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Disruption of the COP9 Signalosome Csn2 Subunit in Mice Causes Deficient Cell Proliferation, Accumulation of p53 and Cyclin E, and Early Embryonic Death

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Csn2 (Trip15/Cops2/Alien) **encodes the second subunit of the COP9 signalosome (CSN), an eight-subunit heteromeric complex homologous to the lid subcomplex of the 26S proteasome. CSN is a regulator of SCF (Skp1-cullin-F-box protein)ubiquitin ligases, mostly through the enzymatic activity that deconjugates the ubiquitin-like protein Nedd8 from the SCF Cul1 component. In addition, CSN associates with protein kinase activities targeting p53, c-Jun, and IκB for phosphorylation. Csn2 also interacts with and regulates a subset of nuclear hormone receptors and is considered a novel corepressor. We report that targeted disruption of *Csn2* in mice caused arrest of embryo development at the peri-implantation stage. *Csn2*$^{-/-}$ blastocysts failed to outgrow in culture and exhibited a cell proliferation defect in inner cell mass, accompanied by a slight decrease in Oct4. In addition, lack of Csn2 disrupted the CSN complex and resulted in a drastic increase in cyclin E, supporting a role for CSN in cooperating with the SCF-ubiquitin-proteasome system to regulate protein turnover. Furthermore, *Csn2*$^{-/-}$ embryos contained elevated levels of p53 and p21, which may contribute to premature cell cycle arrest of the mutant.**

Csn2, also known as *Trip15* or *Sgn2* in humans (19, 26), *Cops2* in mice (33), *Alien* in *Drosophila melanogaster* (15), and *FUS12* in *Arabidopsis thaliana* (36) encodes the second subunit (443 amino acids) of the COP9 signalosome (CSN) (7, 35, 45). CSN is highly homologous to the lid subcomplex of the 26S proteasome, both containing eight subunits (14, 45). Csn2 is one of the most conserved CSN subunits (25, 36) which appears to exist predominantly as an integral part of the CSN complex in HeLa cells (48). CSN associates with protein kinase activities which can phosphorylate cellular regulators including c-Jun, IκB, and p53 in vitro (3, 35, 39). Phosphorylation of p53 by CSN-associated kinases at Thr155 promotes p53 degradation by the ubiquitin-proteasome pathway (3). Another target of the kinase activity is interferon consensus sequence binding protein, which is recruited to CSN through Csn2 (4).

CSN interacts with SCF (Skp1-cullin-F-box protein) E3 ubiquitin ligases and deconjugates the ubiquitin-like protein Nedd8 from Cul1 (deneddylation) (5, 21, 34, 48, 50). This reaction counters the neddylation reaction catalyzed by the Nedd8-specific activating enzymes APP-BP1 and Uba3 (E1) and the Nedd8 conjugating enzyme Ubc12 (E2) (16). Neddylation has been shown to stimulate SCF ubiquitin ligase activities (17, 47). Disruption of *Cul1*, *Cul3*, or the neddylation enzyme Uba3 in mice leads to embryonic lethality and overaccumulation of an SCF substrate, cyclin E (6, 37, 43, 40). It has recently been shown that Cul1 deneddylation enables it to bind CAND1, which subsequently results in dissociation of Skp1-Skp2 from Cul1 (20, 49). As a result, both CSN deneddylase and CAND1 were shown to inhibit SCF activity in vitro (20, 48, 49). However, reducing the level of CAND1 in cells by small interfering RNA caused accumulation of p27^{kip1} (p27) (49). Similarly, genetic data indicate that a fully functional CSN5 is required for optimal activity of SCF in degradation of PSIAA6, a putative substrate of SCF^{TIR1} in *Arabidopsis thaliana* (34), and in degradation of Sic1 in *Saccharomyces cerevisiae* (5). This led to the hypothesis that both neddylation and deneddylation are necessary for the SCF functions.

CSN-mediated deneddylation is believed to center in the metalloprotease motif of CSN5/Jab1, but the manifestation of the activity requires the entire complex (5). Each of the null mutants of CSN subunits from *Arabidopsis thaliana* and fission yeast *Schizosaccharomyces pombe* lacks Cul1 deneddylation activity and preferentially accumulates the Nedd8 modified form of Cul1 (21, 34, 41, 50). In particular, Csn2 plays a critical role in deneddylation in part by direct binding to Cul1 and Cul2 primarily through its N-terminal region (amino acids 1 to 189), while it integrates into the CSN complex via its C-terminal region (amino acids 210 to 443) (48).

Csn2 was initially isolated as a thyroid hormone receptor-interacting protein (19) and was subsequently found to interact with a subset of additional nuclear hormone receptors such as DAX-1 (through human Alien/Csn2, amino acids 1 to 275), COUP-TF1, and ecdysone receptor (2, 9). Transient expression of a fragment corresponding to the N-terminal (amino acids 1 to 300) fragment of Csn2 Alien enhanced thyroid hormone receptor-mediated transcription repression, leading to

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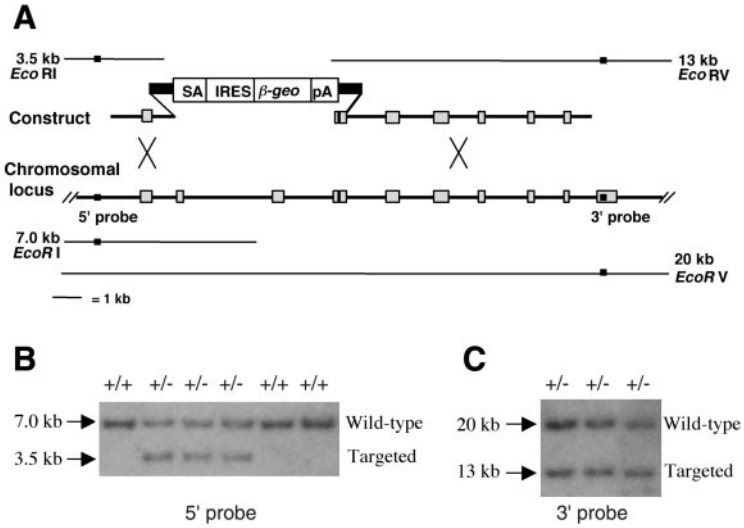

FIG. 1. Targeted disruption of mouse *Csn2*. (A) A diagram of the targeting construct and the genomic structure of mouse *Csn2* locus. The locations of the 5′ and 3′ probes and the relative sizes of the restriction fragments of the wild-type or targeted alleles from the homologous recombination are shown. (B) Identification of homologous recombinants. Genomic DNA from independent ES cell colonies was digested with *Eco*RI and analyzed by Southern blot with the 5′ probe to identify the clones carrying the 3.5-kb fragment specific for targeted allele (+/− lanes). Three out of six colonies shown indicated successful targeting. These three clones were further characterized with the 3′ probe in an *Eco*RV digest to verify the presence of the 13-kb targeting fragment (C).

the hypothesis that Csn2 represents a member of a new class of corepressors (9). The mechanism of this transcription cosuppression is unclear. Interestingly, forced expression of a full-length Csn2 was sufficient to induce neuronal differentiation and a concomitant decrease in Oct3/4 (Oct4) expression in P19 embryonal carcinoma cells in the absence of retinoic acid treatment (1).

The role of CSN in development has been studied only in nonmammalian genetic systems. CSN is involved in photomorphogenesis, flower development, and auxin and defense responses in plants (11, 34, 36, 42) and in oogenesis, embryogenesis, DNA repair, and axon guidance in flies (8, 12, 29, 38). CSN also plays an important role in the S-phase progression of the cell cycle in the fission yeast *Schizosaccharomyces pombe* (25). The CSN-like complex in the budding yeast *Saccharomyces cerevisiae* is involved in mating pheromone response (22). To understand the role of CSN in mouse development, we disrupted the murine *Csn2* gene. Loss of *Csn2* caused embryonic lethality at the peri-implantation stage and a deficiency of cell proliferation in the inner cell mass (ICM). The mutant embryos displayed elevated accumulation of cyclin E, p53, and the cyclin-dependent kinase inhibitor $p21^{Cip1/Waf1}$.

MATERIALS AND METHODS

Targeted disruption of mouse *Csn2*. A mouse *Csn2/Cops2* 22-kb genomic DNA clone was isolated from a 129SvJ/6 bacterial artificial chromosome library. To construct the targeting vector, the 9-kb *Pst*I fragment was subcloned into a pBluescript vector. The 5′ genomic fragment, generated by PCR with AL-A (5′-CCGCCGCTCG AGCTCTGCCTTTACAGTGTTCC) and AL-B (5′-CCG CCGCTCG AGATTTTTCAATGACATCCAATG) was later inserted into *Xho*I sites of the vector. Finally the IRES-βGeo cassette, which has an upstream splicing acceptor, an internal ribosomal entry site, and a 3′ polyadenylation signal was inserted at the *Sal*I site (Fig. 1A). A *Not*I site was used to linearize the construct prior to electroporation. Upon homologous recombination, the *Csn2* locus was expected to be disrupted by the βGeo cassette, from which expression of *lacZ* was placed under the control of the *Csn2* promoter.

The standard embryonic stem (ES) cell gene targeting procedure was followed. For Southern blot analysis of *Eco*RI-digested genomic DNA, a 5′ probe of 500 bp was generated by PCR with the primers ALC (5′-CTTGTTAGTAT GTGGCTGCA) and ALD (5′-AGAGTCTCACTGAGCACAGC). This probe detects a 7-kb band in the wild-type allele and a 3.5-kb band from the targeted allele. The 3′ probe was generated by PCR with primers AL5 (5′-CAACAGTG CAGAGATGTGAC) and AL6 (5′-TTCGGGTTGTCTAACCTTTA) for use on *Eco*RV-digested genomic DNA. This probe detects a 13-kb band in the targeted allele and a 20-kb band in the wild-type allele.

Positive clones were karyotyped and subsequently injected into C57BL/6J blastocysts. Chimera males were mated with C57BL/6J females, and offspring were genotyped by PCR of tail DNA with primers ALA (5′-CCGCCGCTCGA GCTCTGCCTTTACAGTGTTCC) and En2 (5′-CCGCCGCTCGAGTACTTT CGGTTCCTCTTCCC) or the PCR primer sets used for blastocyst genotyping.

Blastocyst isolation, outgrowth, and genotyping. Blastocysts were collected at embryonic development day 3.5 (E3.5) by flushing the uterus with M2 medium (Specialty Media MR-015P-F). The blastocysts were then transferred to ES medium in a 0.1% gelatin-treated multiwell slidechamber slide (Lav-Tek, Nalgene) and cultured at 37°C with 5% CO_2. After photographing or immunostaining, the blastocyst outgrowth colonies were lysed in buffer containing 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 2 mM $MgCl_2$, 0.45% (vol/vol) NP-40, 0.45% (vol/vol) Tween 20, and 60 μg of proteinase K per ml. The samples were boiled for 10 min to inactivate the protease. Half of the lysate (8 μl) was used in a 25-μl PCR, starting with denaturing at 94°C for 3 min, followed by 28 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 1 min. The PCR primers S2I2F (5′-GGGAA CAGATCAATTGATTTCCTGA) and E3R4 (5′-GATCATCTGTTTTAGCGC TTTGAAT) were used to detect the wild-type *Csn2* allele (252 bp). The primers S2I2F and En2 (5′-CCGCCGCTCGAGTACTTTCGGTTCCTCTTCCC) were used to detect the targeted allele (193 bp).

Immunohistochemistry. The pregnant uterus from timed heterozygous matings was surgically removed, and the individual decidua were separated and fixed in 3.7% formaldehyde (or 10% of the commercial 37% solution; J. T. Baker) overnight at 4°C. Samples were then embedded in paraffin blocks and cut into 5-μm sections. For antibody staining, the sections were deparaffinized and placed into a 3% solution of hydrogen peroxide for 5 min. After incubation with the primary antibodies for 1 h followed by washing, biotinylated secondary antibody was applied for 30 min and streptavidin-horseradish peroxidase was applied for 30 min. Diaminobenzidine was used as the peroxidase substrate, and counterstaining was performed with hematoxylin. The antibodies used include anti-CSN2 (1:1,000), anti-CSN1 (1:100), and anti-CSN8 (affinity purified, 0.7 mg/ml,

at 1:250) (Affiniti Research Inc.), anti-cyclin E (M-20 Santa Cruz, 1:150), anti-p53 (Dako), and anti-p21 (C-19 Santa Cruz, 1:250).

Immunofluorescence staining and bromodeoxyuridine incorporation. Bromodeoxyuridine was added to culture media to a final concentration of 10 μM and incubated for 12 h at the time period specified. Cells were then fixed in 4% paraformaldehyde followed by DNA denaturation with 1.5 N HCl for 20 min. For other antibody stainings, the HCl treatment was replaced with permeablization solution (0.4% Triton X-100 in phosphate-buffered saline) for 10 min. The antibodies used include antibromodeoxyuridine (BD Biosciences-PharMingen); anti-Oct4 (Geneka, 1:30), and anti-Fgf4 (R&D Systems, 5 μg/ml). The fluorescein isothiocyanate- or Texas Red-conjugated secondary antibodies were obtained from Molecular Probes, Inc.

Inhibition of deneddylation by anti-CSN2 antibody in vitro. Inhibition of deneddylation by anti-CSN2 antibody in vitro was carried out as previously described (48). The total HeLa cell extract (40 μg) was supplied with ATP and ATP regeneration system and GST or GST-Nedd8 purified from *Escherichia coli* in amounts as indicated. Anti-Csn2 antiserum (1 μl) or preimmune serum (1 μl) was added and incubated at room temperature for 20 min. The reaction was terminated by adding sodium dodecyl sulfate loading buffer. The antibodies used for the immunoblots include anti-Cul1 (1:1,000, H-213, Santa Cruz,), anti-Cul2 (1: 250, Zymed), and anti-glutathione *S*-transferase (GST) (1:1,000, Pharmacia).

RESULTS

***Csn2* gene targeting.** Murine Csn2, previously described as Cops2, is encoded by a single locus on mouse chromosome 2 and is widely expressed in mice embryonic, fetal, and adult tissues (33). The murine *Csn2* gene contains 12 coding exons, with the largest intron of about 10 kb between the first and the second exons (33). The targeting vector was designed to delete exons 3 and 4 by insertion of a *lacZ-neo* (βGeo) cassette (Fig. 1A). Upon homologous recombination, the *Csn2* locus was expected to be disrupted by the βGeo cassette, thereby placing LacZ expression under the control of the *Csn2* promoter. Among the 192 independent embryonic stem (ES) cell colonies examined, we found 88 homologous recombinants. Correct targeting was confirmed in three representative ES clones by Southern blot analyses with a 5′ probe (Fig. 1B) and a 3′ probe (Fig. 1C). One ES clone was injected into C57BL/6J blastocysts to generate chimeras, which subsequently produced germ line transmission. The $Csn2^{+/-}$ line was established by backcrosses with C57BL/6J mice.

***Csn2*$^{-/-}$ embryos arrest at peri-implantation stage.** The $Csn2^{+/-}$ heterozygous mice appeared phenotypically healthy and fertile at up to 1 year of age. The Csn2 expression pattern in embryos, as indicated by LacZ staining of the heterozygous embryos, resembled that revealed by in situ hybridization reported previously (33). These heterozygous mice were intercrossed to produce $Csn2^{-/-}$ mice. However, no nullizygous mice were found among over 86 offspring, indicating that loss of *Csn2* caused recessive embryonic lethality. Embryos were isolated from timed *Csn2* heterozygous intercrosses from embryonic day 11.5 to as early as E7.5 and genotyped. As shown in Table 1, no viable $Csn2^{-/-}$ embryos were detected at any of the postimplantation stages, but abnormally high numbers (more than a quarter of total embryos) of empty decidua were observed. We next isolated and genotyped preimplantation stage embryos (blastocysts) at E3.5 and found that $Csn2^{-/-}$ blastocysts amounted to 16% of the total (Table 1). This result indicates that $Csn2^{-/-}$ embryos survived to blastocyst stage, underwent implantation as the mutants elicited decidual swelling, but died soon after implantation.

$Csn2^{-/-}$ embryos were already arrested at E6.5. At this stage, the layered egg cylinder structure observed in normal embryos (Fig. 2A) was absent in the $Csn2^{-/-}$ embryos (Fig. 2B), which could be identified based on the absence of Csn2 immunoreactivity (Fig. 2D). In addition, the mutant embryos contained very few cells, and these had no discernible cell differentiation and organization (Fig. 2B, 2D, 2F, and 2H). The extraembryonic structure was also missing, though trophoblast giant cells could occasionally be found in the mutants (arrowheads in Fig. 2).

TABLE 1. Genotypes of offspring from $Csn2^{+/-}$ intercrosses

Stage	Total no. of embryos	No. with genotype:			No. of resorptions[a]
		+/+	+/−	−/−	
Live birth	86	30	56	0	NA
E11.5	31	7	11	0	13
E10.5	10	2	3	0	5
E9.5	19	4	7	0	8
E7.5	34	7	18	0	10
E3.5	56	17	30	9	

[a] NA, not applicable.

Csn2 is required for the structural integrity of the CSN complex. To determine whether disruption of Csn2 may also cause structural instability of the CSN complex, we examined the accumulation of Csn1 and Csn8, the two subunits which cannot accumulate in the absence of the complete CSN complex assembly in *Arabidopsis thaliana* (41, 44). Like Csn2, the Csn1 and Csn8 proteins were ubiquitously expressed in normal E6.5 embryos (Fig. 2C, 2E, and 2G), but drastically reduced to background level in $Csn2^{-/-}$ embryos (Fig. 2F and 2H). Thus, disruption of the *Csn2* gene not only abolished Csn2 expression, it also diminished Csn1 and Csn8 accumulation, indicating a crucial role of Csn2 in maintaining integrity and stability of the CSN complex. A similar result has been reported in an *Arabidopsis thaliana* null mutant of *csn2* (*fus12*), where Csn8 protein is undetectable and CSN5/Jab1 accumulates only in low-molecular-weight free forms (18). It is possible, therefore, that the $Csn2^{-/-}$ mutant phenotype, although primarily reflecting the loss of Csn2 activity, may also be due in part to decreases in the activities mediated through Csn1, Csn8, and possibly other subunits of the CSN complex.

***Csn2* is required for cellular proliferation of the inner cell mass.** The newly isolated $Csn2^{-/-}$ blastocysts (day 0) were viable and largely indistinguishable from those of the wild type. These blastocysts were cultured in vitro to allow outgrowth for 5 days (Fig. 3A) and were then genotyped (Fig. 3C). Both $Csn2^{+}$ and $Csn2^{-/-}$ blastocysts hatched from the zona pellucida (day 1), attached onto the culture dish (day 2). Although the $Csn2^{-/-}$ blastocysts seemed to be more sensitive to culture conditions, they can produce apparently normal trophoblast giant cells, a process necessary to induce the decidual reaction during implantation. However, the inner cell mass (ICM), which forms the future embryonic tissues, failed to outgrow in $Csn2^{-/-}$ blastocysts (Fig. 3A).

To further delineate the proliferation defect of the $Csn2^{-/-}$ blastocysts, we carried out bromodeoxyuridine incorporation assays during blastocyst outgrowth (Fig. 3B). Vigorous DNA synthesis was observed in normal ICM cells throughout the outgrowth and in trophectoderm cells in the first 4 days (Fig. 3B-a to 3B-c). The mutant blastocysts underwent DNA synthesis upon attaching to the dish at day 2 (Fig. 3B-g). At day 3,

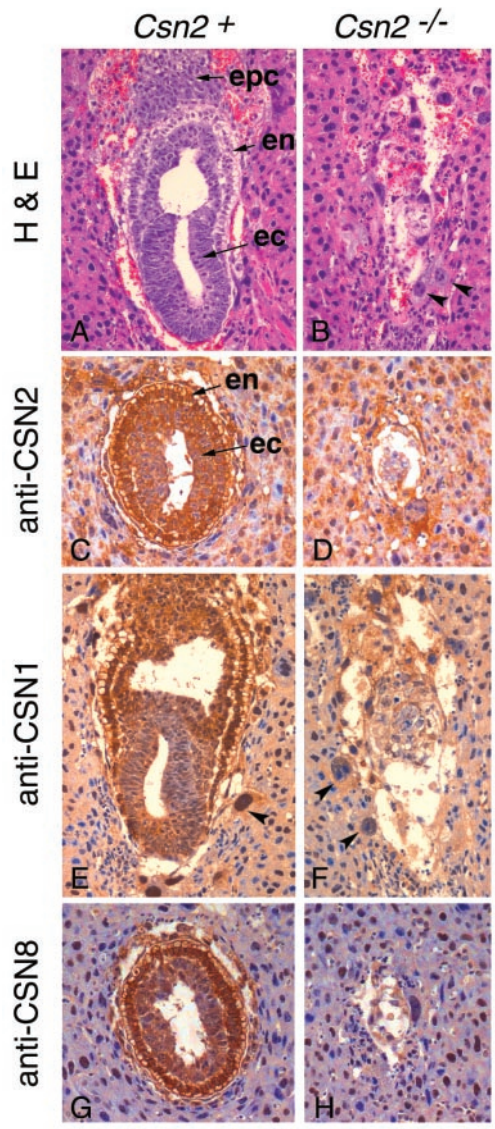

FIG. 2. Developmental arrest of $Csn2^{-/-}$ embryos at E6.5. The sagittal sections were stained with hematoxylin and eosin (A and B). The $Csn2^{+}$ embryos developed highly structured egg cylinder-stage embryos with a central proaminiotic cavity (A), whereas the $Csn2^{-/-}$ embryos had fewer cells and lacked normal architecture and cell differentiation (B). Expression of CSN subunits, as analyzed by immunohistochemical staining with anti-Csn2 (C and D), anti-Csn1 (E and F), or anti-Csn8 (G and H), was present in $Csn2^{+}$ embryos but absent in $Csn2^{-/-}$ embryos. Sagittal sections (A, B, E, and F) and transverse sections (C, D, G, and H) are shown. Abbreviations: epc, ectoplacental cone; en, extraembryonic endoderm; ec, embryonic ectoderm.

however, the presumed ICM cells of the mutant ceased proliferation, while lingering DNA synthesis was still observed in trophoblasts (Fig. 3B-h). After day 4, no cell proliferation was detected in any mutant cells (Fig. 3B-i). Moreover, the mutant displayed enlarged nuclei in the center of the arrested blastocyst outgrowths, which was atypical of ICM cell type (Fig. 3B-l). We did not observe higher apoptotic activity in $Csn2^{-/-}$ blastocyst outgrowths than wild-type blastocysts by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay (not shown). Clearly, the $Csn2^{-/-}$ blastocysts were unable to maintain ICM proliferation in culture.

The ICM failure prompted us to examine the expression of Oct4, a POU domain transcription factor required for establishing and maintaining ICM and other stem cells. The precise level of Oct4 is critical for proper cell differentiation and development in early embryos (28). Disruption of *Oct4* results in the diversion of ICM cells into the trophectoderm cell lineage, leading to early embryonic lethality (27). In the $Csn2^{-/-}$ blastocysts just hatched from the zona (day 1), the ICM-specific expression pattern of Oct4 was largely maintained in the mutant, but the level was slightly reduced (Fig. 3D-b). We also examined an Oct4 downstream target, fibroblast growth factor 4 (Fgf4), whose expression is critical for postimplantation embryo development (10). Fgf4 expression pattern appeared slightly altered in that its level was detectably decreased in the presumptive ICM cells but slightly increased in the trophectoderm cells (Fig. 3D-d). These results indicate that Csn2 is necessary for precise expression of Oct4 pathway components in early embryos.

Loss of Csn2 leads to elevated levels of cyclin E. Csn2 plays a critical role in CSN-mediated deneddylation. Incubation of the anti-Csn2 antibody in HeLa cell extract decreased the proportion of the unneddylated Cul1 and Cul2 with concomitant increase in the neddylated forms (Fig. 4A) (48, 49). This is caused by selective blockage of deneddylation in the extract that was active in the neddylation reactions as confirmed by de novo conjugation of exogenously supplied GST-Nedd8 in the assay (Fig. 4A). Conjugation of GST-Nedd8 appeared less efficient in the presence of anti-Csn2 (Fig. 4A), probably due to steric hindrance of the antibody when bound to CSN-SCF tertiary complex. This result demonstrates that cycles of neddylation and deneddylation are highly dynamic even in the cell extract, and it emphasizes the important role of Csn2 in deneddylation. Although we were unable to directly demonstrate a deneddylation defect in $Csn2^{-/-}$ cells due to early lethality of the mutant, this result combined with the observations that all null *csn* mutants from other genetic systems lack Cul1 deneddylation activity strongly argue that inactivation of *Csn2* would almost certainly lead to inhibition of CSN deneddylase activity.

As in the mutants deficient in SCF components or the neddylation enzyme, we also found increased accumulation of a SCF substrate, cyclin E, in the $Csn2^{-/-}$ cells (Fig. 4B). In E6.5 embryos, almost all of the $Csn2^{-/-}$ cells contained high intensity of cyclin E immunoreaction compared to the normal embryos in which only endoderm-like cells were stained (Fig. 4B-a and 4B-b). Furthermore, examination of the early blastocyst outgrowth showed strong immunofluorescence staining in $Csn2^{-/-}$ blastocysts. This was most evident in the trophectoderm cells that had low cyclin E immunoreaction in normal blastocysts (Fig. 4B-c and 4B-d). The overaccumulation of cyclin E is consistent with the idea that CSN is necessary for degradation of cyclin E by the ubiquitin-proteasome pathway.

***Csn2*$^{-/-}$ cells contain higher levels of p53 and p21.** The tumor suppressor p53 is thought to promote genomic stability, induce growth arrest and apoptosis, and consequently inhibit tumorigenesis. The protein turnover of p53 is tightly controlled in the cell through multiple E3 ubiquitin ligases. In addition, p53 interacts with CSN5/Jab1 and is a phosphorylation substrate of CSN-associated protein kinase (3). We detected p53

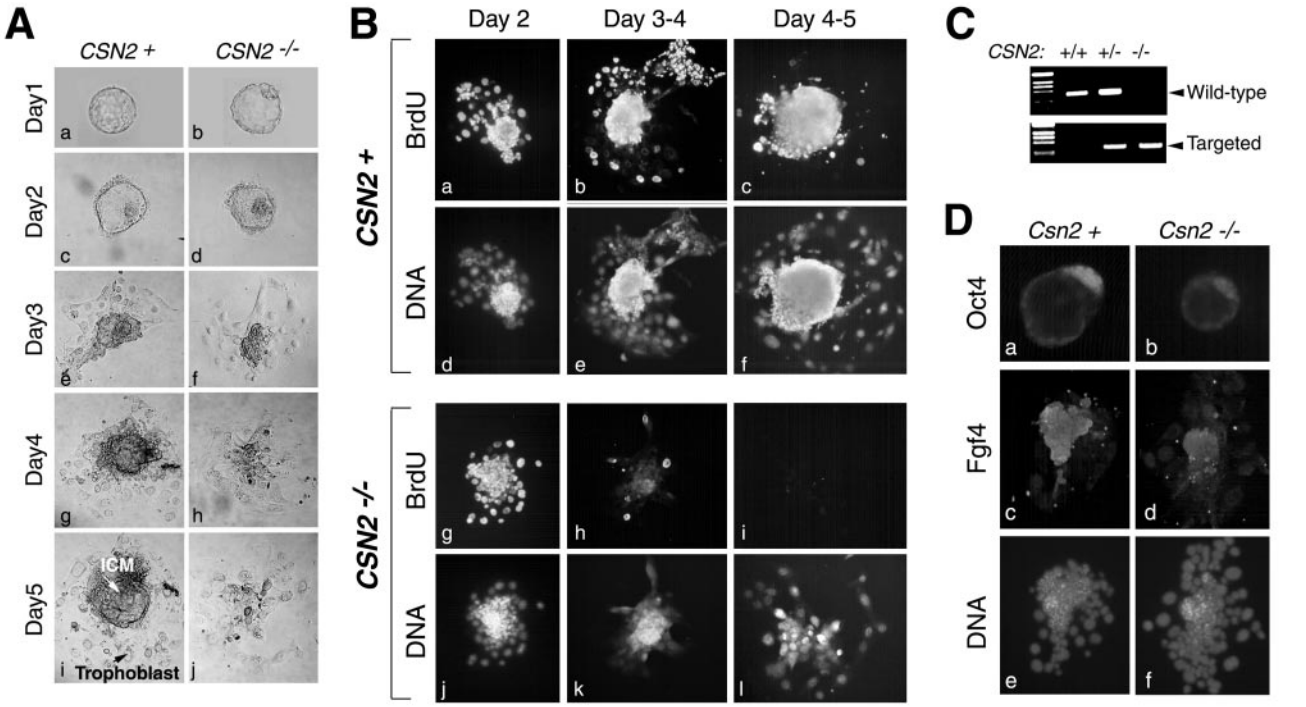

FIG. 3. $Csn2^{-/-}$ blastocysts failed in outgrowth assay and displayed cell proliferation deficiency. (A) E3.5 blastocysts isolated from $Csn2^{+/-}$ intercrosses were cultured to allow outgrowth. Photographs were taken each day from day 1 to day 5 after isolation. The ICM and the trophoblast cells are marked. ICM development occurred in $Csn2^{+}$ blastocysts but failed in $Csn2^{-/-}$ blastocysts. (B) Bromodeoxyuridine (BrdU) incorporation during blastocyst outgrowth. Bromodeoxyuridine was added to the medium and incubated for 12 h from 48 h to 60 h (day 2), from 84 h to 96 h (days 3 to 4), or from 108 h to 120 h (days 4 to 5). Blastocysts were fixed at the end of the 12-h labeling period and immunostained with antibromodeoxyuridine antibody. The corresponding nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) (DNA). Bromodeoxyuridine incorporation ceased by day 4 in $Csn2^{-/-}$ blastocyst cultures but continued in $Csn2^{+}$ blastocysts. (C) PCR genotyping of blastocysts showing the three different genotypes: $Csn2^{+/+}$, $Csn2^{+/-}$ and $Csn2^{-/-}$. (D) Expression of Oct4 and Fgf4 in $Csn2^{-/-}$ blastocysts. Hatched blastocysts after 1 day in culture were used for immunofluorescence staining of Oct4 (a and b). Oct4 expression appeared slightly weaker in the mutant. Blastocysts grown for 3 days were subjected to immunofluorescence staining with Fgf4 (c and d). The mutant appeared to have reduced Fgf4 staining in the ICM region but slightly higher staining in the trophoblasts. DAPI staining shows the nuclei DNA of the corresponding blastocysts (e and f).

expression only in extraembryonic endoderm cells of normal E6.5 embryos (indicated by an arrow, Fig. 5A). In $Csn2^{-/-}$ embryos, in contrast, p53 was intensely stained in essentially all of the mutant cells, including the presumed inner layer cells at E6.5 (Fig. 5B and 5C). Moreover, in vitro-cultured $Csn2^{-/-}$ blastocysts contained strong anti-p53 immunoreactions in the presumed ICM cells compared to the Csn^{+} blastocyst outgrowth (Fig. 5D and 5E). These data indicate that there is a general increase in p53 level in the mutant cells.

$p21^{Cip1/Waf1}$ (p21) is a broad-specificity inhibitor of cyclin/cyclin-dependent kinase and is a target of p53 transcription activity. Induction of p21, by either p53 dependent or independent mechanisms, is essential for the onset of cell cycle arrest in damage response and cell senescence (32). We found that p21 level was elevated in the $Csn2^{-/-}$ mutant cells as evidenced by immunohistochemical staining of E6.5 embryo sections (Fig. 5F and 5G). Immunofluorescence staining also showed a greater amount of p21 in the nuclei of both ICM and trophectoderm cells of $Csn2^{-/-}$ blastocyst outgrowth (Fig. 5I), compared to the background level of staining in normal blastocysts (Fig. 5H). Thus, loss of *Csn2* leads to abnormal accumulation of p53 and p21 in mutant embryos.

DISCUSSION

CSN is a highly conserved protein complex essential for viability in plants and flies (12, 44). Here we show that Csn2 and the CSN complex are essential in mammalian embryo development. Loss of *Csn2* disrupted the CSN complex formation and led to arrest of embryo development soon after implantation. The mutant blastocysts at E3.5 appear indistinguishable from those of $Csn2^{+}$ genotype, but its ICM cells fail to proliferate thereafter. Although this observation seems to suggest that CSN is only important for postimplantation embryo development, it remains possible that successful blastocyst formation and the initial round of cell division of $Csn2^{-/-}$ embryos are supported by maternally deposited CSN complex and mRNAs. Maternal contribution has been shown to play a role in the survival and embryonic patterning in *Csn4* and *Csn5* mutants of *Drosophila melanogaster* (8, 29).

A recent report showed that enforced expression of *Csn2* caused downregulation of Oct4 and was sufficient to convert P19 cells into neurons but not glial cells, and that antisense *Csn2* suppressed neuronal differentiation (1). Our data indicated that the arrest of $Csn2^{-/-}$ embryos at implantation stage

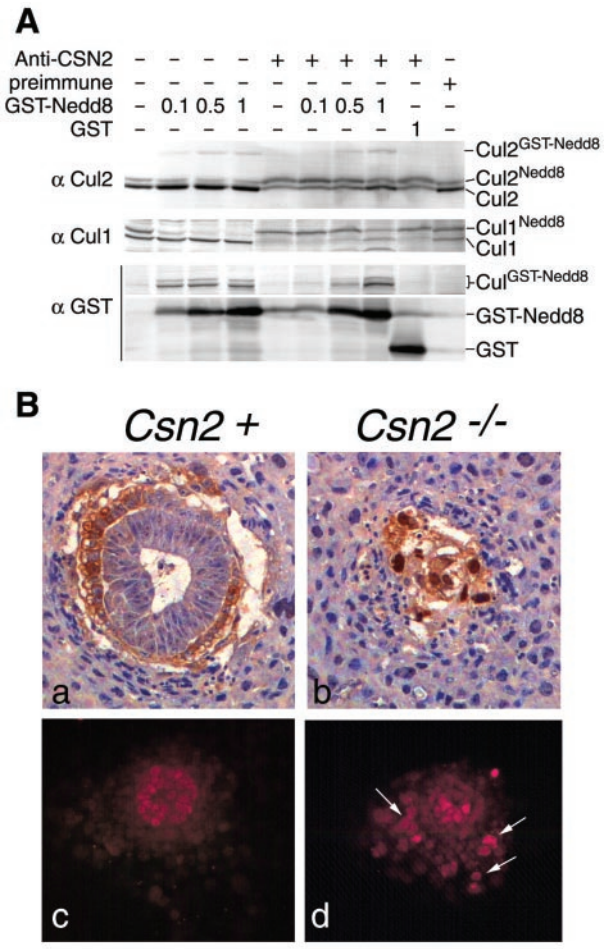

FIG. 4. Inactivation of Csn2 leads to Cul1 and Cul2 deneddylation defect and elevated accumulation of cyclin E. (A) HeLa cell extract was supplied with an ATP regeneration system and additional components as indicated. These included polyclonal antiserum against Csn2 (1 μl) or preimmune serum (1 μl); GST-Nedd8, 0.1, 0.5 or 1.0 μg; and GST, 1 μg. The samples were then analyzed by immunoblot with antibodies against Cul1, Cul2, and GST. The anti-Csn2 serum inhibited deneddylation, allowing neddylated cullins to accumulate as driven by active neddylation reactions in the system. (B) Abnormal accumulation of cyclin E in *Csn2*$^{-/-}$ embryos and blastocysts. Immunohistochemical analysis of E6.5 transverse sections display ubiquitous and high-intensity cyclin E staining in *Csn2*$^{-/-}$ embryos (b) compared to the weak endoderm staining in normal embryos (a). Immunofluorescence analysis of blastocyst outgrowth at day 2 showed higher cyclin E immunoreaction in the mutant (d) compared to normal blastocysts (c), particularly in trophectoderm cells (d, arrows).

was primarily caused by deficient ICM cell proliferation accompanied by a decline in Oct4 level. In addition, since differentiation of the presumed ICM cells was not detected in the mutant embryos (Fig. 2), this implies that *Csn2* is required for both cell proliferation and differentiation during early embryo development.

One of the major activities of CSN is to deneddylate SCF Cul1. Neddylation of SCF has been shown to stimulate the assembly of polyubiquitin chains and the recruitment of an E2 (17, 47). It is also required for ubiquitination of specific SCF substrates such as p27 and IκB (13, 23, 30, 31). Even though the deneddylation pathway components, CSN and CAND1, appear to inhibit SCF activity in vitro (20, 48, 49), our genetic data support the idea that CSN ultimately facilitates SCF activity in vivo. Accordingly, both neddylation and deneddylation seem necessary for proper SCF function. Components of the SCF complex, such as Cul1 and Skp2, have been shown to be ubiquitination targets of its own E3 activity in vitro and in vivo (24, 46, 51). It is conceivable that a mechanism restricting the self-ubiquitination activity is necessary to maintain the sustained SCF activity in vivo. We hypothesize that the control of

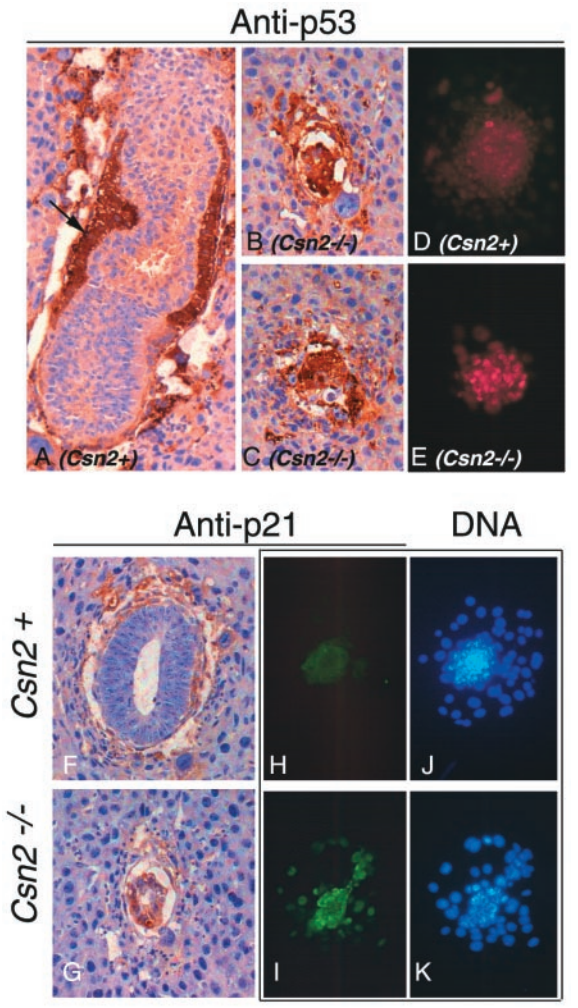

FIG. 5. *Csn2*$^{-/-}$ embryos accumulated elevated levels of p53 and p21. The E6.5 embryos were analyzed by immunohistochemical staining for p53 (A to E), or p21 (F to I). (A) Sagittal section of normal (*Csn2*$^{+}$) embryos stained for p53. The arrow points to the extraembryonic endoderm cells that contain p53 staining. (B and C) *Csn2*$^{-/-}$ mutants contained intense p53 staining in almost all cells of the embryos. (D and E) Blastocysts grown in culture for 2.5 days were used for anti-p53 immunofluorescence staining. The mutant cells (E) contain higher p53 immunoreactions compared to the *Csn2*$^{+}$ blastocyst outgrowth (D). Immunohistochemical analysis of p21 on a transverse section of normal embryo (F) compared with *Csn2*$^{-/-}$ embryos (G) showed more widespread and more intense staining in the mutant. Immunofluorescence analysis of p21 expression on 3-day blastocyst outgrowth (H and I) further confirmed that p21 expression was increased in both presumed ICM and trophectoderm cells of the *Csn2*$^{-/-}$ mutants. Blastocysts were double stained with DAPI to show the nuclear DNA (J and K).

SCF activation-inactivation may be achieved through the neddylation-deneddylation cycle. This is supported by the finding that knockdown of CAND1 by small interfering RNA decreased the level of Skp2, a substrate receptor for SCF, resulting in stabilization of p27 (49). Taken together, our data strongly support the notion that the dynamic cycles of neddylation and deneddylation are necessary for the optimal SCF functions in vivo. Nevertheless, we cannot exclude the possibility that other activities of CSN such as the associated protein kinase activity, which potentially affects substrate phosphorylation and thereby recruitment to the ubiquitin ligases, may be responsible for accumulation of SCF substrate such as cyclin E in the *Csn2*$^{-/-}$ mutant.

Our finding that p53 highly accumulates in *Csn2*-deficient cells is consistent with the previous report that CSN interacts with and phosphorylates p53, leading to ubiquitin-proteasome-dependent degradation (3). Whether the abnormal accumulation of p53 is caused by defects in CSN-mediated phosphorylation or in its ubiquitination through Mdm2-p300, Pirh2, or Cul5 complexes or by other means requires further study. It is likely that the increased abundance of p53 and elevated expression of p21$^{Cip1/Waf1}$ in the *Csn2*$^{-/-}$ embryos are directly relevant to its premature cell cycle arrest and possibly cell senescence. Certainly, other defects caused by CSN deficiency, including decreased expression of Oct4, may also contribute to the early embryonic phenotype. Interestingly, microinjection of the purified CSN complex has been shown to cause cell cycle delay (48). Thus, similar to the scenario of neddylation and deneddylation with regard to SCF function, either gain of function or loss of function of CSN can lead to perturbation of cell cycle progression.

The CSN complex and Csn2 have been implicated in nuclear hormone receptor-mediated gene expression, stress-activated protein kinase cascade, and cell cycle control in mammalian cells (1, 2, 26, 48). In nonmammalian eukaryotic organisms, CSN participates in a variety of developmental processes. We demonstrate here that *Csn2* and the CSN complex are essential for ICM cell proliferation and early embryonic development in mice, underscoring the fundamental role of this conserved protein complex in development and in cellular homeostasis.

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REFERENCES

1. **Akiyama, H., A. Sugiyama, K. Uzawa, N. Fujisawa, Y. Tashiro, and F. Tashiro.** 2003. Implication of Trip15/CSN2 in early stage of neuronal differentiation of P19 embryonal carcinoma cells. Brain Res. Dev. Brain Res. **140:**45–56.
2. **Altincicek, B., S. P. Tenbaum, U. Dressel, D. Thormeyer, R. Renkawitz, and A. Baniahmad.** 2000. Interaction of the corepressor Alien with DAX-1 is abrogated by mutations of DAX-1 involved in adrenal hypoplasia congenita. J. Biol. Chem. **275:**7662–7667.
3. **Bech-Otschir, D., R. Kraft, X. Huang, P. Henklein, B. Kapelari, C. Pollmann, and W. Dubiel.** 2001. COP9 signalosome-specific phosphorylation targets p53 to degradation by the ubiquitin system. EMBO J. **20:**1630–1639.
4. **Cohen, H., A. Azriel, T. Cohen, D. Meraro, S. Hashmueli, D. Bech-Otschir, R. Kraft, W. Dubiel, and B. Z. Levi.** 2000. Interaction between interferon consensus sequence-binding protein and COP9/signalosome subunit CSN2 (Trip15). A possible link between interferon regulatory factor signaling and the COP9/signalosome. J. Biol. Chem. **275:**39081–39089.
5. **Cope, G. A., G. S. Suh, L. Aravind, S. E. Schwarz, S. L. Zipursky, E. V. Koonin, and R. J. Deshaies.** 2002. Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. Science **298:**608–611.
6. **Dealy, M. J., K. V. Nguyen, J. Lo, M. Gstaiger, W. Krek, D. Elson, J. Arbeit, E. T. Kipreos, and R. S. Johnson.** 1999. Loss of Cul1 results in early embryonic lethality and dysregulation of cyclin E. Nat. Genet. **23:**245–248.
7. **Deng, X. W., W. Dubiel, N. Wei, K. Hofmann, K. Mundt, J. Colicelli, J. Kato, M. Naumann, D. Segal, M. Seeger, A. Carr, M. Glickman, and D. A. Chamovitz.** 2000. Unified nomenclature for the COP9 signalosome and its subunits: an essential regulator of development. Trends Genet. **16:**202–203.
8. **Doronkin, S., I. Djagaeva, and S. K. Beckendorf.** 2002. CSN5/Jab1 mutations affect axis formation in the *Drosophila* oocyte by activating a meiotic checkpoint. Development **129:**5053–5064.
9. **Dressel, U., D. Thormeyer, B. Altincicek, A. Paululat, M. Eggert, S. Schneider, S. P. Tenbaum, R. Renkawitz, and A. Baniahmad.** 1999. Alien, a highly conserved protein with characteristics of a corepressor for members of the nuclear hormone receptor superfamily. Mol. Cell. Biol. **19:**3383–3394.
10. **Feldman, B., W. Poueymirou, V. E. Papaioannou, T. M. DeChiara, and M. Goldfarb.** 1995. Requirement of FGF-4 for postimplantation mouse development. Science **267:**246–249.
11. **Feng, S., M. Ligeng, W. Xiping, X. Daoxin, D. P. Savithramma, N. Wei, and X.-W. Deng.** 2003. The COP9 signalosome physically interacts with SCF-COI1 and modulates jasmonate responses. Plant Cell **15:**1083–1094.
12. **Freilich, S., E. Oron, Y. Kapp, Nevo- Y. Caspi, S. Orgad, D. Segal, and D. A. Chamovitz.** 1999. The COP9 signalosome is essential for development of *Drosophila melanogaster.* Curr. Biol. **9:**1187–1190.
13. **Furukawa, M., Y. Zhang, J. McCarville, T. Ohta, and Y. Xiong.** 2000. The CUL1 C-terminal sequence and ROC1 are required for efficient nuclear accumulation, NEDD8 modification, and ubiquitin ligase activity of CUL1. Mol. Cell. Biol. **20:**8185–8197.
14. **Glickman, M. H., D. M. Rubin, O. Coux, I. Wefes, G. Pfeifer, Z. Cjeka, W. Baumeister, V. A. Fried, and D. Finley.** 1998. A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. Cell **94:**615–623.
15. **Goubeaud, A., S. Knirr, R. Renkawitz-Pohl, and A. Paululat.** 1996. The *Drosophila* gene alien is expressed in the muscle attachment sites during embryogenesis and encodes a protein highly conserved between plants, *Drosophila* and vertebrates. Mech. Dev. **57:**59–68.
16. **Hochstrasser, M.** 2000. Evolution and function of ubiquitin-like protein-conjugation systems. Nat. Cell Biol. **2:**E153–157.
17. **Kawakami, T., T. Chiba, T. Suzuki, et al.** 2001. NEDD8 recruits E2-ubiquitin to SCF E3 ligase. EMBO J. **20:**4003–4012.
18. **Kwok, S. F., R. Solano, T. Tsuge, D. Chamovitz, J. R. Ecker, M. Matsui, and X.-W. Deng.** 1998. *Arabidopsis* homologs of a c-Jun coactivator are present both in monomeric form and in the COP9 complex, and their abundance is differentially affected by the pleiotropic *cop*/*det*/*fus* mutations. Plant Cell **10:**1779–1790.
19. **Lee, J. W., H.-S. Choi, J. Gyuris, R. Brent, and D. D. Moore.** 1995. Two classes of protein dependent on either the presence or absence of thyroid hormone for interaction with the thyroid hormone receptor. Mol. Endocrinol. **9:**243–253.
20. **Liu, J., M. Furukawa, T. Matsumoto, and Y. Xiong.** 2002. NEDD8 Modification of CUL1 Dissociates p120 (CAND1), an Inhibitor of CUL1-SKP1 Binding and SCF Ligases. Mol. Cell **10:**1511–1518.
21. **Lyapina, S., G. Cope, A. Shevchenko, G. Serino, T. Tsuge, C. Zhou, D. A. Wolf, N. Wei, A. Shevchenko, and R. J. Deshaies.** 2001. Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome. Science **292:**1382–1385.
22. **Maytal-Kivity, V., R. Piran, E. Pick, K. Hofmann, and M. H. Glickman.** 2002. COP9 signalosome components play a role in the mating pheromone response of S. cerevisiae. EMBO Rep. **3:**1215–1221.
23. **Morimoto, M., T. Nishida, R. Honda, and H. Yasuda.** 2000. Modification of cullin-1 by ubiquitin-like protein Nedd8 enhances the activity of SCF(skp2) toward p27(kip1). Biochem. Biophys. Res. Commun. **270:**1093–1096.
24. **Morimoto, M., T. Nishida, Y. Nagayama, and H. Yasuda.** 2003. Nedd8-modification of Cul1 is promoted by Roc1 as a Nedd8-E3 ligase and regulates its stability. Biochem. Biophys. Res. Commun. **301:**392–398.
25. **Mundt, K. E., J. Porte, J. M. Murray, et al.** 1999. The COP9/signalosome complex is conserved in fission yeast and has a role in S phase. Curr. Biol. **9:**1427–1430.
26. **Naumann, M., D. Bech-Otschir, X. Huang, K. Ferrell, and W. Dubiel.** 1999. COP9 signalosome-directed c-Jun activation/stabilization is independent of JNK. J. Biol. Chem. **274:**35297–35300.
27. **Nichols, J., B. Zevnik, K. Anastassiadis, H. Niwa, D. Klewe-Nebenius, I.**

Chambers, H. Scholer, and A. Smith. 1998. Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell **95:**379–391.
28. **Niwa, H., J. Miyazaki, and A. G. Smith.** 2000. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat. Genet. **24:**372–376.
29. **Oron, E., M. Mannervik, S. Rencus, Harari- O. Steinberg, S. Neuman-Silberberg, D. Segal, and D. A. Chamovitz.** 2002. COP9 signalosome subunits 4 and 5 regulate multiple pleiotropic pathways in *Drosophila* melanogaster. Development **129:**4399–4409.
30. **Podust, V. N., J. E. Brownell, T. B. Gladysheva, R. S. Luo, C. Wang, M. B. Coggins, J. W. Pierce, E. S. Lightcap, and V. Chau.** 2000. Nedd8 conjugation pathway is essential for proteolytic targeting of p27Kip1 by ubiquitination. Proc. Natl. Acad. Sci. USA **97:**4579–4584.
31. **Read, M. A., J. E. Brownell, T. B. Gladysheva, M. Hottelet, L. A. Parent, M. B. Coggins, J. W. Pierce, V. N. Podust, R. S. Luo, V. Chau, and V. J. Palombella.** 2000. Nedd8 modification of cul-1 activates SCF(beta(TrCP))-dependent ubiquitination of IkappaBalpha. Mol. Cell. Biol. **20:**2326–2333.
32. **Roninson, I. B.** 2002. Oncogenic functions of tumour suppressor p21(Waf1/Cip1/Sdi1): association with cell senescence and tumour-promoting activities of stromal fibroblasts. Cancer Lett. **179:**1–14.
33. **Schaefer, L., M. L. Beermann, and J. B. Miller.** 1999. Coding sequence, genomic organization, chromosomal localization, and expression pattern of the signalosome component Cops2: the mouse homologue of *Drosophila* alien. Genomics **56:**310–316.
34. **Schwechheimer, C., G. Serino, J. Callis, W. L. Crosby, S. Lyapina, R. J. Deshaies, W. M. Gray, M. Estelle, and X. W. Deng.** 2001. Interactions of the COP9 signalosome with the E3 ubiquitin ligase SCFTIRI in mediating auxin response. Science **292:**1379–1382.
35. **Seeger, M., R. Kraft, K. Ferrell, D. Bech-Otschir, R. Dumdey, R. Schade, C. Gordon, M. Naumann, and W. Dubiel.** 1998. A novel protein complex involved in signal transduction possessing similarities to the 26S proteasome subunits. FASEB J. **12:**469–478.
36. **Serino, G., H. Su, Z. Peng, T. Tsuge, N. Wei, H. Gu, and X. W. Deng.** 2003. Characterization of the last subunit of the arabidopsis cOP9 signalosome: implications for the overall structure and origin of the complex. Plant Cell **15:**719–731.
37. **Singer, J. D., M. Gurian-West, B. Clurman, and J. M. Roberts.** 1999. Cullin-3 targets cyclin E for ubiquitination and controls S phase in mammalian cells. Genes Dev. **13:**2375–2387.
38. **Suh, G. S., B. Poeck, T. Chouard, E. Oron, D. Segal, D. A. Chamovitz, and S. L. Zipursky.** 2002. *Drosophila* JAB1/CSN5 acts in photoreceptor cells to induce glial cells. Neuron **33:**35–46.
39. **Sun, Y., M. P. Wilson, and P. W. Majerus.** 2002. Inositol 1,3,4-trisphosphate 5/6-kinase associates with the COP9 signalosome by binding to CSN1. J. Biol. Chem. **277:**45759–457564.
40. **Tateishi, K., M. Omata, K. Tanaka, and T. Chiba.** 2001. The NEDD8 system is essential for cell cycle progression and morphogenetic pathway in mice. J. Cell Biol. **155:**571–579.
41. **Wang, X., D. Kang, S. Feng, G. Serino, C. Schwechheimer, and N. Wei.** 2002. CSN1 N-terminal-dependent activity is required for *Arabidopsis* development but not for Rub1/Nedd8 deconjugation of cullins: a structure-function study of CSN1 subunit of COP9 signalosome. Mol. Biol. Cell **13:**646–655.
42. **Wang, X., S. Feng, N. Nakayama, W. L. Crosby, V. F. Irish, X.-W. Deng, and N. Wei.** 2003. The COP9 signalosome interacts with SCFUFO and participates in *Arabidopsis* flower development. Plant Cell **15:**1071–1082.
43. **Wang, Y., S. Penfold, X. Tang, N. Hattori, P. Riley, J. W. Harper, J. C. Cross, and M. Tyers.** 1999. Deletion of the Cul1 gene in mice causes arrest in early embryogenesis and accumulation of cyclin E. Curr. Biol. **9:**1191–1194.
44. **Wei, N., D. A. Chamovitz, and X.-W. Deng.** 1994. *Arabidopsis* COP9 is a component of a novel signaling complex mediating light control of development. Cell **78:**117–124.
45. **Wei, N., T. Tsuge, G. Serino, N. Dohmae, K. Takio, M. Matsui, and X.-W. Deng.** 1998. The COP9 complex is conserved between plants and mammals and is related to the 26S proteasome regulatory complex. Curr. Biol. **8:**919–922.
46. **Wirbelauer, C., H. Sutterluty, M. Blondel, M. Gstaiger, M. Peter, F. Raymond, and W. Krek.** 2000. The F-box protein Skp2 is a ubiquitylation target of a Cul1-based core ubiquitin ligase complex: evidence for a role of Cul1 in the suppression of Skp2 expression in quiescent fibroblasts. EMBO J. **19:**5362–5375.
47. **Wu, K., A. Chen, and Z. Q. Pan.** 2000. Conjugation of Nedd8 to CUL1 enhances the ability of the ROC1-CUL1 complex to promote ubiquitin polymerization. J. Biol. Chem. **275:**32317–32324.
48. **Yang, X., S. Menon, K. Lykke-Andersen, T. Tsuge, D. Xiao, X. Wang, R. J. Rodriguez-Suarez, R. J., H. Zhang, and N. Wei.** 2002. The COP9 Signalosome inhibits p27(kip1) degradation and impedes G_1-S phase progression via deneddylation of SCF Cul1. Curr. Biol. **12:**667–672.
49. **Zheng, J., X. Yang, J. M. Harrell, S. Ryzhikov, E. H. Shim, K. Lykke-Andersen, N. Wei, H. Sun, R. Kobayashi, and H. Zhang.** 2002. CAND1 binds to unneddylated CUL1 and regulates the formation of sCF Ubiquitin E3 ligase complex. Mol. Cell **10:**1519–1526.
50. **Zhou, C., V. Seibert, R. Geyer, E. Rhee, S. Lyapina, G. Cope, R. J. Deshaies, and D. A. Wolf.** 2001. The fission yeast COP9/signalosome is involved in cullin modification by ubiquitin-related Ned8p. BMC Biochem. **2:**7–16.
51. **Zhou, P., and P. M. Howley.** 1998. Ubiquitination and degradation of the substrate recognition subunits of SCF ubiquitin-protein ligases. Mol. Cell **2:**571–580.