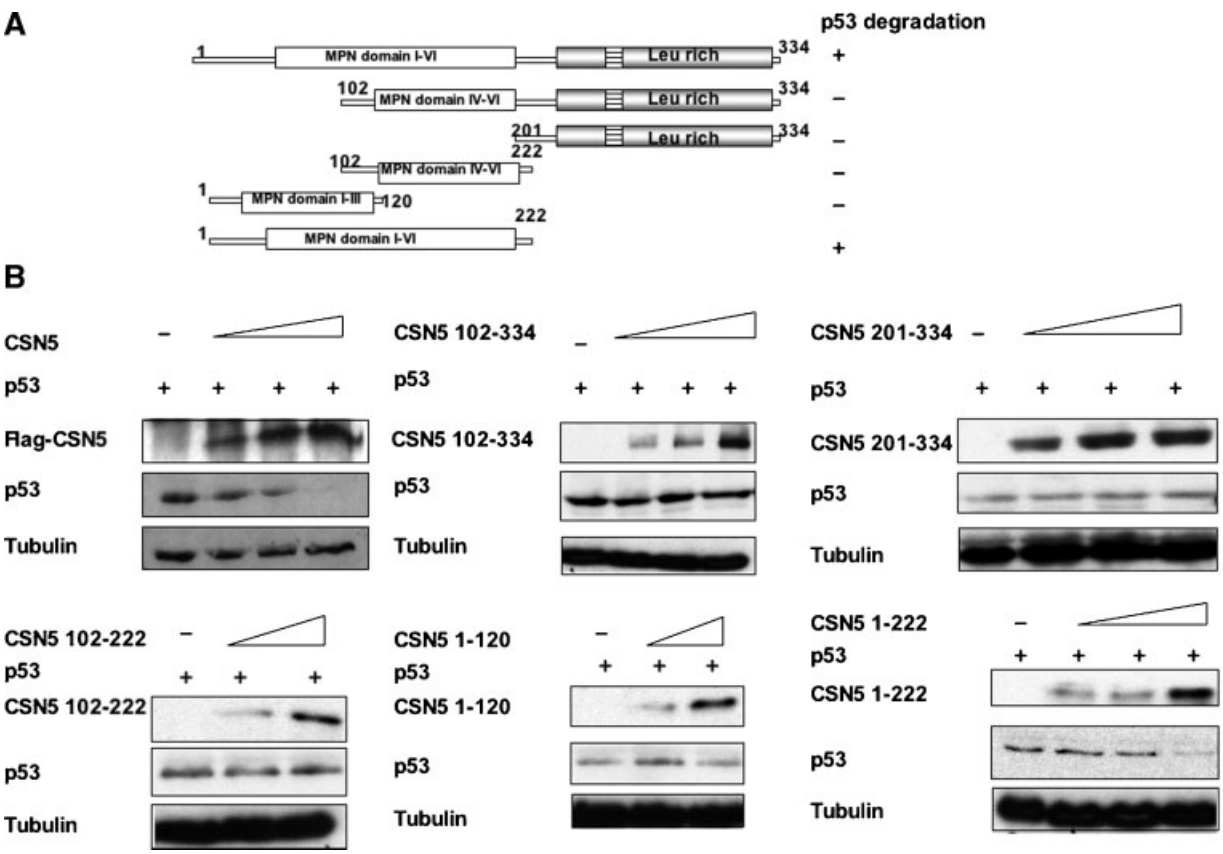

Fig. 2. CSN5 is involved in regulating p53 stability. **A**: Schematic representation of CSN5 deletion constructs. **B**: MPN domain of CSN5 is involved in regulating p53 stability. H1299 cells were transfected with the equal amount of p53 expression vector and increasing amounts of the indicated Flag-tagged CSN5 expression vectors. Equal amounts of protein from cell lysates were IB with anti-p53, anti-Flag, and anti-tubulin. Tubulin served as a loading control.

regulated through proteasome pathway. We demonstrated that the downregulation of p53 protein by CSN5 is mediated via proteasome degradation process, as the downregulation of p53 was prevented when cells were treated with the specific 26S proteasome inhibitor MG132 (Fig. 3A). As expected, more ubiquitinated form of p53 was accumulated in the presence of CSN5 when cells were treated with MG132, suggesting that CSN5 is facilitating the ubiquitination and subsequent degradation of p53 (Fig. 3B).

MDM2 is a known negative regulator involved in p53 degradation; therefore, we determined whether CSN5-mediated p53 degradation requires MDM2. We used p53−/− mouse embryonic fibroblasts (MEF) and p53/Mdm2 double knock-out MEF to assay the impact of CSN5 on MDM2 degradation. As expected, CSN5 is able to degrade exogenous p53 in p53−/− MEF. In contrast, CSN5 is unable to degrade exogenous p53 in p53/Mdm2 double knock-out MEF, suggesting that the presence of MDM2 is required for CSN5 to degrade p53 (Fig. 3C).

Since MDM2 is an E3 ubiquitin ligase for p53, we then further determined whether CSN5

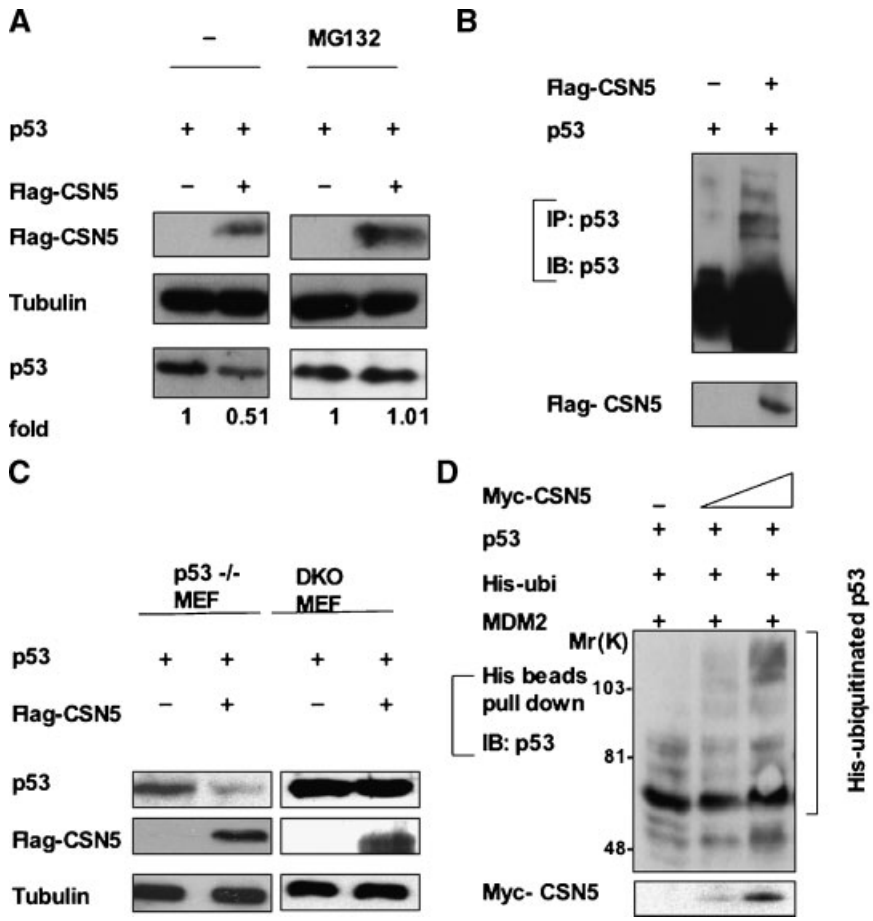

Fig. 3. CSN5 facilitates MDM2-mediated p53 ubiquitination. **A**: Proteasome inhibitor blocks CSN5-mediated degradation of p53. H1299 cells were transfected with the indicated expression vectors in the presence or absence of proteasome inhibitor MG132. Equal amounts of protein from cell lysates were IB with anti-p53, anti-Flag, and anti-tubulin. Tubulin served as a loading control. The expression of p53 was quantitated with a NIH imaging program. **B**: CSN5 facilitates the ubiquitination of p53. H1299 cells were transfected with pCMV-p53 in the presence or absence of pCMV-Flag-CSN5. The cell lysates were harvested and IP with anti-p53 antibody, and the IP were IB with anti-p53. Equal amounts of protein from cell lysates were IB with anti-Flag. Cells were treated with MG132 for 8 h prior to collection. **C**: CSN5-mediated p53 degradation requires MDM2. p53−/− MEF or p53/Mdm2 double knock-out (DKO MEF) was transfected with indicated plasmids. The cell lysates were harvested and IB with anti-p53 or anti-Flag. Tubulin served as a loading control. **D**: CSN5 promotes MDM2-mediated p53 ubiquitination. H1299 cells were cotransfected with pCMV-p53 (1 μg), pCMV-His-ubiquitin (1 μg), pCMV-MDM2 (1 μg), and increasing amounts of pCMV-Myc-CSN5 (0, 1, 5 μg). Cells were treated with MG132 for 8 h prior to collection. The cell lysates were harvested, and the His-ubiquitinated p53 were pulled down using His-bind beads. The protein complexes were probed with anti-p53 antibody to reveal the His-ubiquitinated p53. Equal amounts of cell lysates were IB with anti-Myc to indicate the expression of CSN5.

could accelerate MDM2-mediated p53 ubiquitination, thereby reducing the stability of p53. Cells were cotransfected with MDM2, p53, pCMV-His-ubiquitin, and increasing amount of CSN5 into H1299 cells. Cells were harvested and analyzed by His-Bind resin binding for the presence of His-ubiquitin containing p53. MDM2 can act as an ubiquitin ligase and will add His-ubiquitin onto p53. Thus, His-ubiquitinated form of p53 can be detected in the His-ubiquitin containing protein complexes bound to His-Bind resin by immunoblotting (IB) with anti-p53. Under conditions where CSN5 is increased, the amounts of His-ubiquitinated form of p53 increased, suggesting that CSN5 accelerated MDM2-mediated p53 ubiquitination activity (Fig. 3D).

CSN5 Affects the Subcellular Localization of p53

p53 is exported from the nucleus to the cytoplasm for degradation. It is known that CSN5 can exert its biological function by sequestering its target proteins. For example, CSN5 sequesters p27 in the cytoplasm to cause p27 degradation [Tomoda et al., 1999]. We then determined whether CSN5 has an impact on p53 subcellular localization. We employed live-cell imaging to study the subcellular localization of p53 in the presence of CSN5 in H1299 cells. GFP-p53 was used to indicate the location of p53 in a live cell. The H1299 cells were cotransfected with indicated GFP-p53 and different CSN5 expression constructs. As expected, we found that GFP-p53 was located in the nucleus of control cells (CMV5 vector control) after live-cell images were captured using immunofluorescent microscope (Fig. 4, green). Remarkably, GFP-p53's subcellular location, originally in the nucleus, was detected in the cytoplasm when cells were cotransfected with CSN5 expressing construct (Fig. 4). It is noteworthy that a CSN5 expression construct, which contains just MPN I–VI domain (1–222 aa), can mediate p53 cytoplasmic location. However, a construct encoding MPN I–III region (1–120 aa) is inefficient in affecting the sub-cellular localization of p53 (Fig. 4A,B). Thus, these results highlight the important role of CSN5 in affecting the translocation of p53 from the nucleus to the cytoplasm.

CSN5 is Involved in Regulating MDM2 Stability

As described above, curcumin is able to upregulate p53. It is not clear whether MDM2, a negative regulator of p53, is also affected. Here, we demonstrated that curcumin treatment leads to decreased expression of MDM2 (Fig. 5A). We then determined whether CSN5 interacts with

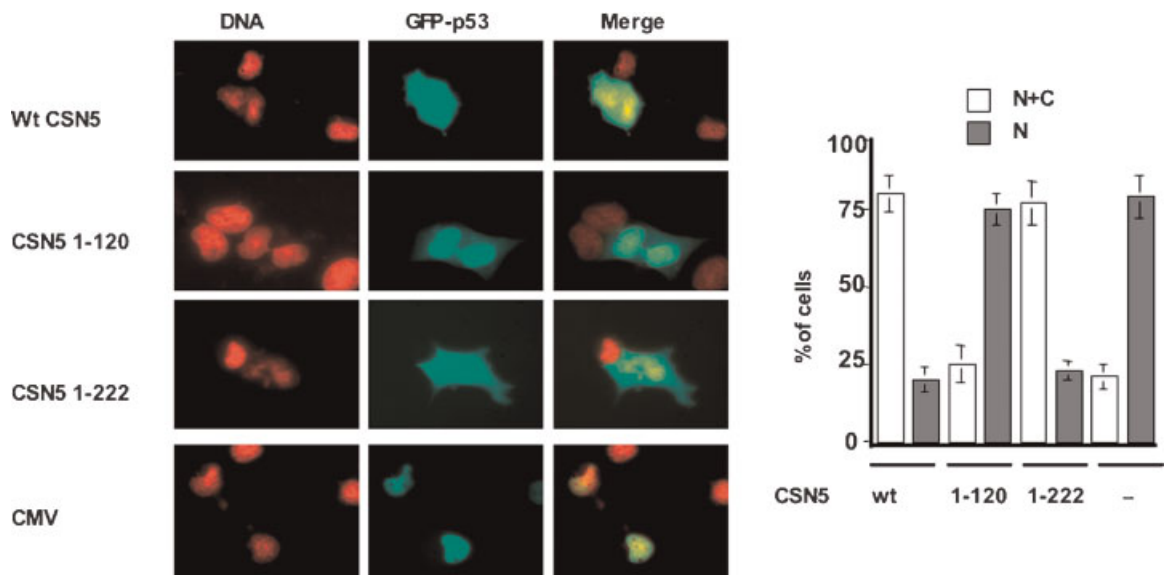

Fig. 4. CSN5 instigates cytoplasmic localization of p53. Localization of GFP-p53 is affected by the expression of CSN5. The H1299 cells were cotransfected with GFP-p53 and the indicated CSN5 expressing constructs. Live-cell images were captured. Subcellular localization of GFP-p53 (green) is shown. Hoechst 33342 (0.5 μg/ml) was added for DNA staining (red). Merged images are also indicated. The location of GFP-p53 was observed under a fluorescence microscope. A total of 300 cells were counted for the location of GFP-p53 in each condition. The percentages of nuclear-positive cells (N) and nuclear and cytoplasmic-positive cells (N + C) are presented. Data shown were from a typical experiment conducted in triplicate. Bars represent standard deviations. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

MDM2. 293T cells were transfected with Flag-tagged CSN5-expressing plasmid in the presence or absence of MDM2-expressing plasmid. Importantly, MDM2 was detected in the anti-Flag IP complex (Fig. 5B). Reciprocally, Flag-CSN5 is detected in the anti-MDM2 IP complex. Additionally, we then tested the specificity of the interaction between CSN5 and MDM2 using GST pull-down assay. GST-MDM2 was able to bind to CSN5 (Fig. 5C). To further confirm the binding between endogenous proteins, we performed the coimmunoprecipitation experiments in A549 cells. Importantly, CSN5 was detected in the anti-MDM2 IP complex (Fig. 5D). Together, these results indicate that CSN5 interacts with MDM2.

We have found that CSN5 associates with MDM2 (Fig. 5). It is not clear whether CSN5 collaborates with MDM2 through interaction, which in turn would potentiate p53 degradation. To determine the biological function of the interaction, we determined whether CSN5 affects MDM2's expression. We examined the role of CSN5's activity in regulating the protein level of MDM2 by transfection, using H1299 lung carcinoma cells (p53 null). Remarkably, the expression level of MDM2 was increased when CSN5 was cotransfected (Fig. 6A), suggesting that CSN5 had a positive impact on MDM2 stability. MDM2 is an E3 ubiquitin ligase for itself and is involved in regulating the stability and half-life of itself through ubiquitin-proteasome pathway [Fang et al., 2000]. To address the stability issue further, we determined whether CSN5 could hinder MDM2-mediated self-ubiquitination, thereby increasing the stability of MDM2. We determined the levels of MDM2 ubiquitination in the cells transfected with or without CSN5. 293T cells transfected with MDM2 and pCMV-His-ubiquitin were harvested and analyzed for the presence of ubiquitin containing MDM2. MDM2 can act as an ubiquitin ligase and will add ubiquitin onto itself. Thus, ubiquitinated form

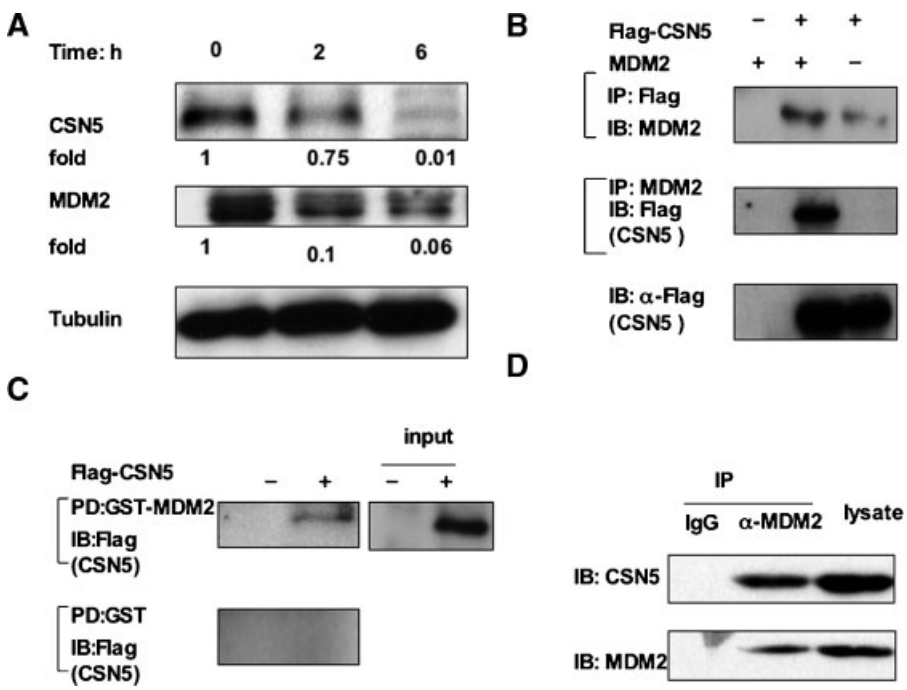

Fig. 5. CSN5 associates with MDM2. **A**: Curcumin down-regulates MDM2. MCF7 cells were treated with curcumin for the indicated hours. Equal amounts of protein from cell lysates were IB with anti-CSN5, anti-MDM2. Tubulin served as a loading control. The expression of CSN5 or MDM2 was quantitated with a NIH imaging program. The decreased fold was expressed as comparison with the value at time 0. **B**: CSN5 associates with MDM2. 293T cells were transfected with the indicated Flag-tagged CSN5 construct and MDM2 expression vectors. Cell lysates were IP with anti-Flag antibody (M2), and the IP were IB with anti-MDM2. Cell lysates were also IP with anti-MDM2 antibody, and then IB with anti-Flag antibody (M2). The cell lysates were IB with anti-Flag antibody (M2, Sigma) to demonstrate the amount of CSN5 expressed. **C**: MDM2 specifically interacts with CSN5. 293T cells were transfected with indicated Flag-CSN5 expression plasmid. Cell lysates were subjected to GST or GST-MDM2 pull-down (PD) followed by IB with anti-Flag antibody. The **right panel** shows 10% of the inputs. **D**: Endogenous interaction. Cells lysates of A549 cells were IP with control IgG or anti-MDM2 antibody, followed by IB with anti-CSN5 or anti-MDM2.

of MDM2 can be detected in the MDM2 IP complexes by IB with anti-ubiquitin. Under conditions where CSN5 is present, the amounts of ubiquitinated form of MDM2 decreased, suggesting that CSN5 blocked MDM2-mediated self-ubiquitination activity (Fig. 6B).

Consistently, we determined the protein turnover of MDM2 by cycloheximide treatment in cells transfected with or without CSN5 and found that cells expressing CSN5 exhibited a reduced MDM2 turnover as compared with control cells (Fig. 6C). The turnover rate of MDM2 was slower in CSN5 expressing cells (more than 50% remained after 1 h) than in control cells (less than 20% remained after 0.5 h) (Fig. 6D), suggesting that the expression of CSN5 leads to reduced degradation of MDM2. Taken together, CSN5 can stabilize MDM2 by decelerating the ubiquitin-mediated proteasome degradation of MDM2.

CSN5 Affects the p53 Transcriptional Activity

To assess the functional significance of the antagonizing effects of CSN5 toward p53, the transcriptional capacity of p53 was determined. We performed a p53 reporter gene assay by cotransfecting the luciferase p53 reporter gene BDS2-3x-luc, CSN5, and p53 into R1B/L17 cells. We found that CSN5 compromises p53 transcriptional activity in cells in a dose-dependent manner, as determined by luciferase activity (Fig. 7A). Since CSN5 (1–220 aa) is capable of degrading p53, we also investigated its role in mediating p53 transcriptional activity. As expected, this construct is able to reduce the transcriptional activity of p53. Thus CSN5 and

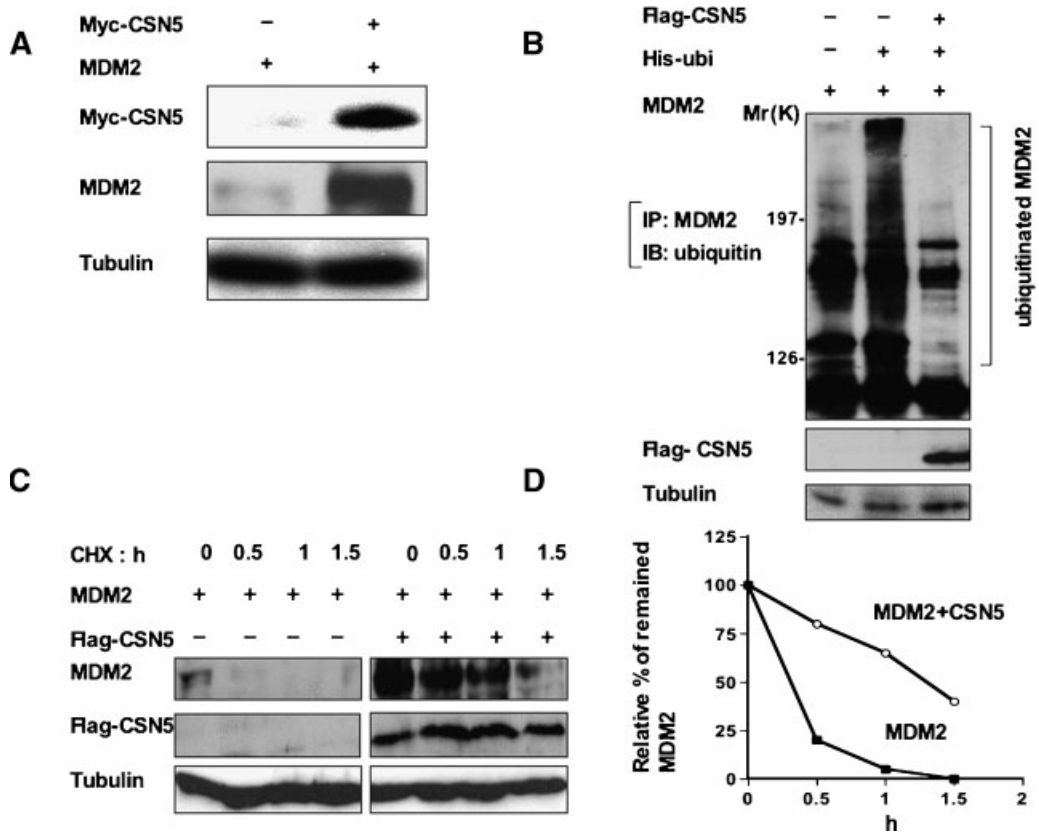

Fig. 6. The expression of CSN5 increases the stability of MDM2. **A**: Ectopic expression of CSN5 can stabilize MDM2. H1299 cells were transfected with the indicated MDM2 expression vector and the Myc-tagged CSN5 expression vector. Equal amounts of protein from cell lysates were IB with anti-MDM2, anti-Myc, and anti-tubulin. Tubulin served as a loading control. **B**: CSN5 blocks self-ubiquitination of MDM2. 293T cells were cotransfected with indicated plasmids. The cell lysates were harvested, and IP with anti-MDM2. The protein complexes were then resolved on 8% SDS-polyacrylamide gel and probed with anti-ubiquitin antibody to reveal the ubiquitinated MDM2. Equal amounts of cell lysates were IB with anti-Flag, anti-tubulin. Cells were treated with MG132 for 8 h prior to collection. **C**: CSN5 reduces the turnover rate of MDM2. 293T cells transfected with the indicated plasmids were treated with cycloheximide (CHX) (100 μg/ml) for the indicated hours. Cell lysates were IB with anti-MDM2, anti-Flag, and anti-tubulin. **D**: Levels of MDM2 at each time point in (C) were measured using a NIH image program. The remaining level of MDM2 at time 0 was set at 100%. MDM2 remaining is indicated graphically.

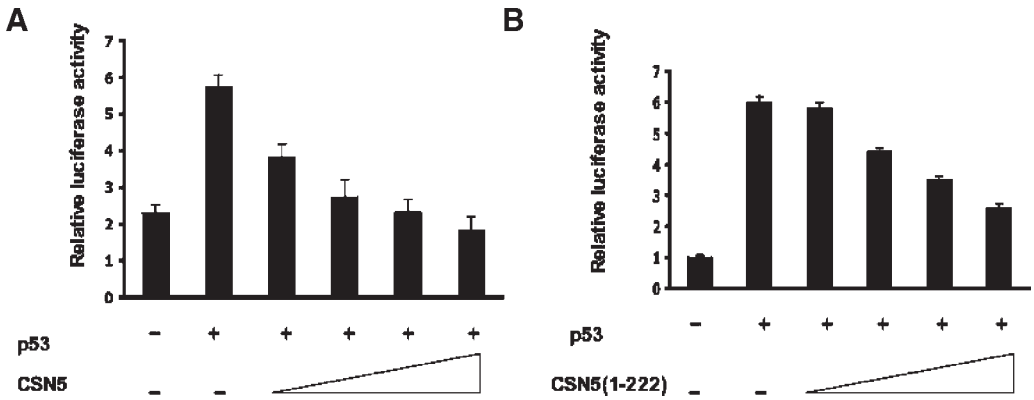

Fig. 7. CSN5 antagonizes the transcriptional activity of p53. **A**: CSN5 antagonizes p53 transcriptional activity. The BDS2-3X-luc reporter containing a p53-responsive element was transfected with the indicated p53 or increasing amounts of CSN5-expressing vectors into R1B/L17 cells. Relative luciferase activity is shown. **B**: Same as (A), except that CSN5 (1–222aa) was assayed for its impact on p53 transcriptional activity.

CSN5 (1–220 aa) block the p53 transcriptional activity (Fig. 7B), possibly via degrading p53.

DISCUSSION

CSN5 plays a pivotal role in cancer formation, and is aberrantly overexpressed in various types of cancer. This oncogenic activity may arise mostly from the elevated activity in degrading important regulators involved in cell proliferation control. Thus, CSN5 can be an important molecular target for rational cancer therapy. Our study shows that curcumin negatively regulates CSN5 and increases the expression of p53 (Fig. 1). Importantly, curcumin, a naturally occurring yellow pigment with anti-proliferative and apoptotic activities, is a potent suppressor of tumor formation [Lin, 2004]. Although curcumin is used in phase I clinical trial for the treatment of cancer [Sharma et al., 2004]. The mechanism by which curcumin mediates tumor suppression remains not well understood. Our results indicate that curcumin can mediate downregulation of both CSN5 and MDM2, which in turn leads to p53 stabilization, offering new clues as to how curcumin may have tumor suppressive activities.

The finding that embryos of CSN5-null mice have an accumulation of p53 suggests that this protein is a crucial regulator of p53 stability [Tomoda et al., 2004]. Three different lines of evidence obtained from our studies demonstrate the activity of CSN5 as a critical regulator of p53 via MDM2. First, CSN5 can potentiate MDM2-mediated p53 ubiquitination, which results in p53 degradation. It is known that CSN can coordinate with ubiquitin ligases to regulate their activities [Cope and Deshaies, 2003]. Here, we showed that for the first time a component of CSN is involved in association with MDM2 ubiquitin ligase. In this case, CSN5 interacts with MDM2 ubiquitin ligase and may bring the MDM2 substrate such as p53 closer to the ubiquitination machinery. It is noteworthy that CSN5 contains a JAMM metalloprotease motif that is critical for cleavage of NEDD8 [Ambroggio et al., 2004], an ubiquitin-like protein, from Cul1, thereby regulating Cul-containing ubiquitin ligase activity [Kawakami et al., 2001]. Recently, MDM2 is known to be self-NEDDylated, and has the capability to neddylate p53 [Xirodimas et al., 2004]. It will be interesting to determine whether CSN5 can regulate the NEDDylation process of MDM2. Second, CSN5 can antagonize MDM2 self-ubiquitination, reducing the turnover rate of MDM2, which can strengthen MDM2 activity toward p53. MDM2 is regulated by negative regulators including ARF [Zhou et al., 2001], PML [Bernardi et al., 2004], and ribosomal proteins L5 [Dai and Lu, 2004], L11 [Lohrum et al., 2003], L23 [Dai et al., 2004; Jin et al., 2004], 14-3-3 σ, but positively regulated by Gankyrin [Higashitsuji et al., 2005], YY1 [Sui et al., 2001], and KAP1 [Wang et al., 2005] (also reviewed in Lee and Lozano [2006]). It is not clear how CSN5 can stabilize MDM2. It is possible that CSN5 has impact on some of the regulators of MDM2, which will result in MDM2 stabilization. Third, CSN5 can mediate the nuclear export of p53, which may facilitate the degradation of p53. CSN5 is known to mediate the translocation of several proteins from the nucleus to the cytoplasm such as p27 [Tomoda et al., 1999]. Here, we also showed that

CSN5 is efficient in facilitating the exportation of p53 from the nucleus to the cytoplasm. Given that CSN5 can stabilize MDM2, a known protein involved in p53 nuclear export, it is conceivable that CSN5 expression leads to the translocation of p53 from the nucleus to the cytoplasm.

MDM2 levels are associated with poor prognosis of several types of human cancer [Momand et al., 1998]. It has been shown that MDM2's negative impact on p53 stability in cancer can compromise the magnitude of p53 activation by cancer therapeutic agents, which causes DNA damaging to eliminate cancer cells, thereby reducing therapeutic effectiveness [Momand et al., 1998]. Obviously, MDM2 is an important molecular target for rational cancer therapy [Zhang and Wang, 2000]. A strategy to activate p53 in tumor by inhibiting MDM2 activity has been the focus in cancer drug discovery. For example, anti-sense oligonucleotides to inhibit the expression of MDM2 have been employed [Bianco et al., 2005; Zhang et al., 2005]. Also, small-molecule inhibitors of MDM2, the Nutlins, have been developed to inhibit the interaction of p53 and MDM2 [Ambrosini et al., 2007; Cao et al., 2006]. Here, we showed that CSN5 can stabilize MDM2, thereby accelerating p53 degradation and compromising p53 transcriptional activity. As described above, CSN5 is overexpressed during tumor progression. Together, it stands to reason that CSN5 is a potential molecular target for cancer therapy, and our studies may provide insight into strategic design for using CSN5 as a target for anti-cancer drug development.

METHODS

Cell Lines and Cell Culture

293T, R1B/L17 (the mink lung epithelial cell line derivative) [Lee et al., 1995], H1299 cells, A549 cells, MCF7 cells (from ATCC), Rat1 and Rat1-akt cells [Yang et al., 2006], p53−/− MEF, and p53/Mdm2 double knock-out (p53−/− MEF and DKO MEF, kind gifts of Dr. Gigi Lozano) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Plasmids, Reagents, Antibodies, and Western Blots

Flag-14-3-3σ has been described previously [Yang et al., 2003]. Plasmids of CSN5 (1–120, 102–334, 102–222, 1–222, 102–222) were constructed by PCR cloning using designed primers. A PCR generated fragment of the cDNA was subcloned into pCMV5 to yield a construct that encodes CSN5 domains with a Flag-tagged sequence. GFP-p53 was kindly provided by Dr. G Wahl [Stommel et al., 1999]. Curcumin, cycloheximide and MG132 were from Sigma. Antibodies against MDM2 (SMP14, Santa Crutz), p53 (DO1, oncogene science), p53 (FL393, Santa Cruz biotechnology), tubulin (B-5-1-2, Sigma), CSN5 (Bethyl), Myc (9E10), and Flag epitope (Sigma), were used for IB or IP. Total cell lysates were solubilized in lysis buffer (20 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP40, 0.5% Triton X-100, 1 mM PMSF, 1 mM NaF, 1 mM sodium orthovanadate, 1 μg each of aprotinin, leupeptin, and pepstatin per ml) and were processed as previous described [Lee et al., 1995]. Western analysis was performed with chemiluminescence (ECL) system (Roche Molecular Biochemicals) according to the manufacturer's instructions. Protein band quantitation analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Ubiquitination Assay

The 293T or H1299 cells were cotransfected with indicated plasmids. After 24 h posttransfection, cells were treated with 200 μM ALLN (*N*-acetyl-Leu-Leu-norleu-AL, Sigma) for 12 h. The cell lysates were harvested with lysis buffer (50 mM Tris pH 7.5, 150 mm NaCl, 0.5% NP40, 0.5% Triton-100, 5 mM NEM). The His-ubiquitinated p53 were brought down using His-Bind resin (Novagen, Germany). The ubiquitinated MDM2 were IP with anti-MDM2. The protein complexes were then resolved by 8% SDS-polyacrylamide gel and probed with anti-p53 or anti-ubiquitin to observe the ubiquitinated p53 or MDM2.

Live-Cell Imaging

Live-cell images of H1299 cells expressing GFP-p53 and CSN5 expressing constructs were captured with a cooled Orca-ER CCD camera (Hamamatsu, Japan) mounted on a Zeiss Axiovert 200 M microscope. It is fully motorized, equipped with the heated stages, and heat and CO2 controlled incubators. The Mark and Find

software allows the stage to move around in a controlled fashion and acquire images in phase contrast and multicolor fluorescent images. Hoechst 33342 (0.5 μg/ml) was added for DNA staining.

Luciferase Reporter Gene Assay

The BDS2-3X-luc reporter containing a p53-responsive element [Hermeking et al., 1997] was transfected with the pCMV-p53- or pCMV-CSN5 expressing vectors into R1B/L17 cells. Luciferase activity was assayed with the dual luciferase assay system (Promega) according to the manufacturer's instructions.

ACKNOWLEDGMENTS

We wish to thank Dr. Vogelstein, Dr. Wahl, Dr. Zhou, Dr. Hung for valuable reagents, and Bill Spohn for live-cell imaging help. This work was supported in part by the NIHRO1CA (089266) and Cancer Center Core Grant (CA16672). M-H Lee is a recipient of the Flemin and Davenport research award.

REFERENCES

Adler AS, Lin M, Horlings H, Nuyten DS, van de Vijver MJ, Chang HY. 2006. Genetic regulators of large-scale transcriptional signatures in cancer. Nat Genet 38:421–430. Epub 2006 Mar 5.

Ambroggio XI, Rees DC, Deshaies RJ. 2004. JAMM: A metalloprotease-like zinc site in the proteasome and signalosome. PLoS Biol 2:E2. Epub 2003 Nov 24

Ambrosini G, Sambol EB, Carvajal D, Vassilev LT, Singer S, Schwartz GK. 2007. Mouse double minute antagonist Nutlin-3a enhances chemotherapy-induced apoptosis in cancer cells with mutant p53 by activating E2F1. Oncogene 26(24):3473–3481. Epub 2006 Dec 4.

Asano K, Vornlocher HP, Richter-Cook NJ, Merrick WC, Hinnebusch AG, Hershey JW. 1997. Structure of cDNAs encoding human eukaryotic initiation factor 3 subunits. Possible roles in RNA binding and macromolecular assembly. J Biol Chem 272:27042–27052.

Bech-Otschir D, Kraft R, Huang X, Henklein P, Kapelari B, Pollmann C, Dubiel W. 2001. COP9 signalosome-specific phosphorylation targets p53 to degradation by the ubiquitin system. Embo J 20:1630–1639.

Bernardi R, Scaglioni PP, Bergmann S, Horn HF, Vousden KH, Pandolfi PP. 2004. PML regulates p53 stability by sequestering Mdm2 to the nucleolus. Nat Cell Biol 6:665–672.

Bianco R, Ciardiello F, Tortora G. 2005. Chemosensitization by antisense oligonucleotides targeting M DM2. Curr Cancer Drug Targets 5:51–56.

Cao C, Shinohara ET, Subhawong TK, Geng L, Woon Kim K, Albert JM, Hallahan DE, Lu B. 2006. Radiosensitization of lung cancer by nutlin, an inhibitor of murine double minute 2. Mol Cancer Ther 5:411–417.

Cope GA, Deshaies RJ. 2003. COP9 signalosome: A multifunctional regulator of SCF and other cullin-based ubiquitin ligases. Cell 114:663–671.

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 298:608–611. Epub 2002 Aug 15.

Dai MS, Lu H. 2004. Inhibition of MDM2-mediated p53 ubiquitination and degradation by ribosomal protein L5. J Biol Chem 279:44475–44482.

Dai MS, Zeng SX, Jin Y, Sun XX, David L, Lu H. 2004. Ribosomal protein L23 activates p53 by inhibiting MDM2 function in response to ribosomal perturbation but not to translation inhibition. Mol Cell Biol 24:7654–7668.

Esteva FJ, Sahin AA, Rassidakis GZ, Yuan LX, Smith TL, Yang Y, Gilcrease MZ, Cristofanilli M, Nahta R, Pusztai L, Claret FX. 2003. Jun activation domain binding protein 1 expression is associated with low p27(Kip1)levels in node-negative breast cancer. Clin Cancer Res 9:5652–5659.

Fang S, Jensen JP, Ludwig RL, Vousden KH, Weissman AM. 2000. Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. J Biol Chem 275:8945–8951.

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

Higashitsuji H, Itoh K, Sakurai T, Nagao T, Sumitomo Y, Masuda T, Dawson S, Shimada Y, Mayer RJ, Fujita J. 2005. The oncoprotein gankyrin binds to MDM2/HDM2, enhancing ubiquitylation and degradation of p53. Cancer Cell 8:75–87.

Iwakuma T, Lozano G. 2003. MDM2, an introduction. Mol Cancer Res 1:993–1000.

Jin A, Itahana K, O'Keefe K, Zhang Y. 2004. Inhibition of HDM2 and activation of p53 by ribosomal protein L23. Mol Cell Biol 24:7669–7680.

Karniol B, Chamovitz DA. 2000. The COP9 signalosome: From light signaling to general developmental regulation and back. Curr Opin Plant Biol 3:387–393.

Kawakami T, Chiba T, Suzuki T, Iwai K, Yamanaka K, Minato N, Suzuki H, Shimbara N, Hidaka Y, Osaka F, Omata M, Tanaka K. 2001. NEDD8 recruits E2-ubiquitin to SCF E3 ligase. EMBO J 20:4003–4012.

Kouvaraki MA, Rassidakis GZ, Tian L, Kumar R, Kittas C, Claret FX. 2003. Jun activation domain-binding protein 1 expression in breast cancer inversely correlates with the cell cycle inhibitor p27(Kip1). Cancer Res 63:2977–2981.

Lee MH, Lozano G. 2006. Regulation of the p53-MDM2 pathway by 14-3-3sigma and other proteins. Semin Cancer Biol 16:225–234. Epub 2006 Apr 6.

Lee MH, Reynisdottir I, Massague J. 1995. Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. Genes Dev 9:639–649.

Li L, Deng XW. 2003. The COP9 signalosome: An alternative lid for the 26S proteasome? Trends Cell Biol 13:507–509.

Lin JK. 2004. Suppression of protein kinase C and nuclear oncogene expression as possible action mechanisms of cancer chemoprevention by Curcumin. Arch Pharm Res 27:683–692.

Lohrum MA, Ludwig RL, Kubbutat MH, Hanlon M, Vousden KH. 2003. Regulation of HDM2 activity by the ribosomal protein L11. Cancer Cell 3:577–587.

Lozano G, Montes de Oca Luna R. 1998. MDM2 function. Biochim Biophys Acta 1377:M55–M59.

Momand J, Jung D, Wilczynski S, Niland J. 1998. The MDM2 gene amplification database. Nucleic Acids Res 26:3453–3459.

Naumann M, Bech-Otschir D, Huang X, Ferrell K, Dubiel W. 1999. COP9 signalosome-directed c-Jun activation/stabilization is independent of JNK. J Biol Chem 274:35297–353300.

Penney M, Wilkinson C, Wallace M, Javerzat JP, Ferrell K, Seeger M, Dubiel W, McKay S, Allshire R, Gordon C. 1998. The Pad1+gene encodes a subunit of the 26 S proteasome in fission yeast. J Biol Chem 273:23938–23945.

Rinaldi T, Bolotin-Fukuhara M, Frontali L. 1995. A Saccharomyces cerevisiae gene essential for viability has been conserved in evolution. Gene 160:135–136.

Sharma RA, Euden SA, Platton SL, Cooke DN, Shafayat A, Hewitt HR, Marczylo TH, Morgan B, Hemingway D, Plummer SM, Pirmohamed M, Gescher AJ, Steward WP. 2004. Phase I clinical trial of oral curcumin: Biomarkers of systemic activity and compliance. Clin Cancer Res 10:6847–6854.

Stommel JM, Marchenko ND, Jimenez GS, Moll UM, Hope TJ, Wahl GM. 1999. A leucine-rich nuclear export signal in the p53 tetramerization domain: Regulation of subcellular localization and p53 activity by NES masking. EMBO J 18:1660–1672.

Sui L, Dong Y, Ohno M, Watanabe Y, Sugimoto K, Tai Y, Tokuda M. 2001. Jab1 expression is associated with inverse expression of p27(kip1) and poor prognosis in epithelial ovarian tumors. Clin Cancer Res 7:4130–4135.

Sun Y, Wilson MP, Majerus PW. 2002. Inositol 1,3,4-trisphosphate 5/6-kinase associates with the COP9 signalosome by binding to C SN1. J Biol Chem 277:45759–45764. Epub 2002 Sep 24.

Tomoda K, Kubota Y, Kato J. 1999. Degradation of the cyclin-dependent-kinase inhibitor p27Kip1 is instigated by Jab1. Nature 398:160–165.

Tomoda K, Yoneda-Kato N, Fukumoto A, Yamanaka S, Kato JY. 2004. Multiple functions of Jab1 are required for early embryonic development and growth potential in mice. J Biol Chem 279:43013–43018. Epub 2004 Aug 06.

Uhle S, Medalia O, Waldron R, Dumdey R, Henklein P, Bech-Otschir D, Huang X, Berse M, Sperling J, Schade R, Dubiel W. 2003. Protein kinase CK2 and protein kinase D are associated with the COP9 signalosome. EMBO J 22:1302–1312.

Wang C, Ivanov A, Chen L, Fredericks WJ, Seto E, Rauscher FJ III, Chen J. 2005. MDM2 interaction with nuclear corepressor KAP1 contributes to p53 inactivation. EMBO J 24:3279–3290. Epub 2005 Aug 18.

Wei N, Deng XW. 2003. The COP9 signalosome. Annu Rev Cell Dev Biol 19:261–286.

Wei N, Tsuge T, Serino G, Dohmae N, Takio K, Matsui M, Deng XW. 1998. The COP9 complex is conserved between plants and mammals and is related to the 26S proteasome regulatory complex. Curr Biol 8:919–922.

Xirodimas DP, Saville MK, Bourdon JC, Hay RT, Lane DP. 2004. Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity. Cell 118:83–97.

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 23:7096–7107.

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 66:3096–3105.

Zhang R, Wang H. 2000. MDM2 oncogene as a novel target for human cancer therapy. Curr Pharm Des 6:393–416.

Zhang R, Wang H, Agrawal S. 2005. Novel antisense anti-MDM2 mixed-backbone oligonucleotides: Proof of principle, in vitro and in vivo activities, and mechanisms. Curr Cancer Drug Targets 5:43–49.

Zhou BP, Liao Y, Xia W, Zou Y, Spohn B, Hung MC. 2001. HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. Nat Cell Biol 3:973–982.