COP9 Signalosome Subunit Csn8 Is Involved in Maintaining Proper Duration of the G1 Phase*

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Background: Among the eight subunits of the COP9 signalosome (CSN), Csn8 is only present in higher eukaryotes.

Results: Decreases in Csn8 accelerate cell growth and shorten G1 duration, whereas decreases in Csn5 impair cell proliferation.

Conclusion: Although Csn5 promotes cell proliferation, Csn8 negatively regulates G1 progression.

Significance: The dynamics of CSN are an important factor influencing cell division and differentiation.

The COP9 signalosome (CSN) is a conserved protein complex known to be involved in developmental processes of eukaryotic organisms. Genetic disruption of a CSN gene causes arrest during early embryonic development in mice. The Csn8 subunit is the smallest and the least conserved subunit, being absent from the CSN complex of several fungal species. Nevertheless, Csn8 is an integral component of the CSN complex in higher eukaryotes, where it is essential for life. By characterizing the CSN complex of several fungal species. Nevertheless, Csn8 is the smallest and the least conserved subunit, being absent from the CSN complex of several fungal species. Nevertheless, Csn8 is an integral component of the CSN complex in higher eukaryotes, where it is essential for life. By characterizing the mouse embryonic fibroblasts (MEFs) that express eukaryotes, where it is essential for life. By characterizing the mouse embryonic fibroblasts (MEFs) that express eukaryotes, where it is essential for life. By characterizing the mouse embryonic fibroblasts (MEFs) that express eukaryotes, where it is essential for life. By characterizing the mouse embryonic fibroblasts (MEFs) that express eukaryotes, where it is essential for life. By characterizing the mouse embryonic fibroblasts (MEFs) that express eukaryotes, where it is essential for life. By characterizing the mouse embryonic fibroblasts (MEFs) that express eukaryotes, where it is essential for life. By characterizing the mouse embryonic fibroblasts (MEFs) that express
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| TABLE 1 |

| Subunit composition of the CSN in various eukaryotic organisms and corresponding paralog of the CSN subunit in the Lid subcomplex of the proteasome |
|----------------------------------|---------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Homo sapiens and Mus musculus    | ✓                  | ✓               | ✓               | ✓               | ✓               | ✓               | ✓               |
| Arabidopsis thaliana             | ✓                  | ✓               | ✓               | ✓               | ✓               | ✓               | ✓               |
| Drosophila melanogaster          | ✓                  | ✓               | ✓               | ✓               | ✓               | ✓               | ✓               |
| Caenorhabditis elegans           | ✓                  | ✓               | ✓               | ✓               | ✓               | ✓               | ✓               |
| Dictostelium discoideum          | ✓                  | ✓               | ✓               | ✓               | ✓               | ✓               | ✓               |
| Aspergillus nidulans             | ✓                  | ✓               | ✓               | ✓               | ✓               | ✓               | ✓               |
| Neurospora crassa                | ✓                  | ✓               | ✓               | ✓               | ✓               | ✓               | ✓               |
| Schizosaccharomyces pombe        | ✓                  | ✓               | ✓               | ✓               | ✓               | ✓               | ✓               |
| Saccharomyces cerevisiae          | ✓                  | ✓               | ✓               | ✓               | ✓               | ✓               | ✓               |
| Paralog in the proteasome Lid    | Rpn7, Csn11 pc18   | Rpn6, Csn10    | Rpn3, Csn3     | Rpn5, Csn5     | Csn5, Rpn11    | Csn6, Csn8     | Csn8, Rpn9     |
|                                  |                    |                |                |                |                |                |                |
| References                        | 57                 | 58              | 59              | 22              | 60              | 61              | 24              |

CSN normally exists as a stable eight-subunit protein complex, whereas its Csn5 subunit exists both as part of the complex and outside of the CSN, a feature that is highly conserved from yeast to human (1, 2). Here we use the term “Csn5-f” to refer to the “free form” of Csn5 protein that is not associated with the CSN holocomplex, regardless of whether Csn5 is monomeric or in association with other effector proteins. Csn5-f has recently been shown to interact with cyclin-dependent kinase CDK2 and act to prevent cell senescence (32). Although small subcomplexes composed of various subsets of CSN subunits have also been reported, their functional relevance remains to be established.

We previously reported germline and conditional disruption of mouse Csn8 (4). Complete ablation of Csn8 in mice led to the destruction of the CSN complex and instability of other subunits (4), similar to the results in higher plants (33, 34) and Drosophila (35). These observations indicate that Csn8 has an important role in maintaining the structural integrity of the complex despite its peripheral position in the complex (16). Germline deletion of Csn8, like germline deletion of other CSN genes in mouse, caused early embryonic lethality (4). Restricted deletion of Csn8 in peripheral T cells abolished the ability of the quiescent cells to enter the cell cycle in response to stimulation, but in cycling T-cells, Csn8 was not required for continued cell division (4). In addition, restricted disruption of Csn8 in liver caused massive cell death and aberrant cell proliferation (8), whereas its disruption in cardiomyocyte of postnatal heart caused heart failure and severe defects in autophagosomal maturation (5, 9).

During the process of generating the Csn8 conditional allele, we have maintained an intermediate allele, Csn8*Neofox or Csn8*Neo. Here we report that this genomic allele expresses Csn8 at a lower level, therefore representing a hypomorphic allele. Through biochemical and cell cycle analyses of this weak Csn8 mutant, we show that Csn8 is involved in maintaining the duration of the G1 phase of the cell cycle. With Csn5 acting to promote cell proliferation, the CSN thus contains both positive and negative regulators of cell cycle and therefore is poised to influence the fate of the cell at the junction of cell division and differentiation during development.

MATERIALS AND METHODS

Primary MEF Cell Culture and Genotyping—To generate h/− mouse embryonic fibroblast (MEF) cells, timed mating between Csn8+/− and Csn8h/h mice was carried out. The resulting embryos, with expected genotype of h/− and h/+ in 1:1 ratio, were used for MEF lines as described previously (4). Genotyping and the generation of Csn8*Neofox conditional and Csn8+/− MEF cells have been described (4). MEF cells from passages 2–4 were used in all of the experiments.

Antibodies—The following antibodies were used in this study: anti-Csn1, anti-Csn2, anti-Csn3, anti-Csn8, and anti-Skp2 (all from Santa Cruz Biotechnology). In addition, anti-p21 was from Cell Signaling Technology, and anti-p27 was from BD Transduction Laboratories.

Tet-inducible Knockdown and Growth Curve Determination—We used the pSuper series of vector (OligoEngine, Seattle, WA) for Tet-dependent shRNA-mediated knockdown of Csn5 and Csn8 in HEK293 cells (38). The shCsn5 vector was kindly provided by the Dr. Deshaies laboratory (38). The same vector was used to express the Csn8-targeting small RNA (GGC UGU GAA AGG CAU AUU) as described previously (39).

HEK293 cells were transfected using Lipofectamine-2000 reagent (Life Technologies) with pshCsn5, pshCsn8, or empty vector together with pCDNA6/TR (Invitrogen/Life Technologies) in a 3:1 ratio. On day 2 after transfection, cells were split and amplified. On day 5, doxycyclin (10 μg/ml) was added to the medium to select for transfected cells. After 4 days in the selection medium, half of the dishes were treated with doxycycline (1 μg/ml) to induce shRNA expression, whereas the other dishes were kept as −Dox control. On the following day, a growth curve experiment (see Fig. 4) was started. Both induced (+Dox) and uninduced (−Dox) cells were counted and seeded on multiple 6-cm dishes at 2 × 10^5 cells/dish in full medium containing doxycyclin (5 μg/ml) with or without Dox. Csn5 and Csn8 proteins started to decline from around day 5, just before the log phase of cell proliferation under our experimental conditions. Cells were counted daily for up to 9 days with a hemocytometer.

Cell Proliferation Assays—Cells of defined number were seeded in a 6-cm dish for growth curve or in 96-well/48-well dishes for ATPLite assays. Controls and Csn8 hypomorphic lines were manipulated identically. At the indicated day after seeding, cells were trypsinized, and the cell numbers were...
RESULTS

The Csn8 Hypomorphic Allele and MEF Line—We previously described generation and characterization of a Csn8 null mutant (Csn8−/−) (4) as well as the Cre/Lox-based Csn8 conditional mouse strain (Csn8flox/flox, Csn8floxflox) (4, 5, 8, 9). During the generation of the flox conditional allele, we have also obtained the intermediate Neoflox allele, which contains a PGK-NEO selection cassette flanked by a pair of FRT sites in intron 3. A DNA gel showing different PCR bands designed to distinguish the three different alleles. B, A DNA gel showing different PCR bands using primer pairs A and B on the genomic DNA of indicated genotypes.

Flow Cytometry, BrdU Labeling, and G1 Length Determination—To determine G1 duration (see Fig. 5C), MEF cells were treated with 100 ng/ml nocodazole (Sigma) for 12 h. The mitotic cells were shaken off, washed to remove the drug, and seeded on coverslips in fresh medium containing 10 μM BrdU (Aldrich). At the indicated hours after replating, cells were fixed with 3.5% paraformaldehyde for 15 min followed by 20 min of treatment with 1.5 N HCl. Cells were immunostained with anti-BrdU monoclonal antibody (BD Pharmingen) followed by FITC-conjugated secondary antibody to identify the BrdU-positive cells. For flow cytometry analysis of cell cycle status (see Fig. 5, A and B), MEF cells were stained with propidium iodide. The fluorescence from the propidium iodide-DNA complex was measured with a FACSCalibur flow cytometer (BD Biosciences).

The Csn8 alleles were also decreased to various degrees (Fig. 2A). Cullin proteins are substrates of the CSN deneddylase, and accordingly set the value of BrdU-positives of Csn8−/− at 24 h as 100%. The percentages of BrdU-positives in earlier time points were calculated relative to the 24-h value. 

For flow cytometry analysis of cell cycle status (see Fig. 5, A and B), MEF cells were stained with propidium iodide. The fluorescence from the propidium iodide-DNA complex was measured with a FACSCalibur flow cytometer (BD Biosciences).

Western blotting (Fig. 2A) were examined by Western blot using the indicated antibodies. Tubulin and Prmt5 are the loading controls. In both panels, the arrows indicate the neddylated form of the respective cullin proteins.

FIGURE 1. Genotype characterization of the Csn8 hypomorphic line. A, graphic illustration of the Csn8 genomic locus in wild type (Csn8+), Csn8Neoflox (Csn8h), and Csn8−/− alleles. The Csn8Neoflox (Csn8h) allele contains the PGK-NEO cassette flanked by a pair of FRT sites in intron 3. The null allele, Csn8−/−, was generated after deletions of the FRT- and LoxP-flanked DNA segments by the Flp and Cre recombinases, respectively. Arrows indicate the PCR primers designed to distinguish the three different alleles. B, A DNA gel showing different PCR bands using primer pairs A and B on the genomic DNA of indicated genotypes.

FIGURE 2. Csn8−/− lines expressed Csn8 at lower levels and exhibited altered cullin deneddylatation pattern. A, representative organs such as brain, heart, and spleen from Csn8−/− mice were examined by Western blot using the indicated antibodies. Tubulin and Prmt5 are the loading controls. B, Western blot of Csn8−/− and its sibling control Csn8+/+ MEF lines using the indicated antibodies. Prmt5 is the loading control. In both panels, the arrows indicate the neddylated form of the respective cullin proteins.

that Csn8 deletion or knockdown can cause instability of other CSN subunits (4, 5, 8, 9), we found that Csn1, Csn3, and Csn5 protein amounts were also decreased to various degrees (Fig. 2A). Cullin proteins are substrates of the CSN deneddylase, and its modification has been widely used as an indicator of CSN activity. Western blots of Cul1 showed that the neddylation or protein accumulation of Cul1 was notably affected in Csn8Neoflox−/− mice (Fig. 2A). This result shows that Csn8Neoflox−/− mice express a lower level of Csn8 and can be
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considered a hypomorphic strain. Hereafter, we will use “Csn8h” or “h” to refer to the Csn8Neoflox− “hypomorphic” (h) genotype for simplicity.

To generate Csn8h MEFs, we first allowed mating between Csn8h−/− and Csn8+/h mice, which would give rise to progenies with the genotype of Csn8h−/− (Csn8h0) and Csn8+/h (Csn8h+) in a 1:1 ratio, and the latter (Csn8+) was conveniently used as a sibling control for the former (Csn8h) in all experiments. The embryos were isolated at 13.5 embryonic days, and MEF lines were generated from each embryo, which were subsequently genotyped (Fig. 1B). Western blot analyses confirmed that Csn8 protein was expressed at a lower level in Csn8h (h) cells when compared with Csn8+ (+) cells (Fig. 2B). Similar to the tissues, most other CSN subunits were found at reduced levels in Csn8h MEFs, further supporting the notion that Csn8 is necessary for the stability of the CSN complex and hence stability of most CSN subunits in mice. Likewise, Western blot of cullin proteins showed that neddylation of Cul1, Cul2, and Cul4A and/or protein accumulation were strongly affected in Csn8h MEFs, with hyperneddylation of Cul2 being most noticeable. This result confirmed that Csn8h MEFs had weaker cullin deneddylation activity (Fig. 2B). We conclude from these results that the Csn8h MEFs are clearly hypomorphic for Csn8 and therefore can be used to study the biochemical and functional characteristics of Csn8 hypomorphism.

**Csn8 Hypomorphic MEF Cells Exhibit a Faster Growth Rate in Culture**—We noticed that the Csn8h MEF cells tended to grow faster than their paired Csn8+ cells because Csn8h cells regularly required greater dilutions on each replating. The growth curve determined by counting the number of cells following reseeding showed that indeed Csn8h cells produced greater numbers of cells in shorter times in culture (Fig. 3A). Similarly, the ATPLite cell proliferation assay showed higher proliferation activity of the Csn8h cells than Csn8+ cells (Fig. 3B). To determine whether Csn8h MEFs exhibit a normal response to growth factor starvation and stimulation, we carried out a serum starvation and readdition experiment and monitored proliferation (S-phase and DNA synthesis) using BrdU incorporation as a read-out (Fig. 3C). The Csn8 hypomorphic MEFs were able to undergo quiescence (<10% BrdU incorporation) after 3 days of serum starvation. This was slower when compared with the Csn8+ control cells, which largely went quiescent after just 2 days of serum removal. Upon the readdition of serum, the Csn8h cells, like the Csn8+ control, were able to re-enter the cell cycle as indicated by increased DNA synthesis activity (Fig. 3C). Consistent with the accelerated growth profile, the Csn8h cells (gray bars) displayed significantly higher BrdU incorporation rates than the Csn8+ control cells (black bars). Taken together, we found that Csn8 hypomorphic MEF cells proliferate faster, require a longer period of serum starvation to exit the cell cycle, and are capable of re-entering the cell cycle upon the readdition of serum. Because the Csn8h−/− (null) cells are completely defective in cell cycle re-entry (4), these results indicate that the amount of Csn8 in the Csn8h cell, although lower than normal cells, is nonetheless sufficient to allow cell cycle reinititation.

**Decreased Expression of Csn8 or Csn5 Causes Opposite Cell Growth Profiles**—Contrary to Csn8, hypomorphism of Csn5, either by RNA silencing (31, 40) or due to genetic heterozygosity (7, 41), has been reported to impair cell proliferation. To confirm that these two subunits indeed behave differently in cell proliferation, we conducted a parallel knockdown study of Csn5 or Csn8 in HeLa cells (Fig. 4). This experiment was to ensure that the results from silencing each of the two different CSN subunits could be directly compared. A tetracycline/doxycycline (Tet/Dox)-inducible shRNA-mediated knockdown system was utilized (38). In our procedure, Western blot of the respective targeted gene products showed that the silencing started around day 6 of Dox induction or days 4–5 after plating the cells for the growth curve assay (Fig. 4, B and D). As clearly shown in Fig. 4, the growth curves of Csn8 knockdown and Csn5 knockdown were drastically different. Upon Dox induction of shCsn8-mediated silencing, the cells exhibited an accelerated growth pattern when compared with the uninduced (−Dox) cells (Fig. 4A), similar to the profile of Csn8h MEFs (Fig. 3A). In contrast, the Csn5 knockdown cells could hardly grow (Fig. 4C), which is in agreement with published studies reporting that a decrease in Csn5 leads to severe cell proliferation defects (7, 31, 41). This result clearly showed that, under identical experimental conditions and in the same cell line, decreases in two different CSN subunits caused contrasting effects on cell proliferation; a decrease in Csn5 drastically

![FIGURE 3. Characterization of cell proliferation properties of Csn8 hypomorphic MEFs. A, the Csn8h MEFs exhibit a faster growth rate in culture. Growth curves of Csn8h and Csn8+ control MEFs at passage 2 were measured by counting of cell numbers at the indicated days following seeding. Similar results were repeated more than four times with different MEF lines. B, the ATPLite assay was used to compare the cell proliferation rates of the Csn8h and Csn8+ MEF cells. Experiments were repeated at least twice at different seeding densities. C, the percentage of BrdU incorporation was used to monitor proliferation of Csn8h and the Csn8+ MEFs grown under: normal growth condition (Control), serum starvation for the indicated days (Serum-Starved), and at the indicated hours after the readdition of serum following a 3-day serum starvation (Serum Re-addition). Cells were pulse-labeled with BrdU for 3 h. The Csn8h is indicated by solid black bars, and the hypomorphic Csn8h is indicated by gray bars in both B and C. Error bars in B and C indicate S.D.](image-url)
diminished cell proliferation, whereas a decrease in Csn8 accelerated the cell cycle progression.

**Csn8 Hypomorphic Cells Have a Shorter G1 Phase**—To further understand the Csn8 function, it is necessary to delineate the specific cell cycle phase that is affected by decreased expression of Csn8. FACS analysis showed that when compared with the Csn8+ control, the Csn8−/− MEF cells exhibited a smaller G1 population and a correspondingly greater S phase population (Fig. 5A). The reduction of G1 population, although not drastic, was highly consistent between experiments (not shown). As shown in Fig. 5B, the same reduction of the G1 population was also observed in the Csn8 knock-out MEFs (Csn8−/−), in which Csn8 was deleted in culture from a Csn8 conditional line, Csn8flox/flox (4). In addition, Csn8−/− cells also displayed a larger G2 population, which could suggest a possible G2 delay in those cells (Fig. 5B). However, the potential G2 abnormality observed in Csn8−/− cells was not a significant issue in Csn8−/− MEFs (Fig. 5A). The diminished G1 population could indicate a shortened G1 phase in Csn8−/− cells, which would also explain its greater S phase population and the faster growth rate.

To measure the length of G1 phase in Csn8−/− and Csn8+ MEFs, cells were first arrested at M phase by nocodazole treatment in a procedure previously described (36) (Fig. 5C). M phase cells were collected and replated in fresh BrdU-containing medium, allowing the cells to enter the G1 phase in a synchronized manner and progress into the S phase. The period from replating of the cells to the time when most of the cells undergo DNA synthesis, or become BrdU-positive, would be considered the length of G1. To determine how long it took the Csn8+ and Csn8−/− MEFs to start synthesizing DNA after replating, we collected the cells in a time course from 6 to 24 h after replating and assayed the percentage of cells with BrdU incorporation. After disregarding dead cells that failed to take up BrdU in 24 h, we found that 82% of Csn8+ MEFs entered the S phase at 10 h after replating, whereas the normal Csn8+ MEFs entered the S phase at about 12 h (82%, Fig. 5B, dotted line). This result demonstrates that Csn8 hypomorphic cells have a shorter G1 phase during their cell cycle progression, which explains why the Csn8−/− cells display an accelerated growth rate.

We next examined the expression level of a number of G1 regulators in Csn8 hypomorphic cells by Western blots (Fig. 6). When comparing different Csn8−/− lines with the Csn8+ sibling control lines, we found that the level of Skp2 F-box protein, a G1-promoting factor, appeared slightly but consistently up-regulated in Csn8−/− lines (Fig. 6A, h lanes). This was clearer upon quantification of Skp2 band intensity shown at the bottom panel of Fig. 6A. This result is opposite to Csn5 knockdown cells, which down-regulate Skp2 (31, 38). On the other hand, we could not definitively detect consistent differences in the level of cyclin E or CDK inhibitor p27kip1, both of which are sensitive to Csn5/Jab1 levels (7, 31, 38). Notably, both cyclin E and p27kip1 can be regulated by multiple mechanisms. We then compared the Csn8 MEFs side-by-side with Csn5−/− MEF cells, which express a lower level of Csn5 (Fig. 6B). Csn5−/− MEFs, cyclin-dependent kinase CDK4, cyclin D1, and cyclin E levels were all drastically decreased. In Csn8 hypomorphic MEFs, we found that in later passages (passage of 3 or greater), CDK4 and cyclin D1 were slightly up-regulated (Fig. 6B). G1 inhibitor p16INK4a plays an important role in cell senescence and has been shown to be up-regulated in Csn5−/− cells (42). Western blot showed that p16, which increased with each passage of MEFs as anticipated, appeared suppressed in Csn8 hypomorphic MEFs (Fig. 6C), although there were variations on the extents of these changes. Overall, the changes in the abundance of the G1 activators and inhibitors are in agreement with the accelerated G1 profile of Csn8 hypomorphism and impaired proliferation of Csn5 hypomorphism.

**Csn8 Hypomorphic Cells Have a Higher Ratio of Csn5-f Relative to CSN—Csn5−/− is unique in partitioning both as part of the 500-kDa CSN holocomplex and as a free form outside of CSN (Csn5-f). Csn5-f was recently shown to interact with cyclin-dependent kinase CDK2 (32). It was reported that in Csn5 hypo-
morphic cells such as siRNA-mediated Csn5 knockdown cells, Csn5-f- or Csn5-containing small complexes were decreased more drastically than the CSN-associated form of Csn5 (31, 43, 44). To investigate how Csn8 hypomorphism affects the distribution of Csn5 proteins between CSN-associated and Csn5-f populations, the Csn8h or Csn8+/H11001 MEF cell extracts were fractionated through a Superose-6 gel filtration column (Fig. 7A). Western blot analyses showed that Csn8 was detected only in 500-kDa fractions containing the CSN complex (Fig. 7, lanes 11–14, Peak-1) in both Csn8h and Csn8+/H11001 lines based on the data shown in the lower panel. The graph in the lower panel shows the percentage of BrdU-positive cells in the time course as illustrated in the upper panel. Based on the rate of BrdU incorporation into the G1, synchronized cells, the timing of S phase entry, which was marked by peaked DNA synthesis, was determined (indicated by arrowheads). All experiments were repeated twice. Error bars indicate S.D.

FIGURE 5. The Csn8 hypomorphic MEF cells exhibit an accelerated G1 progression. A and B, graphs showing cell cycle analysis by FACS of Csn8h (h) and the Csn8+ control (+) MEFs (A) and Csn8+/H11001 and Csn8−/− MEFs (B). C, the upper panel shows a diagram illustrating the timeline procedure used to determine the length of the G1 phase in Csn8h MEF line. The asterisks mark the S phase entry points in Csn8h and Csn8+/H11001 lines based on the data shown in the lower panel. The graph in the lower panel shows the percentage of BrdU-positive cells in the time course as illustrated in the upper panel. Based on the rate of BrdU incorporation into the G1, synchronized cells, the timing of S phase entry, which was marked by peaked DNA synthesis, was determined (indicated by arrowheads). All experiments were repeated twice. Error bars indicate S.D.

FIGURE 6. Expression of G1 regulators in Csn8 hypomorphic MEFs. A, Western blot analysis of Skp2, cyclin E, and p27kip1 in multiple Csn8h (h) and the Csn8+ control (+) MEF lines. Prmt5 was used as a loading control. Quantification of the Skp2 protein relative intensities by ImageJ was shown in the bottom panel. B, Western blot analysis of G1 activators CDK4 and cyclin D and E in Csn5+/H11001 (Csn5 h) MEFs with its control (+) or Csn8h (h) MEFs and its control (+). The passage numbers of the MEF cells are indicated on the top. Tubulin (Tub) was used as a loading control. C, Western analysis of p16Ink4a in MEFs of different passages for Csn8h (h) and its control, Csn8+/H11001 (h) or Csn8h (h) MEFs and its control (+). Tubulin (Tub) was used as a loading control.
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GERMINE ABOLATION of mouse Csn2, Csn3, Csn5, Csn6, and Csn8 all leads to early embryonic arrest and death (3, 4, 7, 30, 46). Similarly, in Arabidopsis, complete loss-of-function mutation of each of the CSN subunits results in early seedling lethality (47, 48). This well documented phenotype can give an illusion that all CSN subunits affect cell proliferation identically and positively. However, because complete ablation of each of the CSN subunits results in destruction of the entire CSN complex, the prevalent pleiotropic developmental phenotypes from losing the complex can obscure the specific functional roles of individual subunits. Through a reduction-of-function approach, we think we have identified a specific function of Csn8: that it negatively regulates cell cycle in MEFs and HeLa cells. Csn8 is probably involved in a brake function in the G1 phase because decreased Csn8 expression resulted in premature transition to the S phase. Nevertheless, Csn8 may not have the same effect on all cell types due to the variations in the regulation and kinetics of the cell cycle.

Csn8 and Csn5 Act Antagonistically in Cell Cycle Regulation—We have previously shown that the Csn8<sup>−/−</sup> MEFs and T-cells can undergo cell divisions in cycling cells but lack the ability to re-enter the cell cycle from the G<sub>0</sub> state (4). In the Csn8<sup>−/−</sup> hypomorphic cells, cell cycle re-entry from the G<sub>0</sub> state was normal, but the cells displayed a faster growth rate, smaller G<sub>1</sub> population, and a shortened G<sub>1</sub>-to-S progression (Fig. 3). Small RNA-mediated silencing of Csn8 in HeLa cells caused a faster growth rate, whereas silencing of Csn5 by the same procedure caused a severe growth inhibition (Fig. 4). Thus the two different subunits of the CSN display opposing function-
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possesses cell proliferation independently of Csn5-f. Csn8 and Csn5 appear to be peripheral subunits of the CSN complex (16). Although Csn8 has not been detected to stably exist outside of the CSN under our mild experimental condition, a low abundant small Csn8 complex has been found by native gel electrophoresis (39) and by gel filtration chromatography (31). Either directly or indirectly, Csn8 seems to affect the expression of cell cycle regulators such as p16\(^{\text{Ink4a}}\), cyclin D1, Cdk4, and Skp2 (Fig. 6), which control the progression of G_{1}. Unlike Csn5\(^{+/+}\) or shCsn5 cells, the growth phenotype of the Csn8\(^{-}\) was not robust. As a result, the amplitude of the observed molecular changes was not dramatic, and it varied depending on the extent of Csn8 reduction and the passage numbers of MEFs. At this point, the precise molecular mechanism of how Csn8 modulates these genes remains to be determined.

Modulation of G_{1} Length in Developmental Regulation—In mammalian cells, G_{1} phase is when the fate of the cell is determined; the cell may undergo quiescence or senescence (G_{0}), differentiation, or cell size expansion or commitment to division (51). In neural, hematopoietic, and embryonic stem cells, shorter G_{1} correlates with division, whereas long G_{1} correlates with differentiation (52–54). In fact, lengthening of G_{1} is not only required, but can also trigger cell differentiation. Overexpression of Cdk4/cyclin D1 is shown to shorten G_{1} and delay neurogenesis (55), whereas loss of Cdk2 and Cdk4 induces a switch from proliferation to differentiation in neural stem cells (56). In this context, Csn8, because of its function in prolonging G_{1} progression, may be relevant in facilitating cell differentiation.

Cell differentiation can be considered as a hallmark of organismal complexity because lower eukaryotes such as unicellular yeast have little cell differentiation. Csn8 is apparently associated with developmental complexity, considering its conserva-

tion profile in evolution; Csn8 is not in the CSN complex of budding yeast, fission yeast, N. crassa, and C. elegans, but is an integral subunit in mammals, higher plants, insects, social amoeba Dictyostelium discoideum, and interestingly, filamentous fungus Aspergillus nidulans (Table 1). It seems plausible that in lower organisms, cell differentiation is so primitive that Csn8 function either is not required or is not linked to CSN. We speculate that, equipped with both pro-proliferative (Csn5 and Csn6) and anti-proliferative factors (Csn3 and Csn8), the CSN plays a dynamic role in the homeostasis of cell differentiation and division during development of higher eukaryotes.

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