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RESEARCH ARTICLE

CSN1 inhibits c-Jun phosphorylation and down-regulates ectopic expression of JNK1

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ABSTRACT

CSN1 is a component of the COP9 signalosome (CSN), a conserved protein complex with pleiotropic functions in many organs and cell types. CSN regulates ubiquitin-proteasome dependent protein degradation *via* the deneddylation and the associated deubiquitination activities. In addition, CSN associates with protein kinases and modulates cell signaling, particularly the activator protein 1 (AP-1) pathway. We have shown previously that CSN1 suppresses AP-1 transcription activity and inhibits ultraviolet (UV) and serum activation of *c-fos* expression. Here we show that CSN1 can inhibit phosphorylation of proto-oncogene c-Jun product and repress c-Jun dependent transcription. Further, CSN1 dramatically down-regulates ectopic expression of c-Jun N-terminal kinase 1 (JNK1) in cultured cells. The decline in JNK1 is not caused by excessive proteolysis or by 3′ UTR-dependent mRNA instability, but by CSN1-dependent repression of one or multiple steps in transcriptional and post-transcriptional mechanisms. Thus, in contrast to CSN5/Jab1, which promotes AP-1 activity, CSN1 displays a negative effect on the AP-1 pathway. Finally, we discuss about the dynamic equilibrium of the CSN complexes in regulation of the AP-1 pathway.

KEYWORDS activator protein 1 (AP-1), c-Jun phosphorylation, COP9 signalosome (CSN), CSN1/GPS1, c-Jun N-terminal kinase 1 (JNK1)

INTRODUCTION

The *c-Jun* proto-oncogene encodes a major component of activator protein 1 (AP-1) transcription factors, which regulate proliferation, survival, differentiation and stress responses in both normal and transformed cells. Upon activation by growth factors, pro-inflammatory cytokines and extracellular stimuli such as UV irradiation, oxidative stress and osmotic shock, *c-Jun* gene expression is induced and c-Jun protein is phosphorylated on Ser-63 and Ser-73, which lead to AP-1 transcriptional activation (Karin 1995; Davis 2000). JNK/SAPK (c-Jun N-terminal kinase/stress-activated protein kinase) are the predominant mitogen-activated protein (MAP) kinases responsible for c-Jun N-terminal phosphorylation (Dérijard et al., 1994).

COP9 signalosome (CSN) is a conserved protein complex consisting of eight subunits, from CSN1 to CSN8 (Wei and Deng, 2003). CSN has an intrinsic isopeptidase activity that deconjugates the ubiquitin-like protein Nedd8/Rub1 from cullin ubiquitin ligases (deneddylation) (Cope and Deshaies 2003). In addition, the complex can associate with protein kinases such as protein kinase CK2 (CK2, formerly casein kinase II), protein kinase D (PKD), and inositol 1,3,4-triphosphate 5/6-kinase (5/6-kinase), which have been shown to phosphorylate c-Jun (Wilson et al., 2001; Uhle et al., 2003). One of CSN subunits, CSN5, also known as Jab1 (c-Jun activation domain binding protein 1), has been identified as a transcription co-activator that enhances transcription activity of c-Jun (Claret et al., 1996) and Myc (Adler et al., 2006). In

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addition, CSN5 functions both as part of the CSN complex and in CSN-free form outside the complex (Wei et al., 2008).

We and others have previously shown that CSN1 inhibits AP-1 and serum response element (SRE) mediated transcription activities (Spain et al., 1996; Tsuge et al., 2001). We showed that CSN1 can repress UV and serum induced expression of *c-fos-lacZ* chimeric gene in mouse fibroblast cells (Tsuge et al., 2001). In addition, CSN1 was shown to mediate interaction of CSN complex with 5/6-kinase, which can phosphorylate c-Jun and ATF-2 (Wilson et al 2001; Sun et al 2002). Moreover, ectopic expression of CSN1 was shown to inhibit the CSN-associated 5/6-kinase activity (Sun et al., 2002).

Human CSN1 contains a large PCI domain at the C-terminal half of the protein (Pick et al., 2009). This C-terminal domain (CTD) 280 amino acid residue (aa) region harbors the binding sites for CSN2, 3, and 4, and is necessary and sufficient for CSN1 to integrate into the CSN complex with other subunits (Tsuge et al., 2001). The N-terminal domain (NTD) 196 aa fragment of human CSN1 (CSN1-N), on the other hand, does not associate with the complex, but it is necessary and sufficient to inhibit AP-1 activity and *c-fos* expression (Tsuge et al., 2001). In *Arabidopsis*, the corresponding CSN1-NTD was similarly found to be dispensable for complex assembly, as the CSN1-NTD deletion mutant (*fus6/C231*) can assemble the mutant form of CSN holocomplex that lacks this domain (Wang et al., 2002). Yet, this mutant dies prematurely and exhibits dramatic gene expression defects, indicating that CSN1-NTD is essential for the viability of the plants (Wang et al., 2002). This genetic study confirmed the biological significance of the CSN1-NTD-dependent functions. Further molecular investigations on CSN1-NTD have identified a protein interacting with CSN1-NTD, SAP130/SF3b-3 (Menon et al., 2008). SAP130 not only is a component of several transcription complexes and the RNA splicing complex, but it also interacts with cullin proteins, the substrate of CSN's deneddylation activity and the scaffold component for cullin-RING ubiquitin E3 ligases (Menon et al., 2008).

Although CSN is best known to regulate ubiquitin-proteasome pathway, it also plays an important role in gene expression (Chamovitz, 2009). Here we show that CSN1 inhibits c-Jun phosphorylation without affecting its protein stability. We found that CSN1 blocks ectopic expression of JNK1, therefore providing another piece of supporting evidence for a role of CSN1 in gene expression.

RESULTS

CSN1 inhibits c-Jun phosphorylation at Ser-63

We previously showed that CSN1 inhibits AP-1 transcription activity (Tsuge et al., 2001). Here we further investigated the effect of CSN1 on c-Jun phosphorylation and transcription activity. Activation of c-Jun was induced in cultured cells by expressing a constitutively active form of upstream kinase MEKK1Δ. As shown in Fig. 1A, CSN1 and CSN1-NTD (Flag-CSN1-N) significantly diminished MEKK1-activated c-Jun transcription activity in a dosage-dependent manner, as determined by the reporter assay. CSN1-CTD (Flag-CSN1-C) and the negative control construct, CSN1-AS, showed

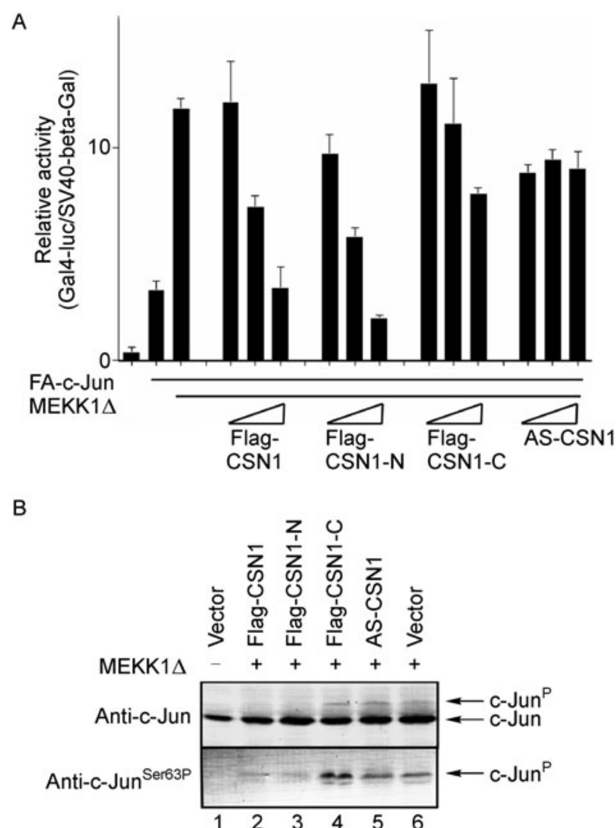

Figure 1. Inhibition of MEKK1 induced c-Jun phosphorylation and transcription by CSN1. (A) The transcription reporter Gal4-luc containing Gal4 binding sites was activated by FA-c-Jun in NIH3T3 cells. Further activation was conferred by expressing a constitutively active form of upstream kinase MEKK1Δ. Increasing amounts of CSN1 expression constructs and CSN1 anti-sense construct (AS-CSN1) were transfected in amount of 0.4, 1.0, and 2.0 μg, respectively. The luciferase activity was normalized against SV40-beta-Gal internal control as described in MATERIALS AND METHODS. Both CSN1 and CSN1-NTD inhibited c-Jun activated transcription in this assay. Data are presented as the mean ± SD. (B) The endogenous c-Jun phosphorylation of NIH3T3 cells was induced by MEKK1Δ (0.5 μg) in lanes 2–6. Constructs (2 μg) were co-transfected, as indicated on the top. Extracts were blotted for anti-c-Jun (upper) and anti-c-Jun^{Ser63P} (lower) antibodies to detect the level of c-Jun phosphorylation. Phosphorylation of c-Jun was reduced by Flag-CSN1 and Flag-CSN1-N, which expressed Flag-tagged full-length CSN1 and CSN1-NTD fragment, respectively.

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minimum or no effect. Next we examined phosphorylation of c-Jun at its active site Ser-63. Phosphorylation of endogenous c-Jun was induced by MEKK1Δ in NIH3T3 cells, and was detected by anti-c-Jun Ser-63-phosphor-dependent (anti-c-Jun^{Ser63P}) or anti-c-Jun immunoblots. Expression of CSN1 or CSN1-NTD clearly reduced c-Jun Ser^{63} phosphorylation (Fig. 1B: lanes 2 and 3 compared to lane 6).

UV irradiation is a natural inducer of c-Jun phosphorylation. To determine the role of CSN1 in this signaling cascade, NIH3T3 cells transiently expressing Flag-CSN1 or its truncated fragments were irradiated with UV light. Phosphorylation of c-Jun was detected by immunofluorescent staining with anti-c-Jun^{Ser63P} antibody, while CSN1 transfected cells were identified by double staining with anti-Flag antibody (Fig. 2A). UV irradiation led to c-Jun phosphorylation in about 85% of normal untransfected cells (Fig. 2B). However, in cells expressing Flag-CSN1 and Flag-CSN1-N, only 25% and 14% of cells, respectively, displayed above background level of anti-c-Jun^{Ser63P} immunoreaction (Fig. 2B), while the immunoreaction against total amounts of c-Jun was not affected by Flag-CSN1 (Fig. 2C). The CTD of CSN1, Flag-CSN1-C, has no detectable effect on c-Jun phosphorylation (Fig. 2B). Immunofluorescence staining with anti-c-Jun antibodies indicated that the total amounts of c-Jun were similar between transfected and untransfected cells (Fig. 2C). We conclude from these results that transient expression of CSN1 or its NTD can suppress UV and MEKK1 stimulated c-Jun phosphorylation.

CSN1 inhibits JNK1 transient expression

It was reported that CSN1 could repress JNK1 kinase activity when an epitope tagged JNK1 was co-expressed with CSN1 in cultured cells, but the expression level of JNK1 was not examined in that study (Spain et al., 1996). In an effort to understand the mechanism of CSN1, we started by performing the same co-transfection experiment. Surprisingly, we found that the expression level of Flag-JNK1 was drastically reduced by Flag-CSN1, and that the reduction was independent of stimulation in the JNK pathway by UV irradiation,

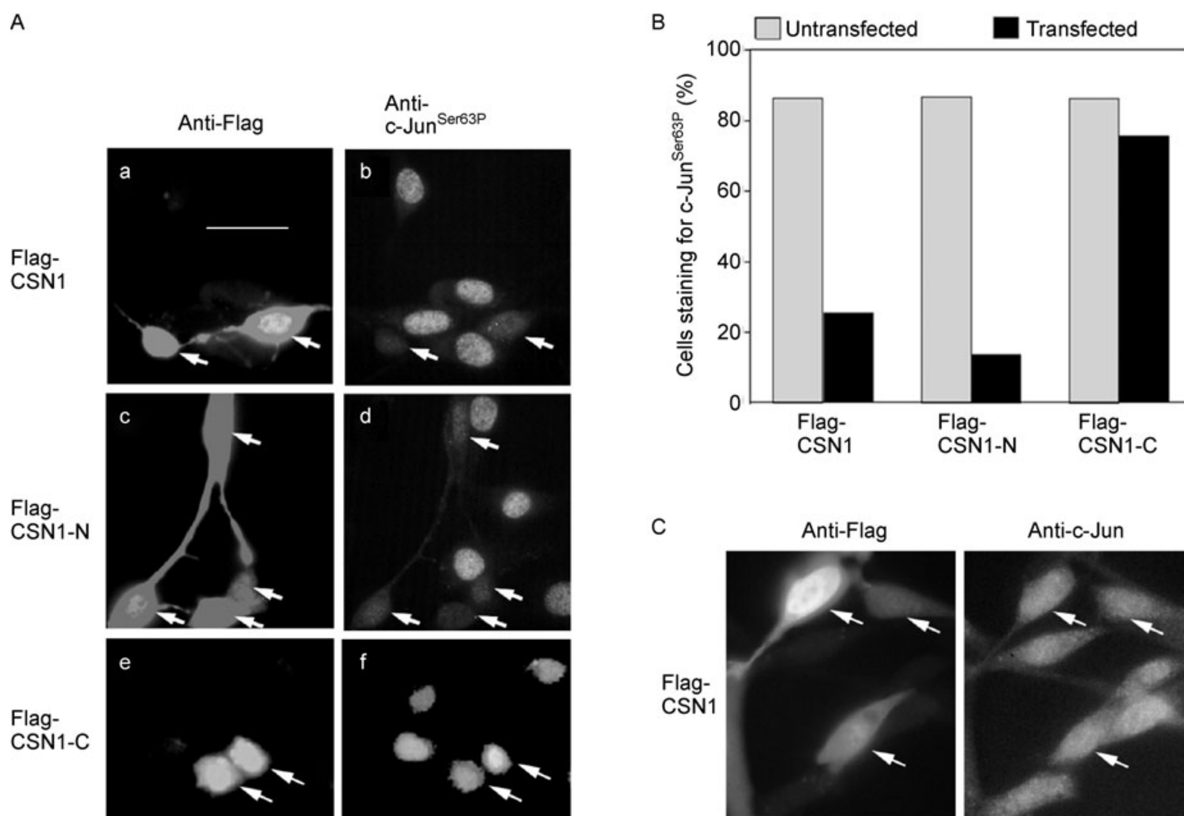

Figure 2. CSN1 blocks UV stimulated c-Jun phosphorylation without affecting its stability. (A) NIH3T3 cells were transfected to express Flag-CSN1 (a and b), Flag-CSN1-N (c and d), and Flag-CSN-C (e and f). Cells were irradiated with UV-C (80 J/m^2), fixed, and immunofluorescently labeled with anti-Flag (a, c, and e) and anti-c-Jun^{Ser63P} antibodies (b, d, and f) in combination with Texas-red conjugated anti-mouse antibodies and FITC-conjugated anti-rabbit antibodies, respectively. Arrows indicate transfected cells, expressing Flag-tagged CSN1 proteins. Cells expressing full-length CSN1 or CSN1-NTD display near background levels of anti-c-Jun^{Ser63P} staining. (B) The graph displays the statistical summary of cells staining positive for anti-c-Jun^{Ser63P} antibody, among transfected or untransfected cells, as percentages in their respective populations. (C) The intensity of total c-Jun immunoreactivity was not affected by expression of CSN1 (comparing arrowed cells to non-arrowed cells).

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sorbitol induced osmotic shock, or by a microtubule toxin, nocodazole (Fig. 3A). Under the same condition, Flag-CSN1 did not affect the protein level of Flag-c-Jun, but it weakened its phosphorylation as noted earlier. The effect of CSN1 on JNK1 expression precluded the suitability of this transient system in accessing possible inhibition of JNK1 kinase activity by CSN1. However, since down-regulation of JNK1 may indicate an important and undefined activity of CSN1, we conducted further investigations on how CSN1 affects JNK1 expression.

To determine whether CSN1 affects only the active JNK1, we utilized in the experiment a kinase-inactive mutant, Flag-JNK1 (APF). This mutant contains point mutations at Thr-183 and Tyr-185 phosphorylation sites that are necessary for JNK1 activation (Dérijard et al., 1994). Similar to wild-type JNK1, the expression of Flag-JNK1 (APF) remained sensitive to CSN1 (Fig. 3B). Therefore, JNK1 activity is not necessary for its down-regulation by CSN1. In addition, CSN1-NTD was similarly effective as the full-length CSN1, in down-regulation of JNK1 (Fig. 4A).

CSN1 mediated down-regulation of JNK1 is not caused by excessive proteolysis

Since CSN has a role in the ubiquitin pathway, we tested the idea that the observed JNK1 reduction could be due to increased protein turnover *via* the ubiquitin-proteasome mediated degradation. In this case, inhibitors of protein degradation pathways would rescue JNK1 from degradation. However, when cells were treated with proteasome inhibitor MG132 (Fig. 4A), down-regulation of Flag-JNK1 was not rescued. On the contrary, MG132 further exaggerated CSN1 effect such that the difference of Flag-JNK1 levels in the presence and absence of CSN1 was further enhanced (Fig. 4A: comparing lanes 2 and 3). This apparent synergistic effect between MG132 and CSN1 was not caused by stabilization of CSN1 proteins, since Flag-CSN1 level was not detectably affected by MG132 application (data not shown). Consistently, Flag-CSN1-C, which expressed at similar level to Flag-CSN1 and Flag-CSN1-N, did not exhibit any effect.

Another well-established pathway of intracellular protein degradation is mediated by lysosomal process in which ubiquitin may also play a role. This pathway can be inhibited by 3-methyladenine (3-MA) (Seglen and Gordon, 1982; Fuertes et al., 2003). As was the case with MG132, 3-MA also failed to rescue the Flag-JNK1 protein level (Fig. 4A). These results suggest that proteolysis is unlikely to be the cause of JNK1 down-regulation.

To determine whether CSN1 affects protein expression, we conducted a protein pulse labeling experiment in which HeLa cells expressing Flag-JNK1 or Flag-c-Jun with or

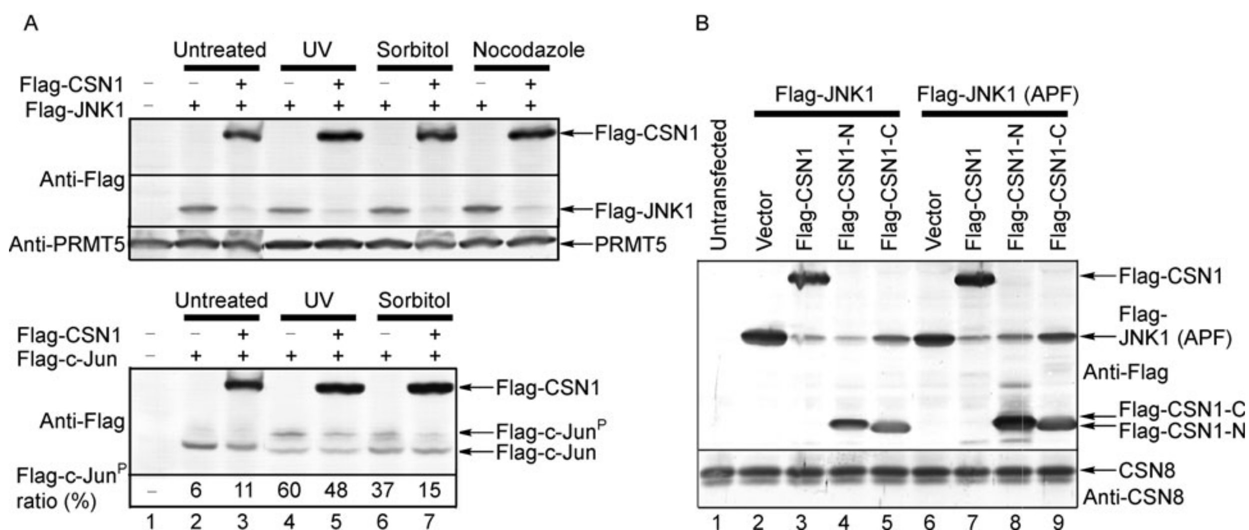

Figure 3. CSN1 inhibits transient expression of Flag-JNK1. (A) HEK293 cells were transfected with constructs expressing Flag-JNK1 (2 µg) (upper panel) or Flag-c-Jun (1 µg) (lower panel) with Flag-CSN1 (1.5 µg) or empty vector (1.5 µg) to test for protein expression. When indicated, cells were treated with UV-C (40 J/m^2) 5 h prior to sampling, or with sorbitol induced osmotic shock (300 mmol/L) or a microtubule toxin nocodazole (2 µmol/L) for 30 min. Expression of Flag-JNK1 and Flag-c-Jun were detected by anti-Flag immunoblots. Anti-PRMT5 immunoblot was shown as a loading control in the upper panel. Note that CSN1 did not affect the protein level of Flag-c-Jun, but it weakened its phosphorylation. Numbers in the lower panel indicate the amount of the phosphorylated c-Jun signals over the respective total (phosphorylated and non-phosphorylated together) c-Jun signals, in percentage. (B) Experiments similar to the above were performed, except that Flag-JNK1 (APF) (2 µg) kinase-inactive mutant was also used in the experiment. Constructs expressing CSN1 and its truncations (1.5 µg) were co-transfected as indicated. Anti-Flag immunoblots detected expression of Flag-JNK1 or the APF mutant form, and anti-CSN8 immunoblot was shown as a loading control. Note that the expression of Flag-JNK1 (APF) remained sensitive to CSN1.

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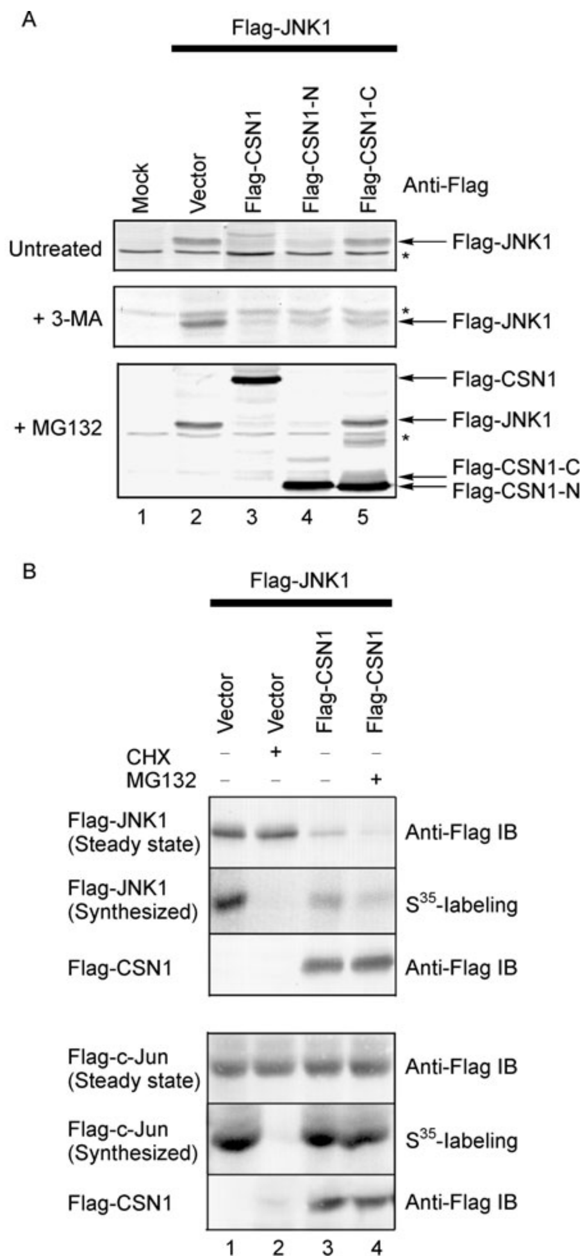

Figure 4. Down-regulation of JNK1 by CSN1 is due to reduced synthesis rather than proteolysis. (A) Proteasomal and lysosomal proteolysis inhibitors, MG132 and 3-MA respectively, did not block CSN1 mediated down-regulation of JNK1. HeLa cells were transfected with constructs expressing Flag-JNK1, together with empty vector or constructs expressing CSN1 or its truncations. MG132 or 3-methyladenine (3-MA) was added to the culture medium 3 h prior to sample collection. Total cell extracts were subject to anti-Flag immunblot analysis to detect the level of Flag-JNK1. Asterisk marks non-specific bands. (B) In experiments similar to the above, new protein synthesis was radio-labeled for 30 min with S^{35} L-methionine and L-cysteine. When indicated, cycloheximide (CHX) or MG132 was added to inhibit protein synthesis or proteasome mediated proteolysis, respectively. Total cell lysates were analyzed by anti-Flag immunoblots (IB). To determine the amount of protein synthesis, Flag-JNK1 or Flag-c-Jun was immunoprecipitated using anti-Flag resin followed by SDS-PAGE and auto-radiography (S^{35}-labeling). Note that CSN1 strongly inhibited protein synthesis of Flag-JNK1 but not Flag-c-Jun.

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without Flag-CSN1 were metabolically labeled with S^{35}-Met for 30 min (Fig. 4B). Newly synthesized Flag-JNK1 and Flag-c-Jun proteins were detected by autoradiogram after immunoprecipitation (Fig. 4B: S^{35}-labeling), while the steady-state protein accumulation was detected by immunoblotting of the whole cell extract (Fig. 4B: IB). As expected, CHX, an inhibitor of protein biosynthesis, completely shut down protein synthesis without affecting the steady-state protein level (Fig. 4B: lane 2), validating the system. In this experiment, CSN1 strongly inhibited protein synthesis of Flag-JNK1 (Fig. 4B: comparing lanes 1 and 3 of S^{35}-labeling, upper panel) but not Flag-c-Jun (Fig. 4B: corresponding lanes of lower panel). Moreover, the extent of inhibition in Flag-JNK1 protein synthesis (S^{35}-labeling) was comparable to its decrease in steady-state levels (Fig. 4B: Total IB), indicating that the reduced protein synthesis can account for the observed decrease of the total Flag-JNK1 protein. In agreement with previous observations, MG132 treatment failed to rescue the diminishing level of Flag-JNK1 (Fig. 4B: lane 4, upper panel). We conclude from these results that the decline in the synthesis, rather than protein degradation, is responsible for down-regulation of JNK1 by CSN1.

The 3′ UTR is not responsible for CSN1 mediated repression

Although expressions of Flag-JNK1 and Flag-c-Jun were both driven by viral CMV promoter (Dérijard et al., 1994), only Flag-JNK1, and not Flag-c-Jun, was affected by CSN1. This suggests that the mechanism of CSN1-mediated down-regulation is probably promoter-independent. We tested different expression vectors for JNK1 and CSN1, and similar results were obtained (data not shown). To examine possible involvement of the 3′ untranslated region (UTR), we deleted from the original Flag-JNK1 plasmid the remaining JNK1 3′ UTR sequence of approximately 100 nucleotides and replaced with the bGH (bovine growth hormone) 3′ UTR/polyadenylation region, forming Flag-JNK1-(3′ UTR) (Fig. 5A). The expression of JNK1 from the resulting construct was still repressed by Flag-CSN1 (Fig. 5A). Similarly, Flag-JNK1 (1–248), which bears a deletion of 3′ UTR as well as 408 nucleotides of 3′ coding sequences corresponding to C-terminal 136 aa, was also down-regulated by Flag-CSN1 (Fig. 5A). Therefore the element responsive to CSN1 was not located at the 3′ UTR or near the C-terminal coding region of JNK1.

The major protein-coding-region determinant of instability (CRD or mCRD) pathways of mRNA decay is a key mechanism responsible for controlling *c-myc* and *c-fos* mRNA stability, and it works by triggering endonuclease cleavage within *c-myc* mCRD (Prokipcak et al., 1994; Lemm and Ross 2002), or by accelerating deadenylation of *c-fos* mRNA (Grosset et al., 2000). Ectopic expression of UNR (Upstream of N-ras), a cold-shock domain-containing RNA-binding protein, was shown to block the mCRD function

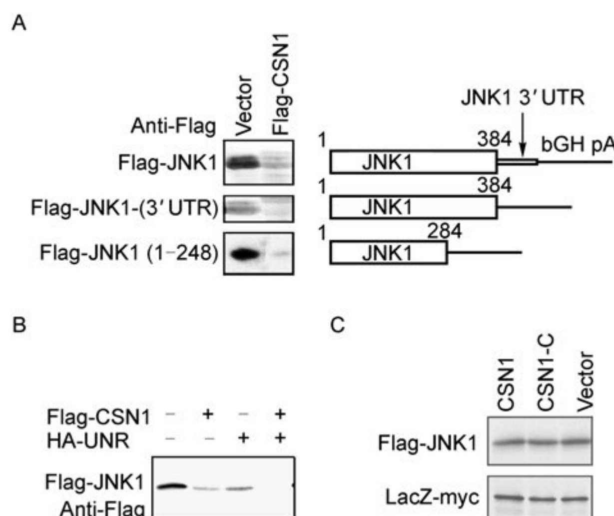

Figure 5. CSN1 inhibits Flag-JNK1 expression independently of JNK1 3′ UTR. (A) Deletion of JNK1 3′ UTR did not prevent its inhibition by CSN1. JNK1 3′ truncation constructs are diagrammed as shown on the right. On the left, the level of JNK1 expression from the corresponding constructs (1.2–1.5 µg) was detected by anti-Flag immunoblots. Empty vectors or constructs expressing CSN1 (0.3 µg) were co-transfected in HEK293 cells. (B) Experiments similar to the above were performed, except that a cold-shock domain-containing RNA-binding protein HA-UNR (1 µg) was co-expressed in the samples to block the mCRD function, as indicated. UNR ectopic expression did not affect the inhibition by CSN1 but reduced overall level of Flag-JNK1. (C) *In vitro* transcription-translation of Flag-JNK1 in rabbit reticulocyte lysate is not affected by CSN1. Flag-JNK1 or lacZ-myc was produced by T7 polymerase *in vitro*. The resulting mRNAs together with those of CSN1 or CSN1-C were translated into proteins in rabbit reticulocyte lysate.

(Grosset et al., 2000). We reasoned that if the observed JNK1 down-regulation was caused by a mCRD-dependent mechanism, then co-expression of UNR might disrupt mCRD and override the CSN1 effect. As shown in Fig. 5B, HA-UNR expression merely reduced the overall expression level of Flag-JNK1 without detectably compromising the effect of Flag-CSN1 on Flag-JNK1, suggesting that CSN1 adopts a mCRD independent pathway to repress JNK1 expression.

Inhibition of JNK1 expression by CSN1 may occur at many different levels of transcription process including transcription elongation and termination, RNA processing, polyadenylation, mRNA stability and nuclear export, or at translation. To test whether the inhibition can be observed in an *in vitro* translation system, a rabbit reticulocyte lysate transcription-translation coupled system was employed. Flag-JNK1 mRNA was synthesized by T7 polymerase and was translated *in vitro*. In this system, co-translation of CSN1 did not affect JNK1 protein production (Fig. 5C). This result argues against the possibility that CSN1 affects JNK1 protein synthesis at least in the *in vitro* system.

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CSN1 acts at the level of transcriptional and/or post-transcriptional regulation

We next directly examined mRNA level of transiently expressed Flag-JNK1 in HeLa cells by Northern hybridization. As shown in Fig. 6, Flag-CSN1 caused a drastic reduction of *Flag-JNK1* mRNA level, while the internal control luciferase mRNA was only slightly affected (Fig. 6: comparing lanes 3 and 4). UNR reduced the overall level of *Flag-JNK1* mRNA without alleviating the repression instigated by CSN1 (Fig. 6: comparing lanes 5 and 6), similar to its effect on the protein level (Fig. 5B). It should be noted that even though *JNK1* cDNA was used as hybridization probe, we were unable to detect endogenous *JNK1* mRNA expression by Northern blot. We have attempted to examine the endogenous *JNK1* mRNA expression by RT-PCR in NIH3T3 cells, but have not detected significant repression by CSN1 (data not shown). It is possible that our transfection efficiency of CSN1 was not high enough to detect a reduction in a small number of transfected cells among mostly untransfected cells. Alternatively, CSN1 may only inhibit over-expression of JNK1 and therefore restrict the amount of *JNK1* mRNA at endogenous level. Regardless, the northern result demonstrates that CSN1 mediated down-regulation of Flag-JNK1 operates at the mRNA level.

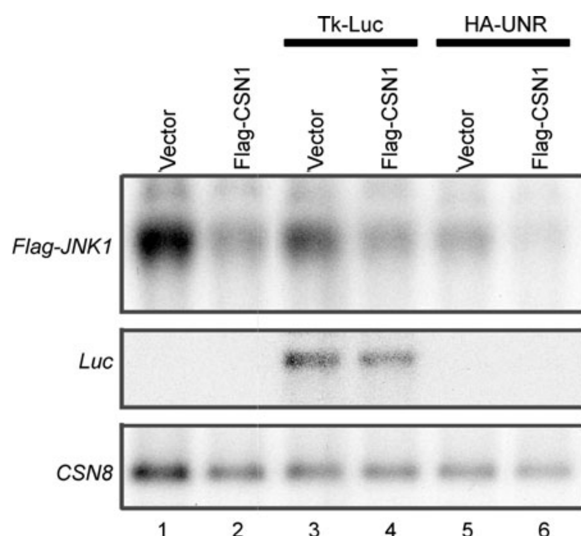

Figure 6. CSN1 inhibits JNK1 expression at the mRNA synthesis level. HeLa cells were transfected with construct expressing Flag-JNK1 DNA, together with empty vector or constructs expressing CSN1, Tk-luc, and HA-UNR, as indicated. Total RNA was analyzed by Northern hybridization using a *JNK1* cDNA probe, or *luciferase* (*luc*) and *CSN8* probes as described in MATERIALS AND METHODS. Tk-luc was included as an internal control. The endogenous *CSN8* mRNA level from each sample serves as a loading control. Note that Flag-CSN1 caused reduction of *Flag-JNK1* mRNA level.

DISCUSSION

Proto-oncogene *c-Jun* and JNK group of MAP kinases are associated with stress and inflammatory responses, and influence cell proliferation, survival, and differentiation of both normal and transformed cells (Davis, 2000). We have previously reported that CSN1 inhibits AP-1 activation and *c-fos* induction (Tsuge et al., 2001). Here we show that CSN1 inhibits UV and MEKK1 induced c-Jun phosphorylation, represses c-Jun dependent transcription activity, and silences ectopic expression of JNK1. In addition to our findings, CSN1 has been shown to bind 5/6-kinase and inhibit its kinase activity in c-Jun phosphorylation (Sun et al., 2002). Together, these results demonstrate that CSN1 has a negative impact on the AP-1 pathway.

CSN1 was not detected outside of the CSN complex by gel filtration chromatography of total cell lysate (Tsuge et al., 2001). However, this cannot exclude the possibility that small amount of CSN1 disassociated from or unassociated with the complex may exist transiently under certain conditions. The finding that CSN1 and CSN2 can function independently of other CSNs in fission yeast is consistent with this speculation (Mundt et al., 1999; Liu et al., 2003). By native gel electrophoresis, Fukumoto et al., (2005) showed that CSN complexes are more dynamic and heterogeneous than previously believed. For example, the CSN5 subunit, also known as Jab1, can stably exist outside the CSN holocomplex (Kwok et al., 1998; Yang et al., 2002; Gusmaroli et al., 2004). Notably, the amount of the CSN5 populations outside of the CSN holocomplex can change in response to cellular environment and signaling cues (Fukumoto et al., 2005; Tomoda et al., 2005). Moreover, over-expression or knock-down of subunits such as CSN3, CSN5, or CSN8 in cultured cells can cause increase or decrease of specific CSN sub-complexes more drastically than others (Fukumoto et al., 2005; Yoneda-Kato et al., 2005; Su et al., 2009). It will be interesting to examine whether and how the dynamics of the CSN holocomplex, subcomplexes, and free CSN5 populations may change upon ectopic expression of CSN1.

JNK/c-Jun functions are regulated by CSN at several levels. The CSN complex associates with multiple protein kinases capable of phosphorylating c-Jun (Uhle et al., 2003; Wilson et al., 2001). CSN2 and CSN5 promote (Claret et al., 1996; Naumann et al., 1999; Pollmann et al., 2001), while CSN1 inhibits AP-1 activation (Tsuge et al., 2001; this study). Taken together, we speculate that through the action of different subunits, CSN modulates the activity of AP-1 in response to cell signaling cues and extracellular stimuli to maintain homeostasis. Although CSN1 causes both repression of JNK1 ectopic expression and inhibition of c-Jun phosphorylation, it remains unclear whether these two events are connected. In the cells where c-Jun phosphorylation was inhibited, various endogenous JNK protein isoforms seemed to accumulate normally (data not shown). It is possible that

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CSN1 is important in guarding against undesired increase in JNK1 level in the cells.

CSN1 is a key component of the COP9 signalosome complex whose role in the ubiquitin-proteasome pathway has been established (Wei and Deng, 2003; Wolf et al., 2003). Meanwhile, compiling studies in recent years show that CSN and its subunits are critically involved in transcriptional regulation (Menon et al., 2007; 2008; Ullah et al., 2007; Adler et al., 2008; Chamovitz, 2009; Su et al., 2009). In particular, CSN1 has been shown to interact with SAP130, a component of transcription complex and RNA splicing complex (Menon et al., 2008). Our observations in this report lend another support to the idea that CSN1 affects the transcriptional and post-transcriptional process(es). CSN1 may manifest the activity at one or multiple steps of transcription such as promoter escape of RNA polymerase II, transcription elongation and termination, polyadenylation or mRNA nuclear export, all of which being extensively coupled (Maniatis and Reed, 2002). Previously we have attributed the repression of chromatin localized c-*fos-lacZ* chimeric gene expression to the blockage of signaling activation by CSN1 (Tsuge et al., 2001). In light of the findings from this study, it seems plausible that direct repression of c-*fos-lacZ* transcription may also contribute to CSN1 dependent silencing of the gene. Our results are consistent with the suggestion that CSN1 may affect the transcription elongation events that involve sequences at the 5′ coding region of JNK1. Nevertheless, it is clear that further experiments are necessary to understand the mechanisms of the CSN and CSN1 in transcription as well as possible connections between deneddylation, the ubiquitin-proteasome pathway, and control of gene expression.

MATERIALS AND METHODS

Cell culture and reagents

NIH3T3, HeLa, and HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Invitrogen, CA) and supplemented with 10% (*v/v*) heat inactivated fetal bovine serum in a 37°C humidified incubator containing 5% CO_2. MG132 (Z-Leu-Leu-Leu-al/benzyloxycarbonylleucyl-leucyl-leucine aldehyde) and 3-MA were purchased from Sigma (Sigma, MO) and kept in the culture media for the duration noted in the legends at 10 μmol/L and 10 mmol/L, respectively.

Plasmids

Constructs expressing Flag-JNK1 and Flag-JNK1 (APF) were kind gifts from Dr. R. Davis (Howard Hughes Medical Institute, University of Massachusetts Medical Center). In the Flag-JNK1 (APF) construct, the JNK1 kinase active site Thr-183 and Tyr-185 were substituted by Ala and Phe, respectively (Dérijard et al., 1994). pFlag-CSN1, pFlag-CSN1-N (expressing 1–196 aa), pFlag-CSN1-C (expressing 221–500 aa), and pMEKK1Δ have been described previously (Tsuge et al., 2001). pFlag-JNK1 (1–248) was constructed by deleting the *Eco*RI fragment from the original pFlag-JNK1 construct. To construct the 3′ UTR deletion clone of Flag-JNK1, we made a PCR primer corresponding to the sequence near the stop codon followed by a *Pst*I site. Another primer upstream of an internal *Pst*I site was used to obtain a PCR fragment of JNK1 3′ region of the ORF. After *Pst*I digestion, this PCR fragment was subcloned to replace the original fragment from the internal *Pst*I site to the one just upstream of bGH polyadenylation sequences, removing about 100 nucleotides of JNK1 3′ UTR from the original pFlag-JNK1 plasmid. The pcDNA3-CSN1 contains human CSN1 coding region in pcDNA3 expression vector. The UNR expression construct was kindly provided by Dr. A. Shyu (Houston Medical School, University of Texas).

Transfection, immunoblots, immunofluorescence staining and antibodies

Transient transfections were performed using Lipofectamine^{TM}2000 (Invitrogen, CA) and OPTI-MEM® (Invitrogen, CA) according to manufacturer's instruction. For most expression studies, cells were seeded on 12-well plates (BD Falcon, NJ), and Flag-JNK1 DNA in the range of 1–1.5 μg and Flag-CSN1 or pcDNA3-CSN1 DNA in the range of 0.2–0.8 μg were used, depending on the batch of plasmid DNA. In those samples where CSN1 was absent, pFlag-CMV2 empty vector was used to ensure that all the samples contained equal amount of total DNA during transfection. Some of the experiments were done in 6-well plates (BD Falcon, NJ) with proportionally increased amount of DNA. At 24 h post transfection, cells were washed with PBS, and directly lysed in SDS-sample buffer. The samples were boiled for at least 5 min, cleared by centrifugation, and the supernatants were loaded and separated by SDS-PAGE for immunoblot analysis.

Immunofluorescence staining was performed as described previously (Tsuge et al., 2001). The primary antibodies used in this study include anti-Flag (M2) monoclonal antibody (Sigma, MO), polyclonal anti-c-Jun and anti-c-Jun^{Ser63P} phosphor-specific antibodies (Cell Signaling, MA), and anti-HA antibody (Santa Cruz, CA). NIH3T3 cells were transfected with Flag-tagged CSN1 constructs. After 24 h, cells were irradiated with UV-C (80 J/m^2). Fifteen minutes later, cells were fixed and immunofluorescently labeled with anti-c-Jun^{Ser63P} antibody to detect c-Jun phosphorylation and anti-c-Jun to detect total level of c-Jun. Anti-Flag antibody was used to identify the transfected cells. The secondary antibodies used were FITC-conjugated anti-rabbit and Texas-red conjugated anti-mouse antibodies (Sigma, MO).

Transcription reporter assays

NIH3T3 cells on 6-well plates were transfected with 0.5 μg of Gal4-luc reporter plasmid, which contains 5 copies of Gal4 binding sequences in the promoter that drives the expression of luciferase. This reporter plasmid was used in conjunction with 0.02 μg of FA-c-Jun DNA (Stratagene, CA), which expresses a fusion protein of Gal4 DNA binding domain (DBD) and c-Jun transcription activation domain (TAD). The CSN1 expressing constructs and anti-sense construct (AS-CSN1) were co-transfected in amount of 0.4, 1.0, and 2.0 μg as indicated in Fig. 1A. Internal control vector pSV40-beta-Gal was co-transfected in all samples. Cells were lysed 24 h post transfection and the luciferase activity was determined using the Stratagene assay kits according to manufacturer's instruction. The relative activity was determined by the ratio of luciferase activity to the beta-galactosidase

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activity of the same sample. At lease three independent repeats was performed for each point.

Metabolic labeling

HEK293 cells were transfected with the DNA described in Fig. 4B in a 6-well plate. At 36 h after transfection, cells were washed and incubated in 1 mL of Met/Cys depleted media (D-MEM without L-methionine and L-cysteine, Invitrogen, CA) for 30 min. Cells were then incubated in depleted media supplemented with S^{35} labeled L-methionine and L-cystein (Perkin-Elmer, MA) at 0.2 mCi/mL with or without 100 μg/mL of cycloheximide (CHX, Sigma, MO) or 10 μg/mL of MG132 (Sigma, MO) for 30 min. Cells were rinsed with PBS and lysed in RIPA buffer [1% TritonX, 1% deoxycholic acid sodium salt, 0.1% SDS, 0.15 mol/L NaCl, 0.01 mol/L sodium phosphate, proteinase inhibitor cocktail (Roche, Basel Switzerland), 1 mmol/L DTT, 1 mmol/L PMSF]. Immuno-precipitation using anti-FLAG® M2 resin (Sigma, MO) was performed overnight at 4ºC. After washing with RIPA buffer, the samples were subjected to SDS-PAGE analyses.

Northern blot and RT-PCR

HeLa cells grown in 60-mm dishes were transfected with plasmid DNA expressing Flag-JNK1 (6 μg), empty vector, or Flag-CSN1 (1.2 μg), Tk-luciferase (5 μg) and HA-UNR (6 μg). After 24 h, cells were lysed and RNA was extracted using Qiagen RNeasy kit (Qiagen, MD). Ten μg of RNA from each sample was separated in a formaldehyde denaturing gel, and transferred to a membrane. The internal *Eco*RI-*Eco*RV DNA fragment (~500 bp) from Flag-JNK1 plasmid was used as a probe to detect JNK1. The *Nco*I-*Xba* I fragment (1.7 kb) from pTk-luc was used to probe luciferase expression, which served as an internal control. The P^{32} labeled probes were generated by random primer labeling (Amersham Biosciences, NJ) according to manufacturer's instructions. Prehybridization was carried out at 42ºC for 4 h in prehybridization buffer (6 × SSC, 50% formamide, 0.5% SDS, 5 × Denhardt's solution, 100 μg/mL denatured salmon sperm DNA). Labeled probe was added to the prehybridization buffer and hybridization was carried out overnight at 42ºC. The membrane was washed 3 times with 2 × SSC, 0.1% SDS for 10 min each at 65ºC followed by twice with 2 × SSC, 0.1% SDS for 30 min each and once with 0.5 × SSC, 0.1% SDS for 30 min. The membrane was then exposed to X-ray film.

RT-PCR was performed using 2 μg of total RNA from each sample and oligo-dT priming of the first strand cDNA, followed by 25 cycles of PCR amplification. The resulting PCR fragments were visualized on a 2% agarose gel with ethidium bromide staining. Gene specific primers were: JNK1, 5'-ATGAGCAGAAGCAAGCGTGAC-3' (forward) and 5'-AAGAACTAGCTCTCTGTAGGC-3' (reverse); and beta-actin, 5'-AAGAGAGGCATCCTCACCCT-3' (forward) and 5'-ATCTCTTGCTCGAAGTCCAG-3' (reverse).

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ABBREVIATIONS

AP-1, activator protein 1; CHX, cycloheximide; CRD or mCRD, major protein-coding-region determinant of instability; CSN,COP9 signalosome; CTD, C-terminal domain; IB, immunoblots; Jab1, c-Jun activation domain binding protein 1; JNK1, c-Jun N-terminal kinase; 3-MA, 3-methyladenine; MAP, mitogen-activated protein; NTD, N-terminal domain; PKD, protein kinase D; SAPK, c-Jun N-terminal kinase/stress-activated protein kinase; SRE, serum response element; UTR, untranslated region

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