The COP9 signalosome-mediated deneddylation is stimulated by caspases during apoptosis

Bettina K.J. Hetfeld^{1*}, Andreas Peth^{1*}, Xiao-Ming Sun², Peter Henklein³, Gerald M. Cohen², and Wolfgang Dubiel¹

¹Department of Surgery, Division of Molecular Biology, ³Institute of Biochemistry, Charité - Universitätsmedizin Berlin, Monbijoustr. 2, 10117 Berlin, Germany, ²MRC Toxicology Unit, Hodgkin Building, University of Leicester, PO Box 138, Lancaster Road, Leicester, LE1 9HN, United Kingdom

*The two authors contributed equally.

Correspondence should be sent to: Wolfgang Dubiel, Tel: +4930-450522305, Fax: +4930-450522928, e-mail: wolfgang.dubiel@charite.de

Running title: **CSN deneddylation is stimulated during apoptosis**

Abstract In concert with the ubiquitin (Ub) proteasome system (UPS) the COP9 signalosome (CSN) controls the stability of cellular regulators. The CSN interacts with cullin-RING Ub ligases (CRLs) consisting of a specific cullin, a RING protein as Rbx1 and substrate recognition proteins. The Ub-like protein Nedd8 is covalently linked to cullins and removed by the CSN-mediated deneddylation. Cycles of neddylation and deneddylation regulate CRLs. Apoptotic stimuli cause caspase-dependent modifications of the UPS. However, little is known about the CSN during apoptosis. We demonstrate *in vitro* and *in vivo* that CSN6 is cleaved most effectively by caspase 3 at D₂₃ after 2 – 3 h of apoptosis induced by anti-Fas-Ab or etoposide. CSN6 processing occurs in CSN-CRL complexes and is followed by the cleavage of Rbx1, the direct interaction partner of CSN6. Caspase-dependent cutting of Rbx1 is accompanied by decrease of neddylated proteins in Jurkat T cells. Another functional consequence of CSN6 cleavage is the enhancement of CSN-mediated deneddylating activity causing deneddylation of cullin 1 in cells. The CSN-associated deubiquitinating as well as kinase activity remained unchanged in presence of active caspase 3. The cleavage of Rbx1 and increased deneddylation of cullins inactivate CRLs and presumably stabilize pro-apoptotic factors for final apoptotic steps.

Keywords COP9 signalosome, deneddylation, caspases, apoptosis, ubiquitin, Rbx1

Introduction

The COP9 signalosome (CSN) of mammalian cells consists of eight polypeptides, CSN1 to CSN8 [1]. The CSN interacts with components of the ubiquitin (Ub) proteasome system (UPS) known as cullin-RING Ub ligases (CRLs) [2]. These enzyme complexes select proteins for ubiquitination and are responsible for substrate specificity of the UPS. Besides a specific cullin, most of the CRLs contain a RING domain protein called ROC1/Rbx1 involved in the Ub ligation reaction as well as substrate recognition components [2, 3]. The CSN removes the Ub-like protein Nedd8 from its covalent linkage to cullins, a process called deneddylation [4-6]. As it has been shown, CSN-mediated deneddylation prevents the assembly of a specific CRL [4]. In cooperation with the UPS the CSN participates in processes such as DNA repair [7], cell cycle [8], angiogenesis [9] and development [10-12]. However, its role in apoptosis is unknown. Although the UPS and the CSN determine the stability of regulatory molecules involved in apoptosis such as p53, Bax, Bak and Smac [13-15], the exact function of the system in programmed cell death is still obscure. Inhibitors of CSN-associated kinases induce accumulation of p53 and apoptosis in tumor cells [16]. The UPS in concert with the CSN controls the degradation of I κ B α , a major regulator of the key surviving factor NF- κ B [17, 18]. The fact that proteasome inhibitors induce apoptosis is already successfully used in tumor therapy [19, 20].

Two major pathways of apoptosis, the intrinsic and extrinsic pathways, have been described [21, 22]. Many of the effects that occur during apoptosis result from activation of caspases. Caspase 8 (Casp8) and 9 (Casp9) are the apical caspases in the extrinsic and intrinsic pathway, respectively [21, 22]. In the intrinsic pathway, mitochondrial perturbation leads to cytochrome c release into the cytosol where it binds to Apaf-1, facilitating the binding of ATP/dATP and oligomerization of Apaf-1 to form the apoptosome. This oligomerization activates Casp9 [23, 24]. The extrinsic pathway is triggered by binding ligands (e.g. FAS, TRAIL) to their respective cell-surface death receptors, which after oligomerization recruits adaptor molecules

and the initiator caspase Casp8 [25]. These two pathways converge with the activation of effector caspases such as caspases 3 and 7 (Casp3 and Casp7) and the activation of endonucleases resulting in DNA fragmentation.

Do the UPS and the CSN exert specific regulatory functions during the apoptotic process? The activity of the 26S proteasome is reduced by caspase-dependent cleavage of the regulatory subunits Rpt5, Rpn10, and Rpn2, resulting in the stabilization of the pro-apoptotic protein Smac. This effect provides a feed-forward amplification loop of apoptosis [26]. We were interested in the role of the CSN complex during apoptosis. Here we demonstrate that apoptotic stimuli cause the caspase-dependent cleavage of subunit CSN6, which is accompanied by the activation of CSN-mediated deneddylation. In addition, CSN6 modification is followed by Rbx1 cleavage resulting in the inactivation of CRLs.

Material and Methods

Cell culture and materials

HeLa cells and Jurkat T cells were cultured in RPMI 1640 supplemented with 10% heat inactivated FCS in 5% CO₂ at 37°C. To induce apoptosis HeLa cells were treated with 1 µg/ml recombinant TRAIL [27]. Jurkat T cells were treated with etoposide (50 µM, Sigma) or anti-Fas antibody (50 ng/ml, CH-11, Upstate) (Fas-Ab). To block apoptosis 20 µM of the caspase inhibitor z-VAD-fmk (z-VAD(OMe)-fluoromethyl ketone, z-VAD, MP Biomedicals) were added. After treatment, the percentage of Annexin V-positive cells (% apoptosis) was determined by FACS analysis as described (Annexin V-FITC, Bender Medsystems) [28].

Cell lysis and Western blotting

Cells were washed twice with ice-cold PBS and lysed in triple-detergent lysis buffer [14]. After centrifugation protein concentration of cell lysates was determined by Bradford Assay (Biorad). Aliquots were subjected to SDS-PAGE and Western blotting. The following antibodies were used in this study: anti-CSN1, anti-CSN4, anti-CSN6 and anti-CSN8 antibodies (Biomol), anti-CSN2, anti-CSN3 and anti-CSN7 antibodies [29, 30], monoclonal anti-CSN5 antibody (supplied by B. Christy), anti-ubiquitin antibody (FK2, Biomol), anti-USP15 antibody [31], anti-Rbx1 antibody (Rockland), anti-Nedd8 antibody (a gift from C. Gordon) and anti-cullin 1 (Cul1) antibody (Calbiochem). Secondary antibodies were purchased from Seramun. All blots were developed by ECL technique (Amersham Biosciences).

Glycerol gradients and non-denaturing electrophoresis

Glycerol gradient centrifugation was performed as outlined before [32]. In brief, 2×10^7 cells were lysed in mono-lysis buffer [33] and equal amounts (3 mg) were loaded onto a 12 ml glycerol gradient (10 - 40%). Fractions of 600 μ l were collected and aliquots used for Western blot analysis with the indicated antibodies.

For non-denaturing electrophoresis 20 μ g of cell lysates were separated on a 4 – 15% (w/v) Phast-gel (Amersham Pharmacia Biotech.) at 300 V/h. Proteins were blotted onto nitrocellulose and probed with anti-CSN6 antibody.

Recombinant CSN6 and site-directed mutagenesis

CSN6 and CSN6 Δ 29 was obtained by PCR using clone IRAUp969A108D (Lib.969) purchased from RZPD as template and cloned into pQE expression vector (Qiagen). Site-directed mutagenesis was performed with CSN6 cDNA in pQE using the Quick-Change

strategy (Stratagene). The cDNA of all constructs was verified by sequencing. Protein purification was carried out with the Ni-NTA purification kit (Qiagen).

Cleavage of CSN complex or recombinant CSN subunits by caspases

The CSN complex was purified from human erythrocytes as described before [32]. The purified CSN complex (200 nM) or 500 nM of recombinant His-tagged CSN subunits were incubated with equimolar amounts of either Casp3, Casp7 or Casp8 with or without 50 μ M z-DEVD-fmk (z-DEVD, Biomol) in a buffer containing 30 mM Tris, pH 7.8, 10 mM KCl and 5 mM DTT for indicated times at 37°C. Recombinant processed Casp3, Casp7 and Casp8 were prepared as described previously [26].

CSN activity assays

Purified CSN complex (750 nM) was pre-incubated with or without Casp3 (750 nM) for 1 h at 37°C in PBS with 10 mM DTT. Casp3 was inhibited with 1.5 μ M z-DEVD (z-DEVD-CHO, Biomol) in a 30 min pre-incubation at 4°C before the CSN was added (inhib. Casp3) or was added to all samples after pre-incubation to block Casp3 during cleavage of the Nedd8-Cul1 peptide. Deneddylating activity was determined by adding 1.6 μ g of a biotin-coupled Nedd8-Cul1 peptide (see below). After 3 h the cleavage of the peptide was terminated by addition of loading buffer. SDS-PAGE and Western blotting with streptavidin-POD (ExtrAvidin-peroxidase, Sigma) was performed. CSN5-dependent deneddylation was inhibited by the addition of 5 mM o-phenanthroline (OPT, Roth).

To determine the deubiquitinating activity of the purified CSN complex, 1 μ g of tetra-Ub (Ub₄, Biomol) was added to the pre-incubated CSN complex. SDS-PAGE and Western blotting using the anti-Ub antibody was performed after 1 h incubation. Kinase activity was

detected by addition of 0.5 µg recombinant c-Jun and [γ - 32 P]-ATP to the pre-incubated CSN complex. Phosphorylation of c-Jun was determined as described previously [30].

Synthesis and biotinylation of the Nedd8-Cul1 peptide

The synthesis of the peptide was performed on an ABI 433A automated peptide synthesizer (Applied Biosystems Darmstadt) on a 0.1 mM scale with 250 mg TentaGel R-RAM-Fmoc-resin (capacity 0.19 mmol/g; RAPP Polymere GmbH Tübingen, Germany) using the Fmoc/tBu strategy. The lysine of Cul1 peptide 719 - 724, position 720, was protected at the ϵ -NH₂ group with ivDde [1-(4,4-dimethyl-2-dioxocyclohex-1-ylidene)-3-methylbutyl]. The lysine residue in position 723 was introduced as Fmoc-Lys(Biotin)-OH. The final deprotection from the resin was performed with 95% TFA in water containing 3% triisopropylsilane and 5% phenol. The crude protein was purified by reverse phase-HPLC on a 7 µ Zorbax SB C18 column (21.2 x 250 mm, Prep HT) with a linear gradient of 40% A to 65% B in 50 min (A: 1000 ml water, 2 ml TFA; B: 500 ml acetonitrile, 100 ml water, 1 ml TFA) at a flow rate of 10 ml x min⁻¹.

Results

Subunit CSN6 is cleaved by effector caspases

In order to study changes of the CSN during apoptosis Jurkat T cells were treated for 6 h with the anti-Fas-antibody (Fas-Ab) or with etoposide to activate the extrinsic or intrinsic pathways of apoptosis, respectively. Under these conditions, more than 50% of cells are apoptotic [28]. Apoptosis was blocked with the caspase inhibitor z-VAD. After 6 h treatment cells were lysed and subunits of the CSN were analyzed by Western blotting. Probing of cell lysate with

antibodies against all eight subunits of the CSN revealed that most of the CSN subunits remained stable after 6 h of exposure to anti-Fas-Ab or etoposide (see Fig. 1A). Cleavage of subunit CSN6 but not of other CSN subunits was clearly observed after both apoptotic stimuli. The cleavage was caspase-specific, since z-VAD inhibited the formation of the CSN6 fragment completely. In Fig. 1B the time course of CSN6 cleavage during apoptosis in HeLa as well as in Jurkat cells is shown. In this case apoptosis in HeLa cells was induced by TRAIL and in Jurkat cells by etoposide. The cleavage of CSN6 was clearly detected after 2 – 3 h in both cell lines. This indicated that cleavage of CSN6 was not an early event in apoptosis and was most likely mediated following the activation of effector caspases. After 6 h of apoptosis more than 50% of CSN6 was cleaved.

The notion that effector caspases cleave CSN6 was confirmed by experiments shown in Fig. 2. The purified CSN complex was incubated with activated recombinant Casp3 or Casp7. CSN6 was completely cleaved after 4 h in the presence of Casp3, whereas it was only partially processed by Casp7 (Fig. 2A). Under these conditions, Casp7 is active as we have recently shown that it can cleave some substrates, such as CENP-C and INCENP more efficiently than Casp3 [34]. Proteolysis was specific, because it was inhibited by z-DEVD, a caspase inhibitor (Fig. 2A). These *in vitro* results demonstrated that complex-bound CSN6 was cleaved and supported our observations with cells (see Fig. 1) where free CSN6 does not occur.

Similar to the complex-bound protein, the recombinant full-length CSN6 can be cleaved by Casp3 or Casp7 (see Fig. 2B). However, we did not see any cleavage of a truncated version of CSN6, the CSN6 Δ 29 mutant protein, missing the N-terminal amino acids 1 to 29. Therefore the consensus sequence for caspase cleavage M₂₀EVD₂₃ was the most likely target for Casp3 and Casp7. By mutating D₂₃ to A the full-length CSN6 protein became completely resistant against caspase cleavage. Interestingly, although CSN subunits possess numerous caspase consensus sequences, M₂₀EVD₂₃ of CSN6 was the only one cleaved during apoptosis.

During the preparation of this manuscript it has been published that CSN6 can be also cleaved in SK-BR3 and in various epithelial cell lines [35]. In these studies Casp8 seems to be critical for CSN6 processing, whereas in our hands Casp8 was less effective as compared to Casp3 (data not shown).

Caspase cleavage of CSN6 is accompanied by cleavage of Rbx1

The CSN assembles with CRLs into super-complexes [33, 36, 37], in which CSN6 interacts with Rbx1 and CSN2 with cullins [5, 6]. We were interested to see whether besides CSN6 additional components of the CSN-CRL complexes were modified by activated caspases during apoptosis. Therefore Jurkat T cells were treated for 6 h with etoposide resulting in 60% apoptosis. After treatment protein complexes were separated by density gradient centrifugation. As seen in Fig. 3A, CSN subunits in apoptotic cells sedimented into the same fractions as in control cells or in cells incubated in the presence of etoposide and the caspase inhibitor z-VAD. These data indicate that the CSN complex does not fall apart during extensive induction of apoptosis. This was confirmed by non-denaturing gel electrophoresis of Jurkat and of HeLa cell lysates after induction of apoptosis (see Fig. 3B). Migrations of complexes that cross-react with the anti-CSN6 antibody were not affected by apoptosis. This was also true for the associated enzyme USP15, which co-sedimented with the CSN into fraction 8 (data not shown) and partially dissociated during glycerol gradient centrifugation as published before [31]. As seen in Fig. 3A, no cleavage of USP15 was observed despite the induction of extensive apoptosis (60%). In contrast, marked cleavage of the CRL component Rbx1 was obtained similar to that seen with CSN6. Obviously Rbx1 cleavage was caspase-specific, because it was inhibited by z-VAD. The ~10 kDa Rbx1 fragment produced by caspases was recognized by the used antibody raised against the C-terminal Rbx1 peptide 98-108 indicating that the caspase-consensus cleavage site is localized in the RING domain of

the protein. As seen in Fig. 3C, significant cleavage of Rbx1 was only observed after 4 – 5 h. These data demonstrate that CSN6 cleavage (see Fig. 1B) preceded caspase-dependent cleavage of Rbx1.

Cleavage of CSN6 by caspases modifies the deneddylation by the CSN but not the activity of the associated USP15 or kinases

The isopeptidase activity catalyzing deneddylation has been localized to CSN5, which exhibits a metalloprotease MPN⁺-domain [38]. In addition, the CSN is associated with a deubiquitinating enzyme called USP15 and with kinases such as CK2 and PKD [30, 31, 39]. To investigate the impact of CSN6 cleavage by caspases on CSN functions, we studied the influence of activated Casp3 on the enzymatic activities of the purified CSN. For this purpose the CSN was pre-incubated with recombinant active Casp3 for 1 h. Under these conditions CSN6 was cleaved almost completely (Fig. 4A, panel Anti-CSN6). In control experiments the CSN and Casp3 were pre-incubated with o-phenanthroline (OPT), an inhibitor of the deneddylase CSN5 [38], which had no influence on CSN6 cleavage. In contrast, pre-incubation in the presence of z-DEVD completely blocked CSN6 cleavage (Fig. 4A, panel Anti-CSN6). Deneddylation was measured with a synthesized peptide (Nedd8-Cul1-pep) consisting of the C-terminal amino acids 31 – 76 of Nedd8, which was linked via an isopeptide bond to the Cul1 peptide 719-724. The Cul1 peptide was biotinylated at K₇₂₃. The isopeptide bond was formed between the C-terminal G₇₆ residue of Nedd8 and the ε-NH₂ group of K₇₂₀ of Cul1 (see **Material and Methods**). The peptide was specifically synthesized, because the MPN⁺-based deneddylating activity of CSN5 prefers isopeptide bonds [7]. The Nedd8-Cul1-pep was added after 1 h pre-incubation together with the caspase inhibitor z-DEVD. The reaction was stopped after 3 h and Western blots were performed with streptavidin-POD. As demonstrated in Fig. 4A, panel Streptavidin-POD, approximately 30%

of the Nedd8-Cul1-pep was cleaved after 3 h by purified CSN. Surprisingly, the cleavage was significantly faster upon CSN treatment with activated Casp3 (Fig. 4A, CSN + Casp3). The Casp3-stimulated CSN-mediated cleavage of Nedd8-Cul1-pep was blocked by OPT as well as by the Casp3 inhibitor z-DEVD (see Fig. 4A, panel Streptavidin-POD) demonstrating that (i) the cleavage was catalyzed by the metalloprotease CSN5 and (ii) the activation of deneddylation was induced by Casp3.

As shown before (see Fig. 3A), the associated USP15 was not affected by etoposide treatment. This was confirmed by measuring the tetra-Ub (Ub₄) cleavage activity after treatment of the purified CSN with activated or with inhibited Casp3. As seen in Fig. 4B, panel Anti-Ubiquitin, the Ub₄ cleavage activity did not change during 1 h (Fig. 4C) indicating that USP15 was not affected by the caspase. In addition, the associated kinase activities were not significantly influenced by Casp3 treatment as demonstrated with c-Jun as substrate (Fig. 4B, panel Autoradiography).

Activation of CSN-mediated deneddylation should reduce the neddylation status of cullins in cells and might subsequently cause the disassembly of CRLs. To test this hypothesis neddylation was analyzed by Western blotting in Jurkat T cells (see Fig. 4C). After 4 - 6 h of apoptosis neddylation of endogenous proteins, most likely of cullins, decreased significantly as indicated by the anti-Nedd8 antibody. The effect was caspase-dependent, since z-VAD stabilized neddylated proteins. To analyze whether the Cul1 neddylation status was affected we induced apoptosis in Jurkat T cells with etoposide for 6 h after transfection with Cul1. As shown in the Western blots in Fig. 4D, Cul1 neddylation was reduced during apoptosis. This effect was completely blocked by z-VAD.

Discussion

Here we demonstrate that the CSN subunit CSN6 is cleaved during apoptosis by activated caspases. This is in agreement with recent findings [35]. Our experiments, however, clearly show that activation of both intrinsic as well as the extrinsic apoptotic pathways result in CSN6 cleavage after 2 - 3 h suggesting that effector caspases such as Casp3 might be responsible for the effect *in vivo*. In fact, our *in vitro* data confirm Casp3 as the most effective protease. The CSN6 cleavage site D₂₃ is conserved in higher eukaryotes, which makes it a target in other species too.

We show for the first time that the processing of CSN6 precedes the caspase-dependent cleavage of Rbx1. It has been published that the RING component of many CRL complexes, Rbx1, directly interacts with CSN6 in the CSN-CRL super-complexes [5, 6]. Presumably CSN6 modification makes Rbx1 accessible for caspase cleavage. Most likely activated Casp3 binds to the CSN-CRL complex and cleaves CSN6 first, which facilitates the modification of the adjacent Rbx1 protein. In CRL complexes Rbx1 is essential for the ligation of Ub moieties to substrate proteins [2]. The irreversible caspase-dependent cleavage of Rbx1 produces a fragment (see Fig. 3A) indicating cleavage inside the RING domain. Therefore, Rbx1 modification during apoptosis most likely causes inactivation of the affected Ub ligase and should significantly reduce CRL-mediated ubiquitination. Interestingly, as demonstrated before Ub conjugates accumulate up to 4 h in Jurkat T cells in the presence of etoposide due to the inactivation of the 26S proteasome by caspases. However, after 4 h the level of Ub conjugates decreases perhaps as a result of the CRL inactivation (see Figure 5B in [26]). Moreover, Rbx1 has been identified not only as a ubiquitinating enzyme in CRL complexes, but also as a ligase for Rub1, the yeast homolog of Nedd8 [40]. The decrease of neddylated proteins including Cul1 during apoptosis shown in Fig. 4C and D could be at least in part a result of Rbx1 inactivation. The RING domain protein Rbx1 is highly evolutionarily

conserved [41] and, therefore, caspase-dependent inactivation of CRLs might be a common event in higher eukaryotes.

Another consequence of CSN6 cleavage caused by apoptosis is the stimulation of the deneddylating activity. In the CSN core complex the MPN-domain protein CSN6 directly interacts with the MPN⁺-domain subunit CSN5 [42]. The increase of CSN5 isopeptidase activity might be explained by a conformational change induced by cleaving off 23 amino acids of CSN6. Since CSN-mediated deneddylation prevents CRL complex assembly [2, 4, 43], it is conceivable to assume that the elevation of this activity is another strategy to knockout CRLs during apoptosis. In fact, it has been shown that the typical Cul1-CRL substrate, p27^{Kip} [44], is accumulated in tumor cells after treatment with etoposide [45]. Another Cul1-CRL substrate, p53^{Kip} [46], promotes the mitochondrial apoptotic pathway [47]. Interestingly, overexpression of the cleavage-resistant CSN6D23A mutant had no significant effect on apoptosis in MCF-7 cells [35] indicating that CSN6 cleavage is not necessary for the process. Nonetheless, cleavage of CSN6 and its consequences seem to be part of the programmed inactivation of the UPS during apoptosis. As a result pro-apoptotic factors such as p27^{Kip}, p53 or Smac are stabilized driving the apoptotic process to its completion. For the first time our data demonstrate that CSN-mediated deneddylation can be regulated by active Casp3 and that the CSN executes a specific function during the apoptotic process.

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References

1. Deng XW, Dubiel W, Wei N, Hofmann K, Mundt K, Colicelli J, Kato J, Naumann M, Segal D, Seeger M, Carr A, Glickman M, Chamovitz DA (2000) Unified nomenclature for the COP9 signalosome and its subunits: an essential regulator of development. *Trends Genet* 16:202-203
2. Petroski MD, Deshaies RJ (2005) Function and regulation of cullin-RING ubiquitin ligases. *Nat Rev Mol Cell Biol* 6:9-20
3. Willems AR, Schwab M, Tyers M (2004) A hitchhiker's guide to the cullin ubiquitin ligases: SCF and its kin. *Biochim Biophys Acta* 1695:133-170
4. Bornstein G, Ganoth D, Hershko A (2006) Regulation of neddylation and deneddylation of cullin1 in SCFSkp2 ubiquitin ligase by F-box protein and substrate. *Proc Natl Acad Sci U S A* 103:11515-11520
5. Schwechheimer C, Serino G, Callis J, Crosby WL, Lyapina S, Deshaies RJ, Gray WM, Estelle M, Deng XW (2001) Interactions of the COP9 signalosome with the E3 ubiquitin ligase SCFTIR1 in mediating auxin response. *Science* 292:1379-1382
6. Lyapina S, Cope G, Shevchenko A, Serino G, Tsuge T, Zhou C, Wolf DA, Wei N, Deshaies RJ (2001) Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome. *Science* 292:1382-1385
7. Groisman R, Polanowska J, Kuraoka I, Sawada J, Saijo M, Drapkin R, Kisselev AF, Tanaka K, Nakatani Y (2003) The ubiquitin ligase activity in the DDB2 and CSA complexes is differentially regulated by the COP9 signalosome in response to DNA damage. *Cell* 113:357-367
8. Liu C, Poitelea M, Watson A, Yoshida SH, Shimoda C, Holmberg C, Nielsen O, Carr AM (2005) Transactivation of *Schizosaccharomyces pombe* cdt2⁺ stimulates a Pcu4-Ddb1-CSN ubiquitin ligase. *EMBO J* 24:3940-3951

9. Bech-Otschir D, Kapelari B, Dubiel W (2005). The COP9 Signalosome: Its Possible Role in the Ubiquitin System, R Mayer; A Ciechanover; M Rechsteiner, eds) Protein Degradation, Volume 1: Ubiquitin and the Chemistry of Life,(Weinheim: WILEY-VCH Verlag GmbH & Co. KGaA), pp 348-369
10. Freilich S, Oron E, Kapp Y, Nevo-Caspi Y, Orgad S, Segal D, Chamovitz DA (1999) The COP9 signalosome is essential for development of *Drosophila melanogaster*. *Curr Biol* 9:1187-1190
11. Wei N, Deng XW (2003) The COP9 signalosome. *Annu Rev Cell Dev Biol* 19:261-286
12. Busch S, Schwier EU, Nahlik K, Bayram O, Helmstaedt K, Draht OW, Krappmann S, Valerius O, Lipscomb WN, Braus GH (2007) An eight-subunit COP9 signalosome with an intact JAMM motif is required for fungal fruit body formation. *Proc Natl Acad Sci U S A* 104:8089-8094
13. Jesenberger V, Jentsch S (2002) Deadly encounter: ubiquitin meets apoptosis. *Nat Rev Mol Cell Biol* 3:112-121
14. 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
15. MacFarlane M, Merrison W, Bratton SB, Cohen GM (2002) Proteasome-mediated degradation of Smac during apoptosis: XIAP promotes Smac ubiquitination in vitro. *J Biol Chem* 277:36611-36616
16. Fullbeck M, Huang X, Dumdey R, Frommel C, Dubiel W, Preissner R (2005) Novel curcumin- and emodin-related compounds identified by in silico 2D/3D conformer screening induce apoptosis in tumor cells. *BMC Cancer* 5:97
17. Schweitzer K, Bozko PM, Dubiel W, Naumann M (2007) CSN controls NF-kappaB by deubiquitinylation of IkappaBalpha. *EMBO J* 26:1532-1541

18. Karin M, Ben-Neriah Y (2000) Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol* 18:621-663
19. Yin D, Zhou H, Kumagai T, Liu G, Ong JM, Black KL, Koeffler HP (2005) Proteasome inhibitor PS-341 causes cell growth arrest and apoptosis in human glioblastoma multiforme (GBM). *Oncogene* 24:344-354
20. Fribley A, Wang CY (2006) Proteasome inhibitor induces apoptosis through induction of endoplasmic reticulum stress. *Cancer Biol Ther* 5:745-748
21. Bratton SB, MacFarlane M, Cain K, Cohen GM (2000) Protein complexes activate distinct caspase cascades in death receptor and stress-induced apoptosis. *Exp Cell Res* 256:27-33
22. Reed JC (2000) Mechanisms of apoptosis. *Am J Pathol* 157:1415-1430
23. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91:479-489
24. Zou H, Henzel WJ, Liu X, Lutschg A, Wang X (1997) Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 90:405-413
25. Peter ME, Kischkel FC, Hellbardt S, Chinnaian AM, Krammer PH, Dixit VM (1996) CD95 (APO-1/Fas)-associating signalling proteins. *Cell Death Differ* 3:161-170
26. Sun XM, Butterworth M, MacFarlane M, Dubiel W, Ciechanover A, Cohen GM (2004) Caspase activation inhibits proteasome function during apoptosis. *Mol Cell* 14:81-93
27. MacFarlane M, Merrison W, Dinsdale D, Cohen GM (2000) Active caspases and cleaved cytokeratins are sequestered into cytoplasmic inclusions in TRAIL-induced apoptosis. *J Cell Biol* 148:1239-1254

28. Sun XM, MacFarlane M, Zhuang J, Wolf BB, Green DR, Cohen GM (1999) Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. *J Biol Chem* 274:5053-5060
29. Seeger M, Kraft R, Ferrell K, Bech-Otschir D, Dumdey R, Schade R, Gordon C, Naumann M, Dubiel W (1998) A novel protein complex involved in signal transduction possessing similarities to 26S proteasome subunits. *FASEB J* 12:469-478
30. 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
31. Hetfeld BK, Helfrich A, Kapelari B, Scheel H, Hofmann K, Guterman A, Glickman M, Schade R, Kloetzel PM, Dubiel W (2005) The zinc finger of the CSN-associated deubiquitinating enzyme USP15 is essential to rescue the E3 ligase Rbx1. *Curr Biol* 15:1217-1221
32. Hetfeld BK, Bech-Otschir D, Dubiel W (2005) Purification method of the COP9 signalosome from human erythrocytes. *Methods Enzymol* 398:481-491
33. Huang X, Hetfeld BK, Seifert U, Kahne T, Kloetzel PM, Naumann M, Bech-Otschir D, Dubiel W (2005) Consequences of COP9 signalosome and 26S proteasome interaction. *Febs J* 272:3909-3917
34. Faragher AJ, Sun XM, Butterworth M, Harper N, Mulheran M, Ruchaud S, Earnshaw WC, Cohen GM (2007) Death receptor-induced apoptosis reveals a novel interplay between the chromosomal passenger complex and CENP-C during interphase. *Mol Biol Cell* 18:1337-1347
35. Correia Jda S, Miranda Y, Leonard N, Ulevitch RJ (2007) The Subunit CSN6 of the COP9 Signalosome Is Cleaved during Apoptosis. *J Biol Chem* 282:12557-12565

36. Gusmaroli G, Figueroa P, Serino G, Deng XW (2007) Role of the MPN subunits in COP9 signalosome assembly and activity, and their regulatory interaction with Arabidopsis Cullin3-based E3 ligases. *Plant Cell* 19:564-581
37. Peng Z, Shen Y, Feng S, Wang X, Chitteti BN, Vierstra RD, Deng XW (2003) Evidence for a physical association of the COP9 signalosome, the proteasome, and specific SCF E3 ligases in vivo. *Curr Biol* 13:R504-505
38. 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
39. Wee S, Geyer RK, Toda T, Wolf DA (2005) CSN facilitates Cullin-RING ubiquitin ligase function by counteracting autocatalytic adapter instability. *Nat Cell Biol* 7:387-391
40. Kamura T, Conrad MN, Yan Q, Conaway RC, Conaway JW (1999) The Rbx1 subunit of SCF and VHL E3 ubiquitin ligase activates Rub1 modification of cullins Cdc53 and Cul2. *Genes Dev* 13:2928-2933
41. Megumi Y, Miyauchi Y, Sakurai H, Nobeyama H, Lorick K, Nakamura E, Chiba T, Tanaka K, Weissman AM, Kirisako T, Ogawa O, Iwai K (2005) Multiple roles of Rbx1 in the VBC-Cul2 ubiquitin ligase complex. *Genes Cells* 10:679-691
42. Fu H, Reis N, Lee Y, Glickman MH, Vierstra RD (2001) Subunit interaction maps for the regulatory particle of the 26S proteasome and the COP9 signalosome. *EMBO J* 20:7096-7107
43. Sakata E, Yamaguchi Y, Miyauchi Y, Iwai K, Chiba T, Saeki Y, Matsuda N, Tanaka K, Kato K (2007) Direct interactions between NEDD8 and ubiquitin E2 conjugating enzymes upregulate cullin-based E3 ligase activity. *Nat Struct Mol Biol* 14:167-168
44. Carrano AC, Eytan E, Hershko A, Pagano M (1999) SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. *Nat Cell Biol* 1:193-199

45. Yu HG, Ai YW, Yu LL, Zhou XD, Liu J, Li JH, Xu XM, Liu S, Chen J, Liu F, Qi YL, Deng Q, Cao J, Liu SQ, Luo HS, Yu JP (2007) Phosphoinositide 3-kinase/Akt pathway plays an important role in chemoresistance of gastric cancer cells against etoposide and doxorubicin induced cell death. *Int J Cancer*
46. Kamura T, Hara T, Kotoshiba S, Yada M, Ishida N, Imaki H, Hatakeyama S, Nakayama K, Nakayama KI (2003) Degradation of p57Kip2 mediated by SCFSkp2-dependent ubiquitylation. *Proc Natl Acad Sci U S A* 100:10231-10236
47. Vlachos P, Nyman U, Hajji N, Joseph B (2007) The cell cycle inhibitor p57(Kip2) promotes cell death via the mitochondrial apoptotic pathway. *Cell Death Differ* 14:1497-1507

Figure legends

Fig. 1 Caspase-dependent cleavage of endogenous CSN6 accompanies apoptosis in Jurkat T and in HeLa cells. **(A)** Intrinsic- and extrinsic-induced apoptosis leads to cleavage of CSN6 in Jurkat T cells. Jurkat T cells were treated with either anti-Fas antibody (50 ng/ml, Fas-Ab) or etoposide (50 μ M) in the presence or absence of 20 μ M z-VAD for 6 h. After incubation cells were lysed and equal protein amounts loaded onto gels for SDS-PAGE. Western blotting was performed with antibodies against the eight individual CSN subunits as indicated. The positions of CSN subunits in the gel are marked by arrows. The CSN6 cleavage product is labeled with an asterisk (*), unspecific bands with circles ($^{\circ}$). **(B)** Time course of endogenous CSN6 cleavage. HeLa or Jurkat T cells were incubated for 0 – 6 h either with TRAIL (1 μ g/ml) or with etoposide (50 μ M) in the presence or absence of z-VAD (20 μ M). At the indicated times, cells were analyzed for apoptosis and lysed. Western blotting was performed with an anti-CSN6 antibody. The CSN6 cleavage product is labeled with an asterisk (*).

Fig. 2 Complex-bound as well as recombinant CSN6 is cleaved predominately by Casp3. **(A)** Purified CSN complex (200 nM) was incubated with or without 200 nM Casp3 or Casp7 for 4 h in the presence or absence of z-DEVD (50 μ M). Western blotting was performed using the anti-CSN6 antibody. The CSN6 cleavage product is labeled with an asterisk (*). **(B)** Recombinant His-CSN6, His-CSN6 Δ 29 or His-CSN6D23A (500 nM) was incubated with or without 500 nM Casp3 or Casp7 for 4 h. Recombinant CSN6 versions and the CSN6 cleavage product (*) were detected by Western blotting using the anti-CSN6 antibody.

Fig. 3 In apoptotic cells CSN6 and its binding partner Rbx1 are cleaved within super-complexes consisting of the CSN and CRLs. **(A)** Jurkat T cells were incubated for 6 h with or without etoposide (50 μ M) in the presence or absence of 20 μ M z-VAD. Apoptosis was

determined by Annexin V-binding assay. Cell lysate was prepared and 3 mg aliquots loaded onto a 10 - 40% glycerol gradient. Western blotting with aliquots of fractions 1 - 19 of the gradient and cell lysate (Input) was performed using antibodies against CSN6, CSN3, CSN8, USP15 and Rbx1. Cleavage products are labeled with an asterisk (*). In fractions 3 and 5 unspecific bands with the anti-CSN6 antibody are seen. **(B)** CSN complex integrity is maintained for 6 h after apoptosis-induced cleavage of CSN6. Western blotting using the anti-CSN6 antibody after non-denaturing gel electrophoresis with aliquots (20 µg) of Jurkat T cell lysate (as in (A)) and HeLa cell lysate was carried out. Apoptosis was induced with etoposide or TRAIL in the presence or absence of z-VAD as indicated. **(C)** Time course of endogenous Rbx1 cleavage. Jurkat T cells were incubated for 0 – 6 h with etoposide (50 µM) in the presence or absence of z-VAD (20 µM). At the indicated times, cells were analyzed for apoptosis and lysed. Western blotting was performed with the anti-Rbx1 antibody. Tubulin was used as a loading control. The cleavage product of Rbx1 is labeled with an asterisk (*).

Fig. 4 Casp3 cleavage of CSN6 stimulates the deneddylating activity of the CSN complex. **(A)** Purified CSN complex (750 nM) was pre-incubated with or without equimolar Casp3 (CSN + Casp3) or with Casp3 in the presence of 1.5 µM z-DEVD (inhib. Casp3) for 1 h (-1 – 0 h) at 37°C. Biotin-coupled Nedd8-Cul1 peptide (Nedd8-Cul1-pep) was added after pre-incubation (0 h) to detect deneddylating activity of the CSN. As an inhibitor of CSN5 deneddylase, the metalloprotease inhibitor OPT (5 mM) was used. Western blotting was performed using the anti-CSN6 antibody as well as streptavidin-POD. CSN6 cleavage products were labeled with an asterisk (*). Nedd8-Cul1-pep cleavage was quantified by densitometric analysis. The density at 0 h was 100% and the peptide decay was monitored for 3 h. **(B)** Casp3 treatment of the CSN complex has no impact on its associated deubiquitinating or kinase activities. Purified CSN complex was pre-incubated as in (A). To detect deubiquitinating activity Ub₄ was added (left panel). Western blotting was performed using

the anti-ubiquitin antibody. Kinase activity was visualized by adding recombinant c-Jun (0.5 μ g) and [γ - 32 P]-ATP for 1 h (right panel). In the control experiment (Contr.) CSN alone was incubated demonstrating that no additional autophosphorylation occurred during apoptosis. Aliquots were separated by SDS-PAGE and stained with Coomassie. The dried gel was autoradiographed. **(C)** Apoptosis stimulates deneddylation in Jurkat T cells. Apoptosis was induced in Jurkat T cells by etoposide (50 μ M) in the absence or in the presence of z-VAD. Neddylated proteins were detected using the anti-Nedd8 antibody. **(D)** Apoptosis induces deneddylation of Cul1. Five μ g Cul1 per 5×10^6 Jurkat T cells were transfected. Apoptosis was induced by etoposide 24 h after transfection. After 6 h of apoptosis cells were lysed and Western blots were performed with the anti-Nedd8 and the anti-Cul1 antibodies. Tubulin was probed as a loading control.

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