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Report

Regulation of the anaphase-promoting complex by the COP9 signalosome

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The COP9 complex (signalosome) is a known regulator of the proteasome/ubiquitin pathway. Furthermore it regulates the activity of the cullin-RING ligase (CRL) families of ubiquitin E3-complexes. Besides the CRL family, the anaphase-promoting complex (APC/C) is a major regulator of the cell cycle. To investigate a possible connection between both complexes we assessed interacting partners of COP9 using an in vivo protein-protein interaction assay. Hereby, we were able to show for the first time that CSN2, a subunit of the COP9 signalosome, interacts physically with APC/C. Furthermore, we detected a functional influence of the COP9 complex regarding the stability of several targets of the APC/C. Consistent with these data we showed a genetic instability of cells overexpressing CSN2.

Introduction

The temporal regulation of degradation of damaged or misfolded as well as short-lived proteins in a specific manner is an essential feature of eukaryotic growth and development. Hereby, the SCF (Skp1/Cullin/F-box) complex, an E3 ubiquitin ligase, marks specific proteins with ubiquitin (Ub) for destruction by the 26S proteasome.[1] As a protein complex, that possesses structural similarities to a subunit of the 26S proteasome, the COP9 signalosome was discovered.[2] The COP9 signalosome (also named CSN) was first identified in Arabidopsis and comprises eight conserved subunits, CSN1-CSN8, in all eukaryotic cells.[3] These subunits bear remarkable homologies to the 19S lid of the 26S proteasome as well as to the translational initiation complex eIF3 and are postulated recently to possess an as yet undetermined function in protein degradation.[4,5] Further, there are suggestions that the COP9 complex is able to substitute the 19S lid functionally.[6] The COP9 complex is able to interact with the 19S regulatory complex, replace or interact with the 19S lid resulting in a supercomplex containing 26S proteasome and COP9 signalsosome and E3 ligases.[7] There are data available indicating that the CSN subunits interact directly with the 26S proteasome. In Arabidopsis as well as in Drosophila, CSN1 interacts with Rpn6, a subunit of the 19S lid.[8,9] Furthermore, the COP9 signalosome competes with the 19S lid for binding to the 26S proteasome in vitro.[10] Additionally, it has been shown that subunits of COP9 interact with different subunits of cullin dependent E3 ligases. Hereby, the CSN5 subunit which possesses a metalloproteinase activity is able to remove Nedd8 from cullins.[11] Cycles of neddylation and deneddylation of cullins seem to regulate the ubiquitinating activity of the cullin-based Ub ligases.[12] As yet, there are no data available showing an interaction of the COP9 signalosome with the cullin like protein APC2. Besides the SCF complex, the anaphase promoting complex (APC/C) is the second major E3 ligase mediating degradation of cyclins during cell cycle.[13] Since APC/C especially regulates the mitosis and the segregation of centrosomes it has a great impact on the genomic stability.[14-16] Recently, it was revealed that COP9 is essential for cell cycle progression and genomic stability in Arabidopsis.[17]

In a former study, we identified interacting proteins of the co-repressor Alien, an isoform of the CSN2 subunit of COP9 signalosome, under in vivo conditions.[18] The aim of the present study was to detect and analyze functional interactions between the COP9 complex and proteins involved in cell cycle as well as maintenance of genomic stability to get a detailed insight in cell cycle regulation and cell proliferation.

Results

CSN2 interacts with the anaphase-promoting complex in vivo. CSN2 as a subunit of the COP9 signalosome was shown to interact with several factors of the ubiquitin-proteasome pathway.[7] Recently, it was shown that the signalosome binds to cullin 1–4 and affects their function.[19-23] To extend our knowledge about this network, it is necessary to detect further interaction partners of COP9. For this reason, we performed a protein-protein interaction assay. Therefore, we precipitated an endogenously expressed subunit of COP9, CSN2, from crude U2OS cell extract using a specific antibody against CSN2. In order to recognize unspecific precipitated proteins we used as a negative control a rabbit

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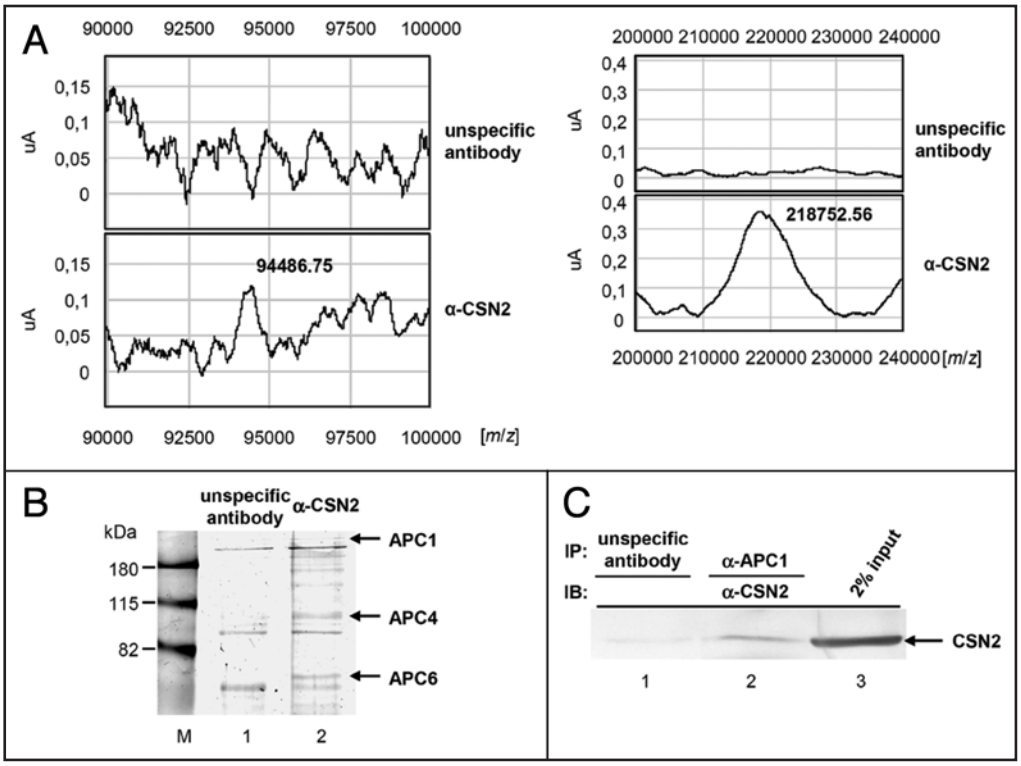

Figure 1. Endogenous CSN2 interacts with several subunits of the APC/C in vivo. (A) In protein-protein complex detection assays, a specific anti-CSN2 antibody was coupled on IDM beads and incubated with crude U2OS cell extract. Bound protein complexes were eluted and analyzed by SELDI-MS. A number of specific signals were detectable in the assay using the specific CSN2 antibody compared to an unspecific antibody. Among other specific signals two signals possessing 218.7 kDa and 94.5 kDa, respectively, were detected which correspond well to the molecular mass of APC1 and APC4, respectively. (B) The eluate was separated using an SDS-PAGE. Three specific protein bands (labeled with arrows) were excised from the coomassie stained gel and subjected to tryptic in-gel digestion. The generated peptide mass fingerprints (PMF) were analyzed by SELDI-MS and compared to an online database. Hereby, APC1 (at approx. 220 kDa), APC4 (at ~100 kDa) and APC6 (at ~70 kDa) were obtained as the best candidates. (C) The CSN2–APC1 protein interaction was confirmed by reverse CoIP experiments. CSN2 (labeled with an arrow) was co-immunoprecipitated (IP) from crude U2OS cell extract by a specific APC1 antibody as detected by an immunoblot (IB) (lane 2) compared to a negative control using an unspecific antibody (lane 1). A faint band in the negative control (lane 1) was unspecific precipitated by the non-specific antibody. Using a densitometrical analysis, this unspecific band represents only 19% of the protein band corresponding to CSN2 (lane 2) which was specific co-precipitated by the anti-APC1 antibody.

pre-immune serum. Captured proteins were eluted and analyzed using SELDI-MS (Fig. 1A). A specific precipitated signal possessed an *m/z* of 218,752 which roughly corresponds to the molecular weight of the anaphase-promoting complex subunit APC1. This signal was absent in the negative control. To identify the 219 kDa signal we subjected the eluted proteins to SDS-PAGE and detected a specific band in the range of ~220 kDa. Beside this specific band we detected further specific bands at ~100 kDa and at ~70 kDa (Fig. 1B). The negative control using a rabbit pre-immune serum did not reveal bands at these positions. Thus, we confirmed the presence of specific CSN2-interacting proteins. These specific bands were excised from the gel and subsequently subjected to an in-gel digestion by trypsin and protein identification. As control, an empty gel piece underwent the same treatment. The digest yielded solution was spotted on a Au array and the peptide mass fingerprints (PMF) were determined by SELDI-MS. Database searches (Profound; prowl.rockefeller.edu/prowl-cgi/profound.exe) revealed APC1 (Z-score: 2.39; supplemental Fig. 1; theoretical MW: 216,500 Da), APC4 (Z-score: 0.76; supplemental Fig. 2; theoretical MW: 92,116 Da), and APC6, also named CDC16 homolog (Z-score: 0.73; supplemental Fig. 3; theoretical MW: 71,656 Da) as the best candidates for the three detected interaction partners. APC1, APC4 as well as APC6 are subunits of the anaphase-promoting complex (APC/C). The APC/C is involved in protein degradation of cell cycle regulating factors. To confirm the presence of a protein complex containing CSN2 and APC1 we performed a co-immunoprecipitation experiment (CoIP). In line with the previous results, we were able to co-precipitate CSN2 using a specific APC1 antibody from U2OS cells (Fig. 1C). Additionally, we were able to coprecipitate APC1 by a specific antibody against CSN2 in a reciprocal CoIP (Fig. 2, lanes 1 and 2). The CSN2/APC1 complex appeared in a cell cycle dependent manner (Fig. 2). These data suggest that endogenous CSN2 exists together with endogenous APC/C, at least transiently, in one and the same stable protein complex.

CSN2 binding to APC/C but not to the base of the 19S proteasome is independent by interaction to COP9. The base, a sub-complex of the 19S proteasome, seems to interact with different subunits of the COP9 signalosome.[9,10] In order to uncover the biological function of the interaction of COP9 with

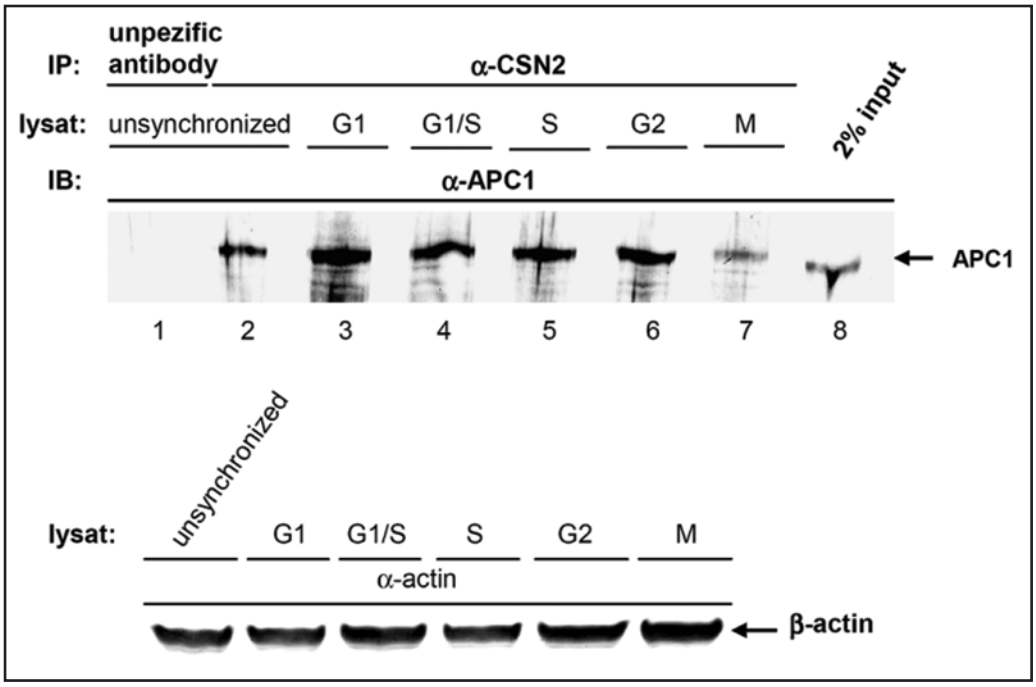

Figure 2. Interaction between CSN2 and APC1 is cell cycle dependent. U2OS cells were synchronized at different cell cycle stages. Synchronization in specific phases was achieved by following treatments. G_1 phase: 0.5 mM mimosine 24 h. G_1/S phase: 2 mM hydroxy urea over night. S phase/G_2 phase: U2OS cells were blocked in G_1 by serum starvation for 24 h following addition of fresh medium containing FBS supplemented with 2.5 mM thymidine over night. M phase: cells were blocked in mitosis by incubation with 100 ng/ml nocodazol for 16–18 h. Cells were lysed using lysis buffer and cleared by centrifugation (15 min; 15,000 rpm) at 4°C. The supernatants corresponding to protein extracts of different cell cycle stages were immediately used for CoIP experiment using a specific antibody against CSN2 for precipitation. APC1 was detected in an immunoblot using a specific anti-APC1 antibody (lanes 2–7), or as negative control, an unspecific antibody (lane 1). As a control for equal protein loading corresponding actin levels were shown.

the proteasome as well as APC/C, we performed additional protein-protein interaction assays. Hereby, we found that endogenously expressed CSN2 interacts with at least four subunits of the base of the 19S proteasome namely SUG1 [proteasome subunit p54/SUG; also named thyroid hormone receptor-interacting protein 1 (TRIP1)], TBP1 (TATA-box binding protein), 26S proteasome subunit S5B and S4. Additionally, an interaction between the COP9 subunit CSN2 and the 20S proteasome subunit alpha 6 was also detectable using the protein-protein complex identification assay (Suppl. Figs. 4–9). To further confirm these interactions, we performed a number of CoIP experiments using several antibodies that recognized different subunits of the proteasome (Suppl. Fig. 10). These results strongly suggest that endogenously expressed CSN2 is integrated in vivo in a protein complex containing the 19S sub-complex base.

Since CSN2 interacts with both the 26S proteasome and the APC/C we asked whether the COP9 signalosome is necessary to connect these two protein complexes resulting in degradation of APC/C in the proteasome. N-ethylmaleimide (NEM) is able to completely disrupt the COP9 complex.[20] In this case, a complete disruption of the COP9 complex using NEM would prevent the APC/C from binding to the proteasome. For that purpose, U2OS cell extract was preincubated with either 5 mM NEM or an equal volume ethanol as the solvent control for 1 hour followed by capturing the COP9 and 19S base containing protein complexes using specific antibodies (Fig. 3). In CoIP experiments with a SUG1-specific antibody, treatment of the cells with NEM led to a strong reduction of the co-precipitated CSN2 signal compared to untreated control cells as quantified by a densitrometric assessment (Fig. 3A; compare lane 7 with lane 3). Hereby, the co-precipitated signal from cells treated with NEM corresponding to CSN2 was reduced for approx. 75% compared to the CSN2 signal co-precipitated from control cells. This loss of interaction between CSN2 and the 19S subunit SUG1 after treatment of U2OS cells with NEM which results in the disruption of COP9 signalosome is further confirmed in similar intensity by a reverse CoIP with a specific CSN2 antibody used for co-precipitation of SUG1 (Fig. 3B). In contrast, there was only a little effect on the binding of APC1 to CSN2 or SUG1, respectively (compare Fig. 3A, lane 6 with Fig. 3B, lane 6). In summary, the binding of CSN2 to the 19S base sub-complex of the proteasome seems to be dependent on its integration in the COP9 complex. In contrast, the interaction between APC1 and CSN2 is NEM resistant which has the notion that CSN2 could bind directly to APC/C. As APC1 interacts with the base of the 19S proteasome even in the presence of NEM, we concluded that the COP9 complex is not exclusively necessary to recruit the APC/C to the proteasome.

The COP9 signalosome specifically affects stability of APC/C target proteins. In the past few years there were several reports available that COP9 influences the stability of many different targets of the ubiquitin/proteasome pathway.[21-23] This activity is mediated by different factors bound to the complex. For

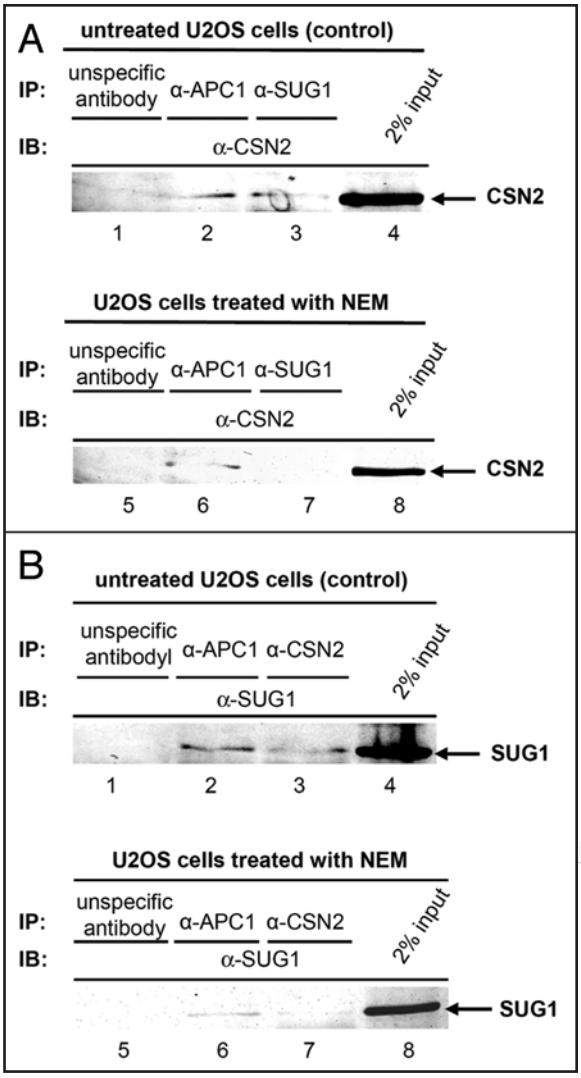

Figure 3. The interaction of CSN2 with APC1 and SUG1, respectively, is differentially affected by NEM. (A) Left panel: In CoIP experiments using crude U2OS cell extract, specific antibodies against SUG1 and APC1, respectively, co-precipitate (IP) CSN2 as detected in an immunoblot (IB) (lane 2 and 3). Right panel: The APC1 antibody was capable to co-precipitate CSN2 (lane 6) after preincubation of the U2OS cells with 5 mM NEM for one hour. In contrast, the SUG1 antibody failed to precipitate CSN2 after the mentioned treatment with NEM (lane 7). (B) Left panel: Antibodies against APC1 as well as CSN2 were both able to co-precipitate SUG1 from crude U2OS lysate (lane 2 and 3) as shown by immunoblot with a specific SUG1 antibody. right panel: A specific APC1 antibody was able to co-precipitate SUG1 from crude U2OS lysate preincubated with 5 mM NEM for one hour (lane 6). A specific CSN2 antibody failed to co-precipitate SUG1 from U2OS cells treated as described above (lane 7). An unspecific antibody which was used as negative control was not able to precipitate neither CSN2 nor SUG1 from untreated U2OS cells or treated U2OS cells (A, lane 1 and 5; B, lane 1 and 5).

example, the attached deubiquitinase USP15 mediates degradation of marked proteins.[20] For this reason, we hypothesized that the signalosome affects protein stability of APC/C targets. It was shown that overexpression of CSN2 leads to a de novo assembly of the COP9 complex.[10] Therefore, we transfected U2OS cells with an expression plasmid coding for CSN2 or, as control, the empty vector. Cells were harvested, lysed and subjected to SDS-PAGE. Thereafter, known APC/C target proteins including cyclin A, cyclin B, CDC6 and SnoN were analyzed in the differentially transfected U2OS cells by immunoblotting. In three independent experiments we detected decreased protein signals for CDC6 and SnoN in cells transfected with the CSN2 expression plasmid compared to mock-transfected control cells. In contrast, an increased signal corresponding to cyclin A was detected in cells overexpressing CSN2 due to the specific expression plasmid. No differences were found regarding cyclin B signal in specific transfected cells and control cells (Fig. 4A).

In order to proof these findings, we knocked down CSN5 by RNA interference using a specific CSN5 siRNA. As control, an unspecific non-silencing siRNA (nsc) was used. In contrast to CSN2 when a downregulation of this subunit is lethal, the CSN5 subunit of the COP9 is dispensable for complex formation but recruits several enzymes, e.g, a deubiquitinase to the signalosome.[24-26] We manipulated the activity of CSN2 and CSN5 as both subunits are located in different sub-complexes of the COP9 signalosome.[27] The COP9 is active if the complex is complete. Cells were harvested 72 h after siRNA transfection and proteins were separated on a SDS gel and analyzed by immunoblotting using specific antibodies. Signal intensities of immunoblots corresponding to CDC6, cyclin A, SnoN and cyclin B were compared between U2OS cells treated with the specific CSN5 siRNA and control cells treated with unspecific, non-silencing siRNA (Fig. 4B). Thereby, we found an increase of both SnoN and CDC6 in CSN5 downregulated U2OS cells. By contrast, there was a lower signal corresponding to cyclin A detectable in cells treated with specific CSN5 siRNA. No difference in signal intensity of cyclin B was detectable between CSN5 downregulated cells and controls. It is obvious that both CDC6 and SnoN were downregulated if a subunit of COP9 was overexpressed. In line with this both CDC6 and SnoN were up-regulated when a COP9 subunit was depleted. The results regarding cyclin A were completely opposite. The overexpression of a COP9 subunit correlates well with cyclin A upregulation well as the depletion of COP9 subunit downregulates the cyclin A expression.

Thereafter, we were interested whether the half life of the proteins was altered by transfection with a vector encoding CSN2 or, as a control, an empty vector. Cycloheximide was applied 48 h after transfection and the cells were harvested 1, 4, and 8 hours, respectively, after incubation. The protein stability of APC/C targets is influenced by CSN2 as shown in Figure 5. Both, SnoN and CDC6 were more rapidly degraded in cells over-expressing CSN2 due to a specific vector encoding CSN2 compare to control cells as densitometrical determined. A significant decreased of SnoN and CDC6, respectively, was detectable even four hours after cycloheximide induction. The half life of cyclin B was not affected by CSN2 overexpression at all. Surprisingly, we detected a decreased kinetic of cyclin A degradation in cells over-expressing CSN2 compared to control cells. These results confirm our findings regarding to the alteration of protein levels of APC/C targets after over-expression of CSN2 or down-regulation of CSN5 as

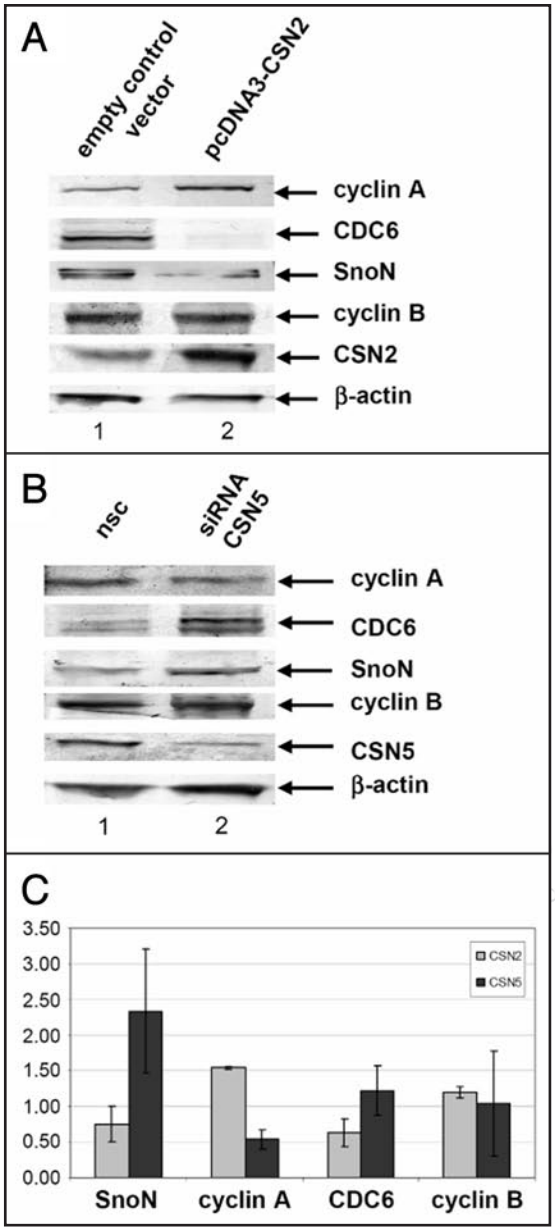

Figure 4: Overexpression of CSN2 as well as downregulation of CSN5 influenced expression of targets of the APC/C. (A) CSN2 was transiently overexpressed in U2OS cells by transfection with a pcDNA3-CSN2 plasmid (lane 2) and compared with cells transfected with the empty vector (lane 1). Transfected cells were lysed and several targets of the APC/C including cyclin A, CDC6, SnoN, and cyclin B were analyzed by immunoblot using specific antibodies. (B) Protein extracts of U2OS cells transfected with either a specific CSN5 siRNA (lane 2) or an unspecific non-silencing siRNA (nsc; lane 1) were subjected to immunoblotting against cyclin A, CDC6, SnoN, and cyclin B using specific antibodies. (C) The results of three independent experiments of CSN5 knock down and over-expression of CSN2 were densitometrically determined and summarized. The Y-axes shows the fold expression compared to controls transfected with the empty vector which were set to 1.

well as disintegration of the COP9 signalosome. Based on these findings, it seems that the COP9 signalosome influences stability of several APC/C targets on protein level.

CSN2 overexpression leads to genomic instability. Both, the COP9 signalosome and the APC/C are well characterized regulators of the cell cycle. For this reason, we asked if overexpression of CSN2 causes an altered cell cycle distribution. Fluorescence activated cell sorting (FACS)-technique was applied using U2OS cells fixed 48 h after transient transfection with the CSN2 encoding vector or, as a control, with the empty vector followed by staining of the DNA with propidium iodide and analysis of DNA content. Thereby, we did not detect any significant changes of cell cycle transition of cells expressing higher levels of CSN2 (Fig. 6A).

Consistently, there were only very slight differences in cell viability determined by colony forming assay (data not shown). Recently, it was shown that APC/C is involved in the segregation of chromatides and is necessary for accurate DNA replication.[16,28] The temporally regulated degradation of its targets seems sufficient to enable genetic stability. Since our data suggest that CDC6 protein level is altered by CSN2 overexpression, we hypothesized that this could lead to genetic instability. Using a 50K microarray analysis from Affymetrix to investigate single nucleotide polymorphism (SNP), we detected a higher rate of both deletions and duplications of genes in a pool of U2OS cells stably transfected with a vector coding CSN2 compared to a pool of control cells stably transfected with the empty vector (Fig. 6B). DNA instability was not detectable at specific sites in the genome, but they were randomly distributed over the whole genome (data not shown). These results suggest that overexpression of CSN2 leads to genetic instability and indicate that the proper regulation of the APC/C dependent protein degradation by the signalosome seems important for genetic stability. Interestingly, we did not detect an effect on the cell cycle phase distribution, although cells revealed an increase in genome instability. A possible explanation could be the lack of functional p53 in these cells. The rate of apoptosis of CSN2 over-expressing cells was analyzed by monitoring the caspase 3/7 activity. Hereby, we found only a slight reduction of the apoptotic cell fraction of CSN2 overexpressing cells compared to cells treated with the empty vector (data not shown).

Taken together, the data suggest that CSN2 interacts with the APC/C and influenced functionally the stability of APC/C targets. Further, in cells overexpressing CSN2 the genomic stability of these cells massively perturbed.

Discussion

In the past few years many publications discussed about the influence of the COP9 signalosome on the ubiquitin/proteasome pathway. It could be shown the direct interaction of Flag-CSN2 with the 26S proteasome in mouse B8 fibroblasts.[10] In the present study, we extended the panel of interacting partners of COP9 in vivo by the detection of several protein interactions between CSN2, a subunit of COP9, and subunits of the base of the 19S proteasome as well as of the 20S proteasome. These protein interactions seemingly depend on COP9 as CSN2 did not bind to the base of the 19S proteasome after disruption of the signalosome in vitro by NEM. Furthermore, we found that CSN2 binds at least three proteins of the anaphase promoting complex/cyclosome (APC/C). Beside the SKP1 Cullin F-box (SCF) complex, APC/C

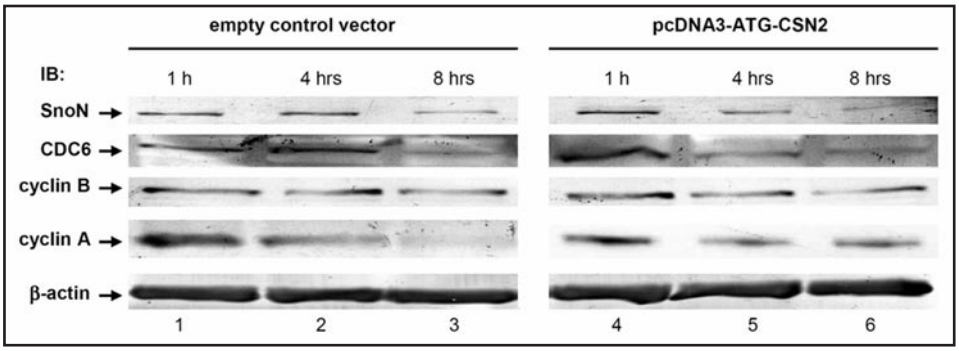

Figure 5. CSN2 deregulates stability of APC/C targets. U2OS cells were transiently transfected either with pcDNA3-CSN2 or the empty vector. 48 hours after transfection fresh medium with 50 µg cycloheximide/ml was applied and the cells were harvested after the indicated time points (1 hour: lane 1 and 4; 4 hours: lane 2 and 5; 8 hours: lane 3 and 6) and lysed using a lysis buffer. Lysates of the transfected cells treated with cycloheximide were analyzed by western blotting with antibodies specific for SnoN, Cdc6, Cyclin A and Cyclin B, respectively.

is responsible for the temporally degradation of cyclins during cell cycle.[13] As CSN2 is known to interact with cullins we suggested that it interacts directly with the cullin-like protein APC2.[24,25] Consistent with that, we found CSN2 to be bound to the APC/C even in the absence of an intact signalosome. Whether the COP9 complex recruits the APC/C to the proteasome or if these interactions are independent remains unknown. Our data clearly show that APC/C is able to bind to the proteasome even if the COP9 complex was disrupted. In our opinion, there are two possible explanations for this. On the one hand, the signalosome and the lid of the 19S proteasome compete for the APC/C mediating substrate specifity. Such a competitive binding of both complexes was shown for the binding to the base of the 19S proteasome.[10] On the other hand, it might be possible that the APC/C directly binds to the base. In this case, the COP9 would be able to influence the APC/C independently of proteasome binding.

The specificity of the APC/C for its multiple substrates is regulated by the competitive binding of Cdh1 and Cdc20 to the E3 ligase dependent on the cell cycle phase.[29-32] We found that degradation of cyclin A, SnoN and CDC6 was regulated by the COP9 complex while protein levels of cyclin B which is degraded at the end of mitosis was not affected.[33] Consistent with these data, we found that CSN2 did not bind to the APC/C during mitosis. Because of these reasons, we speculate that the regulation of APC/C by the COP9 is both high substrate and cell cycle specific.

Because of its role as a regulator of DNA duplication and chromatide separation, the APC/C was shown to mediate genomic stability.[16] Otherwise, a stabilization of APC/C targets may initiate a perturbation of these processes causing a p53 response by deregulating G_1 phase.[30] Consistently, we showed that overexpression of CSN2 and the resulting influence on the APC/C targets led to genomic alterations. This instability seems not to affect cell cycle distribution or cell viability. An explanation for unaffected cell cycle despite deregulation of APC/C targets stability might be as U2OS cell possess an inactivated form of p53. In a large genetic screen in yeast, the COP9 was shown to have a great impact on cell cycle without complete explanation for the results.[34] Our presented data show, that the signalosome regulates both the SCF and also the second important pathway for degradation of cyclins by APC/C. Additionally, it seems that COP9 is involved in regulation of the cell cycle. Taken together, CSN2 overexpression seems to lead to genomic instability. On the other hand, these cells pass cell cycle checkpoints without being arrested and are prevented from apoptosis leading to further accumulation of genomic alterations.

Materials and Methods

Cell culture and cell cycle synchronization. Human U2OS osteosarcoma cells were cultured in DMEM supplemented with 10% fetal bovine serum. Cells were harvested at 70–90% confluence using PBS with 0.05% EDTA and trypsin. In order to synchronize U2OS cells, cells were submitted to different treatments as described elsewhere.[35] Mitosis: Cells were blocked in mitosis by incubation with 100 ng/ml nocodazol (Sigma) for 16–18 h. Mitotic cells were detached by mitotic shake-off and cleared from the medium by centrifugation for 5 min on 1500 rpm at room temperature. G_1 phase: Synchronization in G_1 was achieved by mimosine treatment (0.5 mM, for 24 h, Sigma). G_1/S phase: to accumulate cells at the G_1/S transition hydroxy urea (Sigma; 2 mM, overnight) was used. S phase/G_2 phase: In a first step, U2OS cells were blocked in G1 by serum starvation for 24 h. Afterwards fresh medium with FBS supplemented with thymidine (Sigma; 2.5 mM over night) was added. The cell cycle block was released by washing cells twice with PBS and normal medium was applied. Cells were harvested after 4 h reflecting to S phase or after 8 h corresponding to G_2 phase, respectively.

Cells were lysed in a buffer containing 100 mM sodium phosphate pH 7.5, 5 mM EDTA, 2 mM MgCl2, 0.1% CHAPS, 500 µM leupeptin, and 0.1 mM PMSF. After centrifugation (15 min; 15,000 rpm; 4°C) the supernatant was immediately used.

Protein-protein complex identification assay. The protein-protein interaction assay was performed as described before.[36] In short, 4 µl protein A (Sigma) was bound to 20 µl of Interaction Discovery Mapping (IDM) beads (Bio-Rad) overnight at 4°C. After discarding the supernatant the pellet was washed twice with a buffer containing 50 mM sodium acetate pH 5.0. Afterwards, unspecific binding sites were blocked by incubation with a buffer

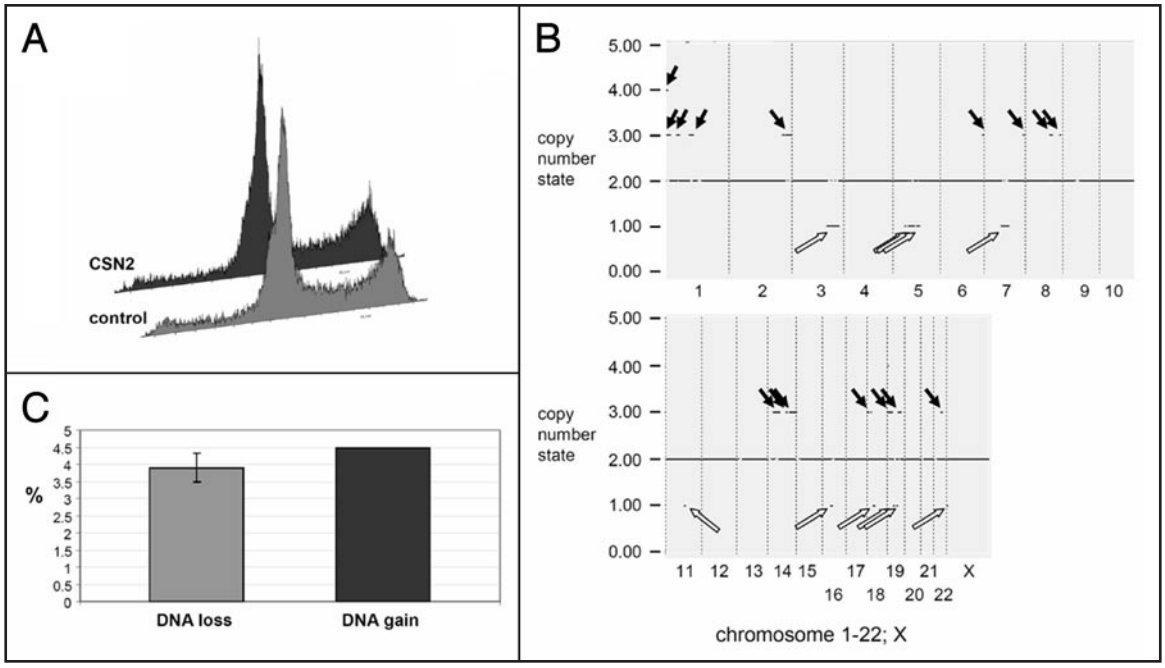

Figure 6. CSN2 overexpression promotes genetic instability. (A) U2OS cells were transfected with pcDNA3-CSN2 or the empty vector and ethanol fixation following staining of DNA with propidium iodide and measuring cell cycle distribution by FACS analysis. (B) Stably transfected U2OS cells with pcDNA3-CSN2 or the empty vector, respectively, were analyzed with a 50K microarray (Affymetrix), which shows the copy number of genomic sequences. CSN2 overexpressing cells exhibit loss as well as well as gain of allels compared to cells transfected with the empty vector control. The DNA changes in the control cells transfected with the empty vector were normalized to a copy number of 2 chromosomes. Dots above the black line imply DNA amplifications in cells transfected with the pcDNA3-CSN2 vector (copy number of 3; black arrow); dots below the black line shows DNA losses in CSN2 over-expressing cells (copy number 1; white arrow). The experiment was done twice. (C) Percentage DNA loss or DNA gain, respectively, of the whole genome of the CSN2 over-expressing cells.

containing 0.5 M Tris/HCl pH 9.0 and 0.1 % Triton X-100 for 1 hour at room temperature. The beads were washed three times with 1x PBS. Thereafter, a specific antibody which recognized human CSN2 (rabbit polyclonal), or normal rabbit IgG (Pepro Tech Inc.; Rocky Hill, NJ) as negative control, in 50 mM sodium acetate pH 5.0 was applied to the beads and allowed to bind at room temperature for 1 hour in an end-over-end mixer. The specific anti-CSN2 antibody was described before.[37] Unbound antibodies were removed by washing in 50 mM sodium acetate. Following two washes with 1 x PBS the beads were incubated with at least 100 µl of crude U2OS cell extract for two hours at 4°C in an end-over-end mixer. Unbound proteins were removed by sequential washes in 0.5 M sodium chloride, 0.1 % Triton X-100 and PBS. Afterwards proteins were eluted from the IDM beads by 25 µl 50% acetonitrile/0.5% trifluoroacetic acid and gently vortexed for 30 minutes. Five microliters of the eluted samples were subjected on an activated H50 ProteinChip Array (Ciphergen Biosystem, Inc., Fremont, CA) and the array was analyzed in a ProteinChip Reader (series 4000; Ciphergen, Bio-Rad) according to an automated data collection protocol by SELDI-MS. This includes an average of 265 laser shots to each spot with a laser intensity of 2300 nJ and 3500 nJ (20–200 kDa), respectively, dependent on the measured region (low = 2.5–20 kDa and high = 20–200 kDa, respectively) and an automatically adapted detector sensitivity.

The volume of the eluted samples was reduced to a maximum of 10 µl using a speed-vac (ThermoServant) and subjected to SDS-PAGE for separation of containing proteins followed by staining with Simply Blue Safe Stain (Enhanced Coomassie, Invitrogen). Specific gel bands were excised, destained, and dried followed by rehydration and digestion with 10 µl of a trypsin solution (0.02 µg/µl; Promega) at 37°C overnight. The supernatants of the in-gel digestions were applied directly to a gold (Au) arrays (Bio-Rad). After addition of the matrix (CHCA), peptide fragment masses were analyzed by SELDI-MS. A standard protein mix (all-in-1 peptide standard mix; Bio-Rad), including Arg8-vasopressin (1082.2 Da), somatostatin (1637.9 Da), dynorphin (2147.5 Da), ACTH (2933.5 Da), and insulin beta-chain (3495.94 Da) was used for calibration. Proteins were identified using the fragment masses searching in a publicly available database (Profound; prowl.rockefeller.edu/prowl-cgi/profound.exe).

Co-immunoprecipitation. Specific antibodies which recognise CSN2 (rabbit polyclonal), S4 (rabbit polyclonal; Abcam), SUG1 (25D5, mouse monoclonal; Abcam), p42 (p42-23, mouse monoclonal; Calbiochem), TBP1 (TBP1-19, mouse monoclonal), TBP7 (TBP7-27, mouse monoclonal), proteasome α 6 subunit (MCP20, mouse monoclonal; Abcam), APC1 (H-300, rabbit polyclonal; Santa Cruz) or, as negative control, normal rabbit IgG (Pepro Tech Inc.) were bound on protein A-agarose beads. The antibody loaded beads were incubated with 150 µl of crude U2OS cells extract for 1 hour at 4°C. Unspecific bound proteins were removed by three washes with CoIP buffer containing 20 mM HEPES/KOH pH 8.0, 50 mM KCl, 0.1 mM EDTA and 0.05% CHAPS.

Afterwards beads were boiled in 4 x SDS loading buffer (200 mM Tris-Cl pH 6.8, 4% SDS, 30% Glycerol, 10% β-mercaptoethanol, 0.002% Bromophenol blue) and bound proteins were subjected to a 10% SDS–PAGE and analyzed by immunoblotting. For detection in immunoblots following antibodies apart from the ones mentioned above were used: CSN2 (goat polyclonal; Abcam), β-actin (A266, rabbit polyclonal; Sigma), cyclin A (C-19, rabbit polyclonal; Santa Cruz), cyclin B1 (M-20, rabbit polyclonal; Santa Cruz), CDC6 (0.T.17, mouse monoclonal; Santa Cruz), SnoN (H-317, rabbit polyclonal; Santa Cruz) and CSN5 (FL-334, rabbit polyclonal; Abcam).

For CoIP experiments using crude U2OS cell extract preincubated with N-ethylmaleimide (NEM), lysates were split into two aliquots. The sample was treated either with 5 mM NEM or with an equal volume of ethanol at 4°C for 1h in an end-over-end mixer.

Small interfering RNA-mediated knockdown of CSN5. For knockdown by RNA interference the following small interfering RNA (siRNA) duplex oligonucleotides was used in this study that was based on the human cDNA encoding CSN5. CSN5: 5'-GCAAUCGGGUGGUAUCAUAdTdT-3'(sense), 5'-UAUGAUACCCGAUUGCdAdT-3' (antisense) (QIAGEN GmbH, Hilden, Germany); nonsilencing control siRNA: 5'-UUCUCCGAACGUGUCACGUdTdT-3' (sense), 5'-ACGUGACACGUUCGGAGAAdTdT-3' (antisense) (QIAGEN GmbH, Hilden, Germany). U2OS cells (3 x 10^5) were seeded in a six-well plate 24–48 h before transfection and were 50% confluent when siRNA was added. The amount of siRNA duplexes applied was 1.5 µg/well for CSN5. Transfection was performed using the amphiphilic delivery system SAINT-RED (Synvolux Therapeutics B.V., Groningen, The Netherlands) as described.[38] Briefly, siRNA was complexed with 15 nmol of transfection reagent and added to the cells for 4 hours. Subsequently, 2 ml of culture medium was added and incubation proceeded for 72 h.

Transfection. U2OS cells were seeded into 6-well plates at a density of 2.5 x 10^5 cells per well 24 h prior to transfection. Fresh DMEM supplemented with 10% FCS was added four hours before transfection. Afterwards, transfection was performed with $CaPO_4$ as described.[22] Hereby, one microgram of pcDNA3-Linker or pcDNA3-CSN2 were used per well. Cells were washed three times with 2 ml PBS and new medium was applied after 24 h.

In case of transient transfection, cells were harvested 48 h after initial transfection. In case of cycloheximide chase, 50 µg cycloheximide per ml medium were applied and cells were collected after 1, 4, and 8 hours, respectively. For stable transfection, cells were selected with 0.5 mg/ml geneticin sulphate (G418). Medium and hygromycin were replaced every 2–3 days until all cells were died in a transfection control experiment. All experiments were performed at least three times.

Densitometrical assessment. Signal intensities of corresponding proteins were densitrometrical assessed using the Lab Image 1D program (Kapelan Bio-Imaging, Leipzig, Germany) according to the manufacturer's instructions.

FACS. Cell cycle distribution of U2OS cells were shown by fluorescence activated cell sorting (FACS) as described.[39] Harvested transiently transfected cells and medium were collected together to obtain all cell cycle phases and apoptotic cell population. After centrifugation (5 min, 1500 rpm, RT) the resulting pellet was washed twice in PBS. For fixation, 1 ml of ice-cold 70% ethanol was added slowly to the cells. Afterwards, the cells were resuspended carefully and incubated for 1 h on ice. After centrifugation and washing in PBS, the cells were resuspended in 300 µl PBS with 1 mg/ml RNase A (Fermentas). The samples were incubated for 10 min at room temperature before staining the DNA with 50 µg propidium iodide (Sigma) for 10 min at RT. Fluorescent labeling was measured with a FAC-Scan using Cell Quest Software (Becton Dickinson).

Apoptosis assay. Changes of the apoptotic population of U2OS cells transiently transfected with either pcDNA3-CSN2 or the empty vector, respectively, were analyzed with the Apo-ONE Homogeneous Caspase-3/7 Assay (Promega) according to manufacturer´s instructions. Hereby, the amount of total protein of cell lysates was measured with a NanoDrop device (ND-1000 spectrometer; Peqlab, Erlangen, Germany) and samples were diluted to same concentrations. Afterwards, samples were measured on a fluorescence plate reader with excitation maximum at 498 nm and emission maximum of 521 nm.

Genetic stability. The gene chip copy number analysis was based on the detection of single nucleotide polymorphisms (SNP). Therefore, a 50K microarray from Affymetrix was used to detect differences of chromosomal imbalance between U2OS cells overexpressing CSN2 and untreated U2OS cells. The procedure was performed according to the Mapping 100K Assay manual from Affymetrix (www.affymetrix.com). First, total DNA was isolated from U2OS cells using a Qiagen Mini Kit following by digestion with XbaI restriction enzyme and amplification by one-primer PCR. After amplification the genomic DNA was purified, fragmented and labeled. Finally, the DNA fragments were hybridized to the 50.000 SNPs on the XbaI microarray surface. After 16 h hybridization, the DNA was stained with streptavidin phycoerythrin (SAPE), washed and scanned. The difference in fluorescence intensity was caused by variation in concentration of bound DNA. This difference indicates a loss or gain of chromosomal material. Following primers were used. XbaI adaptor sequence: 3'-AATACTCGTGCTGTCTGCGGACTAGAGATCT-5'; PCR primer, 001: 5'-ATTATGAGCACGACAGACGCCTGATCT-3'.

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/KobCC8-13-Sup.pdf

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