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CSN5/Jab1 controls multiple events in the mammalian cell cycle

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ABSTRACT

The COP9 signalosome (CSN) complex is critical for mammalian cell proliferation and survival, but it is not known how the CSN affects the cell cycle. In this study, MEFs lacking CSN5/Jab1 were generated using a CRE-flox system. MEFs ceased to proliferate upon elimination of CSN5/Jab1. Rescue experiments indicated that the JAMM domain of CSN5/Jab1 was essential. CSN5/Jab1-elimination enhanced the neddylation of cullins 1 and 4 and altered the expression of many factors including cyclin E and p53. CSN5/Jab1-elimination inhibited progression of the cell cycle at multiple points, seemed to initiate p53-independent senescence and increased the ploidy of cells. Thus, CSN5/Jab1 controls different events of the cell cycle, preventing senescence and endocycle as well as the proper progression of the somatic cell cycle.

Structured summary: MINT-8046253: *Csn1* (uniprotkb:Q99LD4) *physically interacts* (MI:0914) with *Csn5* (uniprotkb:O35864), *Csn8* (uniprotkb:Q8VBV7), *Csn3* (uniprotkb:O88543), *Csn7b* (uniprotkb:Q8BV13) and *Csn6* (uniprotkb:O88545) by *anti bait coimmunoprecipitation* (MI:0006)

1. Introduction

The COP9 signalosome (CSN) complex is conserved basically in all eukaryotic cells, and plays a pivotal role in cell proliferation and survival [1,2]. In plants, the CSN acts as a repressor for photomorphogenesis, and is also critical for later development [3]. In mice, the CSN is essential, and animals deficient in CSN components die at a very early stage of embryonic development [4].

The CSN consists of eight subunits (CSN1–8) and has multifunctional roles [1], including regulation of the stability of cellular regulators through the control of the cullin-RING-E3 ligase (CRL) by deneddylation [5–9]. The fifth subunit (CSN5) is essential for this activity. CSN5 contains a JAMM domain, critical for the isopeptidase catalytic activity by which the CSN removes the ubiquitin-like modifier Nedd8/Rub1 from the cullin subunit (deneddylation), thereby regulating the activity of CRLs. However, CSN5 alone is incapable of this activity, indicating that the holo-CSN complex is required for this reaction.

Among the eight components of the CSN, CSN5 is unique in many ways. CSN5 was originally identified as a protein binding to the transcription factors c-Jun and JunD, and so also termed Jun-activation-domain-binding protein (Jab) 1 [10]. CSN5/Jab1 was repeatedly isolated as an interactor of many factors regulating signal transduction and cell proliferation/survival [4]. Besides the CSN holo-complex, CSN5/Jab1 is also suggested to function as a monomer or a smaller complex outside the CSN complex. However, the identity of the smaller form remains to be investigated.

During the last decade, substantial evidence has accumulated showing that CSN5/Jab1 is an onco-protein [4]. First, CSN5/Jab1 is overexpressed in many human cancers. Among these tumors, the CSN5/Jab1 gene itself is rarely mutated except for gene amplification in some cases, suggesting that the genetic target for malignant transformation is the upstream regulators of CSN5/Jab1. A high level of CSN5/Jab1 is often correlated with a poor prognosis. Second, knockdown of CSN5/Jab1 inhibits the proliferation of human tumor cells [11,12], suggesting that overexpression of CSN5/Jab1 not only serves as a marker of malignant transformation, but also actually contributes to tumor cell proliferation. In fact, it was shown that the CSN's integrity was required for the growth of Ras-transformed cells [13]. Third, ectopic expression of a stable form of CSN5/Jab1 in mice induces the development of myeloproliferative disorders with expansion of the stem cell population [14]. Thus, CSN5/Jab1 functions in favor of cell proliferation/survival and eventual tumorigenesis.

From the results obtained using CSN5/Jab1 knockout cells/mice [15,16], it is clear that CSN5/Jab1 is essential for the proliferation and survival of mammalian cells. CSN5/Jab1-null embryos ceased to survive at a very early stage of development, in which elevated levels of cyclin E, Cdk inhibitor p27, and tumor suppressor p53

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were observed, suggesting cell cycle arrest by the G1 and G2 checkpoint control. Conditional knockout of CSN5/Jab1 in T cells inhibited progression through the S phase without significant effects on the G1 phase. Therefore, it remains to be clarified how CSN5/Jab1 functions for the proper progression of the mammalian cell cycle.

In this study, we isolated MEFs harboring a conditional allele (floxed) [16] of the CSN5/Jab1 gene. Introduction of the CRE recombinase into these cells efficiently inactivated the CSN5/Jab1 locus and thereby eliminated the expression of CSN5/Jab1 protein. Using this system, we analyzed the effect of CSN5/Jab1-elimination on the progression of the cell cycle.

2. Materials and methods

2.1. Mice and generation of MEFs

Mice harboring conditional (floxed, f) [16] and null (−) [15] alleles of the CSN5/Jab1 gene were generated as described previously. CSN5/Jab1$^{+/f}$ mice and CSN5/Jab1$^{+/-}$ mice were crossed and CSN5/Jab1$^{-/f}$ mice were obtained. Male and female CSN5/Jab1$^{-/f}$ mice were crossed and at day 13.5, embryos and embryonic fibroblasts were isolated according to the standard procedure [15]. Genotypes were determined by PCR using high molecular DNA as a template and a set of primers described previously [15,16].

2.2. Cell culture, transfection, and retroviral infection

Mouse embryonic fibroblasts (MEFs) and 293T human embryonic kidney (HEK) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin (GIBCO/BRL). For viral production, MSCV-based expression vectors were co-transfected via the calcium phosphate-DNA precipitation method [17] into 293T cells together with a plasmid encoding an ecotropic helper virus containing a defective virion-packaging (ψ2) sequence. Culture supernatants containing retroviruses harvested 48–72 h after transfection were used to infect proliferating MEFs in the presence of polybrene (4 μg/ml) [18]. For synchronization in G0/G1, cells were incubated in medium containing 0.5% FBS for 48 h.

2.3. Generation of CSN5/Jab1-mutants and plasmid construction

Nuclear export signal (NES)-mutant of CSN5/Jab1 was generated by converting three conserved leucine residues (positions 237, 240, and 241) in the NES motif to alanine residues (L237/240/241A) by PCR-based mutagenesis as described [19]. JAMM domain mutant was generated by changing the conserved aspartic acid residue in the His-X-His-X10-Asp motif (151st Asp in mice) (codon GAT) [20] to asparagine (codon AAT) (D151N) by PCR using following primers; sense 5′-CTC CGG GAT TAA TGT TAG TAC-3′ and anti-sense 5′-GTA CTA ACA TTA ATC CCG GAG-3′. cDNA fragments containing the coding sequence of CRE and wild-type/mutant CSN5/Jab1 were subcloned into the pMSCV-ires-GFP vector (a gift from Dr. Owen Witte).

2.4. Flow cytometric analysis

Cells were tripsinized and suspended in PBS. GFP signals were measured with a FACScan flow cytometer (Becton Dickinson). GFP-positive cells were sorted with a FACSVantage cell sorter. For the cell cycle analysis, cells were stained with a 1 ml solution of 0.1% sodium citrate and 0.1% Triton X-100 containing 50 μg/ml of propidium iodide and treated with 1 μg/ml of RNase for 30 min at room temperature. Fluorescence from the propidium iodide-DNA complex was measured with a FACScan flow cytometer (Becton Dickinson), and the percentage of cells in the sub-G1 population was determined with Modifit cell cycle software. The populations of dead cells were confirmed by the dye exclusion assay.

2.5. Protein analyses

Cell lysis, immunoprecipitation, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and immunoblotting were performed as described [18,21]. In short, cells were washed with PBS and lysed for 30 min on ice in modified EBC buffer (50 mM Tris–HCl pH8.0, 120 mM NaCl, 1 mM EDTA, and 0.5% NP40) containing 2000K IU/ml of aprotinin, 1 mM PMSF, 0.1 mM NaF, 0.1 mM Na_3VO_4, and 10 mM β-glycerophosphate. The cell debris was removed by micro centrifugation at full speed. The antibodies were reacted with cell lysate for 2 h to overnight at 4 °C. Immune complexes were collected by incubation with protein A or protein G Sepharose beads for 2 h at 4 °C, and boiled in SDS-sample buffer (40 mM Tris–HCl, pH 6.8, 0.1 M DTT, 1% SDS, 10% glycerol, and 0.05% bromophenol blue) for 4 min. To prepare direct lysate for immunoblotting, cell lysate in a EBC buffer was mixed with the same amount of 2X SDS-sample buffer (80 mM Tris–HCl, pH 6.8, 0.2 M DTT, 2% SDS, 20% glycerol, and 0.1% bromophenol blue) and boiled for 4 min. Protein samples were separated on SDS–polyacrylamide gels under reducing conditions (SDS–PAGE), transferred to a nitrocellulose membrane or a PVDF membrane (Milipore), and immunoblotted with the antibodies indicated. Proteins were detected with the ECL blotting system (Amersham) according to the manufacturer's instructions. Developed films were quantitatively analyzed with a densitograph (ATTO, Japan).

For non-denaturing gel electrophoresis (native-PAGE) [22], cells were lysed in a digitonin-lysis buffer (50 mM Tris–HCl, pH 8.0, 120 mM NaCl, 1 mM EDTA, and 0.1% digitonin) at 1×10^5 cells/μl (more than 1×10^7 cells were required for quantitative, reproducible native-PAGE analysis). Equal amounts (ca. 100 μg) of total protein were separated on precast native gradient gels (5–15%; BioCraft, Tokyo, Japan) without sodium dodecyl sulfate (SDS) at 5 mA for 16 h followed by 10 mA for 6 h, and analyzed by immunoblotting using antibody to CSN5/Jab1.

Rabbit polyclonal antibodies to the CSN subunits, Cul1 and 4, p53, p21, and Skp2 were generated using bacterially produced polypeptides in our laboratory. A rabbit polyclonal antibody to Geminin was provided by Dr. Yoshihiro Takihara. Mouse monoclonal antibodies to cyclin D1 (72–13G) and Cdc2 (17) and rabbit polyclonal antibodies to Skp2 (H-435), p16 (M156), cyclin E (M20), cyclin A (C19), Cdk2 (M2), and Cdk4 (C22) were obtained from Santa Cruz. A mouse monoclonal antibody to γ-tubulin (GTU88) was purchased from Sigma.

2.6. Immunofluorescence staining

Cells grown on coverslips were fixed in 4% paraformaldehyde. Chromosomal DNA was stained by incubation in 1 μg/ml of Hoechst 33342 for 2 min. The samples were viewed by phase-contrast or fluorescence microscopy.

For the determination of BrdU incorporation, cells incubated in 10 μM bromodeoxyuridine (BrdU) for 15 min were treated with 1.5 M HCl and stained with anti-BrdU mouse monoclonal antibody (Amersham Biosciences) and fluorescein isothiocyanate-linked anti-mouse IgG. The cell samples were viewed by phase contrast or fluorescence microscopy. More than 500 cells were enumerated for each sample.

2.7. β-Galactosidase assay

Cells were fixed in 0.25% glutaraldehyde, incubated for 16–24 h in X-gal solution containing 0.2% X-gal, 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$, and 5 mM $K_3Fe(CN)_6$, and viewed with a phase-contrast microscope.

3. Results

3.1. Conditional knockout of the CSN5/Jab1 gene in mouse embryonic fibroblasts (MEFs)

Because previous studies showed that CSN5/Jab1$^{-/-}$ mice died in the embryonic stage [15] and the depletion of CSN5/Jab1 prevented cell proliferation [11,12], we decided to utilize the conditional allele (floxed) of CSN5/Jab1 [16] (Fig. 1A) to analyze the effect of CSN5/Jab1 depletion simultaneously. CSN5/Jab1$^{+/-}$ mice were crossed with CSN5/Jab1$^{+/f}$ mice, generating CSN5/Jab1$^{-/f}$ mice. From the embryos obtained by crossing CSN5/Jab1$^{-/f}$ males and females, we isolated MEFs harboring the CSN5/Jab1$^{-/f}$ genotype.

To achieve a high efficiency of gene transduction, we generated a CRE retroviral vector by inserting cDNA encoding a CRE recombinase into a retroviral vector, MSCV-ires-GFP (a gift from Dr. Owen Witte, UCLA), and transfected it into 293T cells together with helper viral DNA lacking the packaging signal sequence. The resulting retrovirus was used to infect CSN5/Jab1$^{-/f}$ MEFs. Uninfected cells were removed by cell sorting.

Fig. 1B shows that the introduction of CRE efficiently inactivated the floxed allele of the CSN5/Jab1 gene. Because CSN5/Jab1-depletion effectively prevented cell proliferation and survival (see below), and it is reported that conditional knockout of the CSN5/Jab1 gene in mice allowed cells in which CSN5/Jab1-knockout was not successful to prevail in the end [16], we used the CSN5/Jab1$^{-/f}$ allele rather than the CSN5/Jab1$^{f/f}$ allele to achieve higher efficiency, but basically the same results were obtained when either allele was used. Upon conversion of the CSN5/Jab1 floxed allele, the expression of CSN5/Jab1 protein was eliminated as well (Fig. 1C). At the later stage (ca. 1 week) after CSN5/Jab1-depletion, other CSN subunits were also down-regulated (see below). However, at the very early stages (2–4 days) of infection by the CRE virus, expression of the most CSN subunits were sustained (Fig. 1D). Our antibody to CSN1 efficiently recognizes the holo-CSN complex and, in fact, anti-CSN1 immunoprecipitates contained other CSN components (Fig. 1D, left panel, and see Ref. [19]). Interestingly, CSN5/Jab1-depletion at the very early stages minimally affected the amount of other CSN components in the anti-CSN1 immunoprecipitates (Fig. 1D), indicating that CSN subunits formed a complex without CSN5/Jab1. However, this complex seemed unstable and CSN5/Jab1-depletion eventually led to the decrease in expression of other CSN subunits (see below).

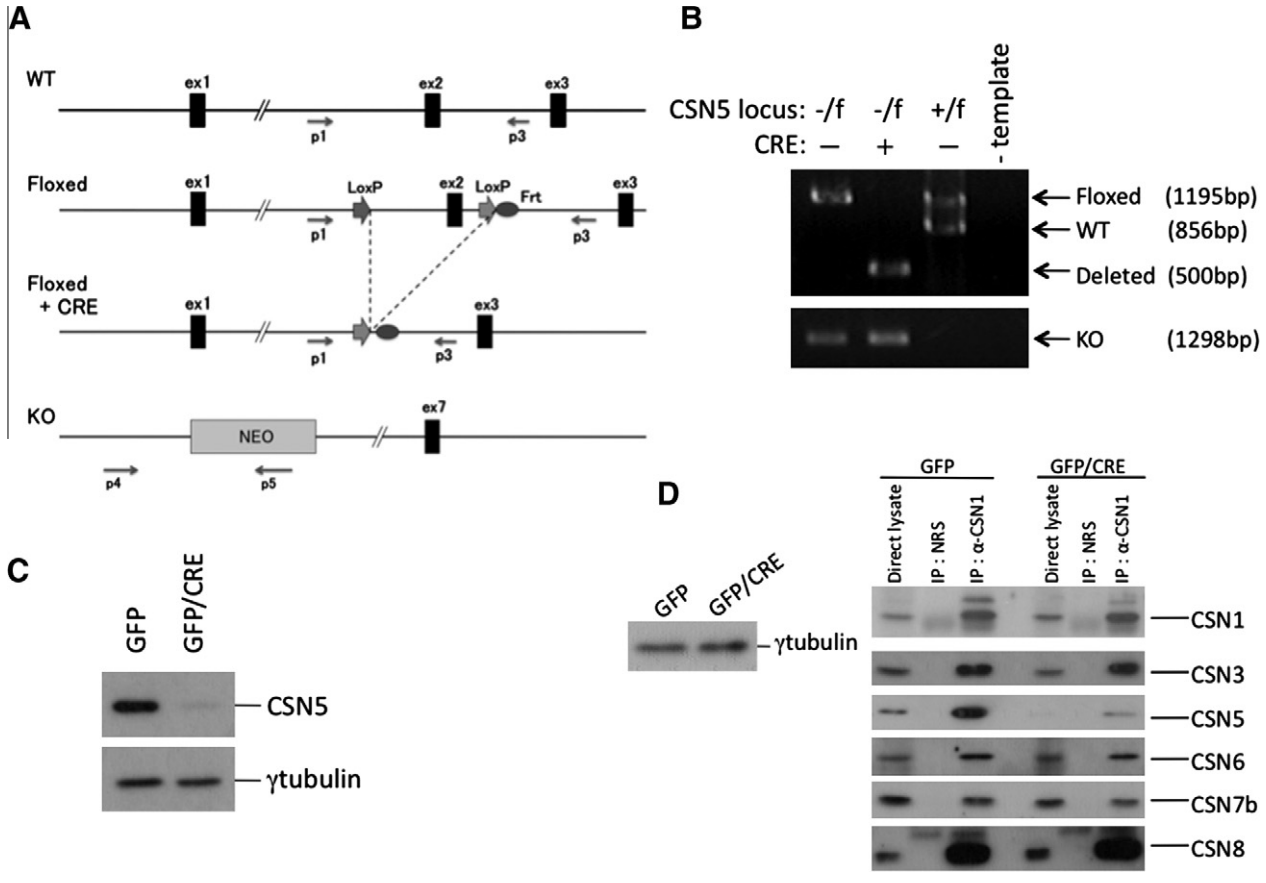

Fig. 1. Conditional knockout of the CSN5/Jab1 locus. (A) Schematic diagram of the wild-type (WT) and modified (Floxed, Floxed + Cre, KO) CSN5/Jab1 loci. (B) High molecular weight DNA was isolated from MEFs harboring the CSN5 locus shown at the top and used as a template. Genomic PCR was performed using primers as described previously [15,16] to determine the genotype. (C) Lysates were isolated from CSN5/Jab1$^{-/f}$ MEFs infected with control (GFP) and CRE (GFP/CRE) viruses and analyzed by immunoblotting using antibodies against CSN5/Jab1 and γ-tubulin. (D) Right panels; Lysates were isolated from CSN5/Jab1$^{-/f}$ MEFs infected with GFP and GFP/CRE viruses at day 3 post-infection. The CSN complex was immunorecipitated from the lysate using antibody to the CSN1 subunit and analyzed by immunoblotting using antibodies to the CSN subunit shown at the right of the panels. (Left panel) The direct lysates were immunoblotted with antibody to γ-tubulin to show the equal loading.

3.2. CSN5/Jab1-depletion inhibited cell proliferation, and the integrity of the JAMM domain is important

CSN5/Jab1-depletion (CSN5/Jab1$^{-/f}$ allele + CRE) markedly inhibited cell proliferation, whereas introduction of the CRE recombinase itself had little effect because wild-type MEFs infected with the CRE virus grew normally compared with control MEFs (Fig. 2A and B). The negative effect of CRE on cell growth was specific to the elimination of CSN5/Jab1 because ectopic introduction of wild-type CSN5/Jab1 rescued the proliferative defect of CSN5/Jab1$^{-/f}$ + CRE (Fig. 2B–D). In this assay, the JAMM-mutant (D151N) form of CSN5/Jab1 [20] failed, whereas the NES-mutant (L237/240/241A) form of CSN5/Jab1 [19] was as effective as the wild type. In these transfectants, the excision of endogenous floxed alleles was equally effective (Fig. 2C) and expression levels of ectopic CSN5/Jab1 proteins (wild type, and JAMM and NES mutants) were equivalent (Fig. 2D). Analysis by native-PAGE and immunoblotting (Fig. 2D, the bottom panel) revealed that ectopically-expressed CSN5/Jab1 was efficiently incorporated into the CSN-holo complex (Only the results with wild-type and NES mutant CSN5/Jab1 were shown. Because the amount of cells expressing both CRE and JAMM mutant CSN5/Jab1 was not sufficient for the analysis, we were unable to perform the native-PAGE analysis). These results imply that the isopeptidase activity of the CSN-holo complex is critical for mammalian cell proliferation.

After the introduction of CRE into CSN5/Jab1$^{-/f}$ MEFs, a small number of GFP-positive colonies sometimes grew up after a long interval. These cells, however, retained the undisturbed floxed allele of CSN5/Jab1, suggesting that expression of and/or recombination by CRE had somehow failed in these cells. We were unsuccessful in the isolation of revertant clones from the CSN5/Jab1-inactivated population of MEFs even after several trials, implying that loss of the CSN activity affects many factors and pathways, and few cellular mutations can recover the loss of the CSN.

3.3. Depletion of CSN5/Jab1 prevented proper progression of the cell cycle at multiple points

Consistent with the growth suppression induced by the loss of CSN5/Jab1, CSN5/Jab1-depleted cells eventually died (mostly from 2 weeks after depletion) and ectopic expression of wild-type CSN5/Jab1 rescued the defect (Fig. 3A). At the relatively early stage (4–6 days) of infection by the CRE virus, however, most cells were still alive and we analyzed their DNA content by flow cytometer. Fig. 3B summarizes that introduction of CRE slightly decreased the population of cells in the G1 and S phases, slightly increased that of cells in the G2/M phase and markedly increased that of cells with higher ploidy (>4n) (see Fig. 3C, right panel for example). Microscopic observation revealed that cells with higher ploidy frequently contained more than one nucleus (Fig. 3C). (They also exhibited a senescence-associated heterochromatin foci (SAHF)-like structure (Fig. 3C, middle panel). See below.) Interestingly, serum-deprived medium failed to cause cells to markedly accumulate in the G0/G1 phase (Fig. 3B, 4th (31%) versus 6th (37%) columns), whereas wild-type cells were efficiently accumulated into the G0/G1 population (Fig. 3B 3rd (46%) versus 7th (86%) columns), indicating that CSN5/Jab1-depletion inhibited progression of the cell cycle at

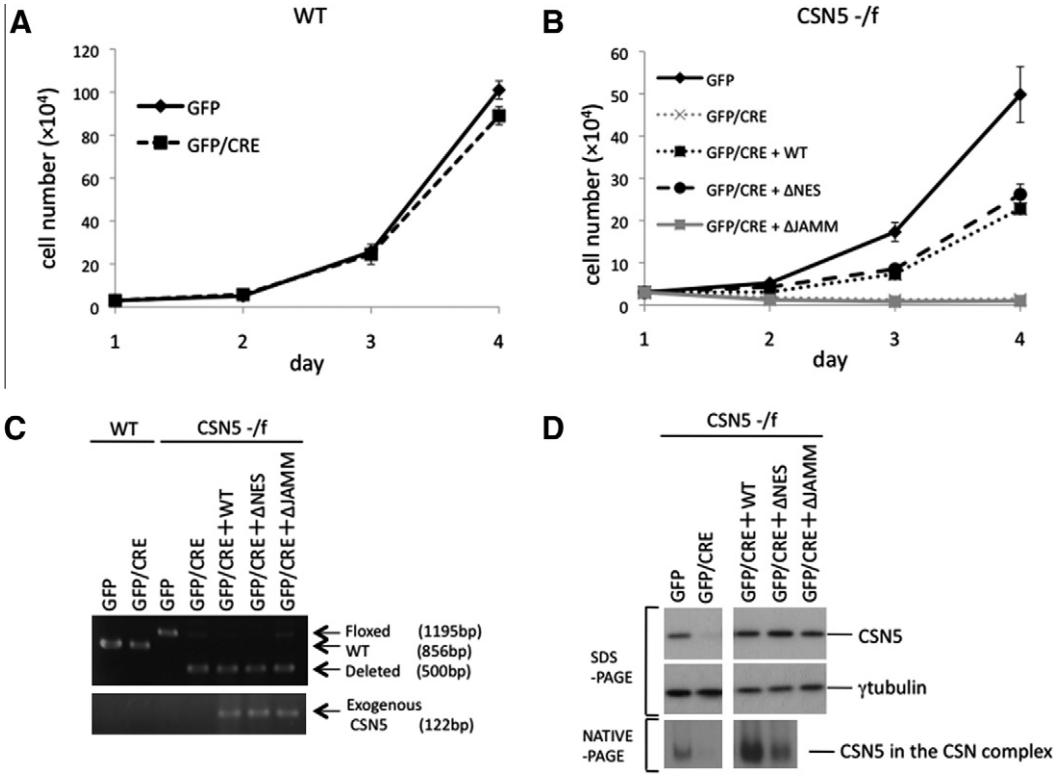

Fig. 2. CSN5/Jab1-depletion prevented cell proliferation. (A) and (B) Wild-type (A, WT) and CSN5/Jab1$^{-/f}$ (B, CSN5-/f) MEFs were infected with retroviruses encoding control GFP, CRE (GFP/CRE), wild-type CSN5/Jab1 (WT), and CSN5/Jab1 containing mutations in the NES sequence (ΔNES, L237/240/241A) or in the JAMM domain (ΔJAMM, D151N), plated (1×10^4 cells), and enumerated daily for 4 days. (C) HMW DNAs isolated from MEFs infected with retroviral vectors were subjected to a genomic PCR analysis as shown in Fig. 1B. (D) Lysates were isolated from CSN5/Jab1$^{-/f}$ MEFs infected with the viruses indicated, separated by SDS–PAGE (top and middle panels) and native-PAGE (bottom panel) and analyzed by immunoblotting using antibodies against CSN5/Jab1 (top and bottom panels) and γ-tubulin (middle panel). Native-PAGE: the position of the CSN-holo complex (containing CSN5/Jab1) was shown. Because the amount of cells was not sufficient, we were unable to perform the native-PAGE analysis for the cells doubly infected with CRE- and ΔJAMM-viruses.

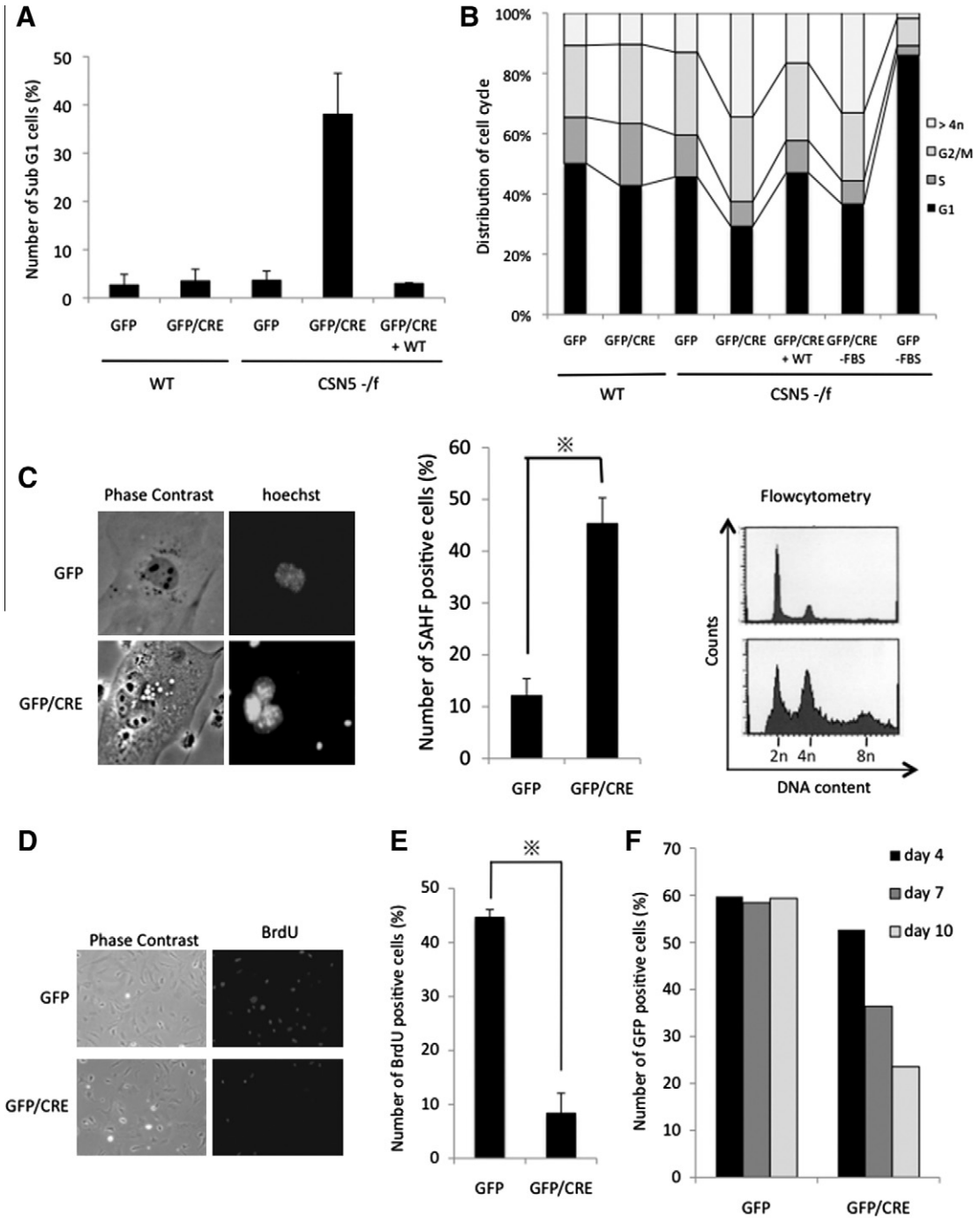

Fig. 3. CSN5/Jab1-depletion inhibited progression of the cell cycle at multiple points. (A) Wild-type (WT) and CSN5/Jab1$^{-/f}$ (CSN5−/f) MEFs were infected with viral vectors encoding GFP, CRE (GFP/CRE), and wild-type CSN5/Jab1 (WT). At 2 weeks post-infection, cells were analyzed by flowcytometer. Percentages of apoptotic cells were determined from the population of cells in subG1. (B) MEFs infected as in panel A were analyzed by flowcytometer at day 6 post-infection. In some cases (-FBS), cells were cultured in serum-deprived (0.5% FBS) medium for 48 h before analysis. (C) MEFs infected and cultured as in panel B were photographed after staining with Hoechst 33342 (left panels), or treated with PI and subjected to a flowcytometric analysis (right panels). The cells stained with Hoechst 33342 and exhibiting a SAHF-like structure were enumerated (middle panel) ($^{*}P < 0.0005$). (D) and (E) CSN5/Jab1$^{-/f}$ (CSN5−/f) MEFs infected with viral vectors encoding GFP and CRE (GFP/CRE) were subjected to BrdU incorporation assays at day 6 post-infection. Photos (D) and a summary of the results (E) are shown. $^{*}P < 0.0001$. (F) CSN5/Jab1−/f MEFs infected with control GFP and CRE (GFP/CRE) viruses were cultured with GFP-negative cells at a 1:1 ratio and the GFP-positive population was enumerated at days 4, 7, and 10.

multiple points. Again these phenotypes were recovered by ectopic expression of wild-type CSN5/Jab1.

To examine whether the elimination of CSN5/Jab1 affects progression through the S phase, we performed a pulse-labeling BrdU incorporation assay using cells at the relatively early stage (6 days) of infection by the CRE virus. Fig. 3D and E shows that CSN5/Jab1-depletion markedly inhibited BrdU incorporation (basically little incorporation above the background), indicating that cells with a DNA content of between 2n and 4n did not progress through the S phase after the inactivation of CSN5/Jab1.

Finally, we investigated the effect of the surrounding environment on suppression of the cell growth of CSN5/Jab1-depleted cells. CSN5/Jab1$^{-/f}$ MEFs infected with the CRE virus were cultured with CRE-negative cells at a 1:1 ratio and the percentage of GFP-

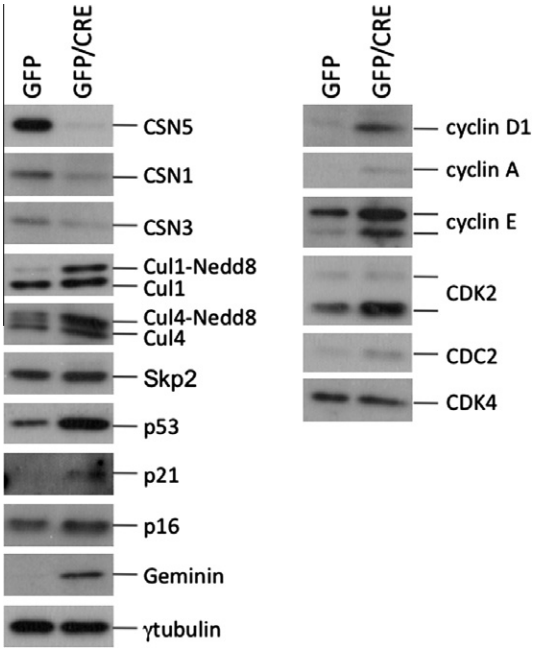

Fig. 4. CSN5/Jab1-depletion altered the expression level of many cell cycle regulators. Lysates from CSN5/Jab1$^{-/f}$ MEFs infected with control (GFP) and CRE (GFP/CRE) viruses were analyzed at day 6 post-infection by immunoblotting using the antibodies shown above the panels.

positive cells in the culture at days 4, 7, and 10 was analyzed (Fig. 3F). GFP-control cells proliferated at the same rate as the surrounding GFP-negative cells for 10 days, while CSN5/Jab1-depleted cells were gradually eliminated from the culture. These results indicate that it is not due to the insufficient release of the factors from the CSN5/Jab1-depleted cells into the culture medium (or insufficient contact between cells), but most probably due to a defect of the intracellular mechanism regulating progression of the cell cycle.

3.4. CSN5/Jab1 affected the expression of many cell cycle regulators

To explore the mechanism by which CSN5/Jab1-depletion prevents progression of the cell cycle, we analyzed the lysates isolated from CSN5/Jab1$^{-/f}$ MEFs infected with control GFP and GFP/CRE viruses at day 6 post-infection by immunoblotting with antibodies against various cell cycle regulators (Fig. 4). Accompanying the elimination of CSN5/Jab1, levels of other CSN subunits markedly declined (CSN1 and 3 are shown in Fig. 4). However, the reduction in levels of other subunits was significantly less than that for CSN5/Jab1, suggesting the existence of a CSN subcomplex that does not contain CSN5/Jab1, or alternatively, the existence of a small complex/monomeric form outside the CSN-holo complex. In fact, at the very early stages (2–4 days) after CSN5/Jab1-depletion, CSN subunits formed a subcomplex without CSN5/Jab1 (Fig. 1D, see above).

Significant amounts of Cul1 and Cul4 proteins were converted to a neddylated form (slower-migrating form) in the absence of CSN5/Jab1 (Fig. 4), consistent with the fact that CSN5/Jab1 is a catalytic subunit for the isopeptidase (deneddylase) activity in the CSN complex. In CSN5/Jab1-depleted cells, we observed the upregulation of many factors partly due to inactivation of CRL, including cyclins D1, A and E, Cdk2, Cdc2, p16, and Geminin (Fig. 4). The level of Skp2 was not significantly changed.

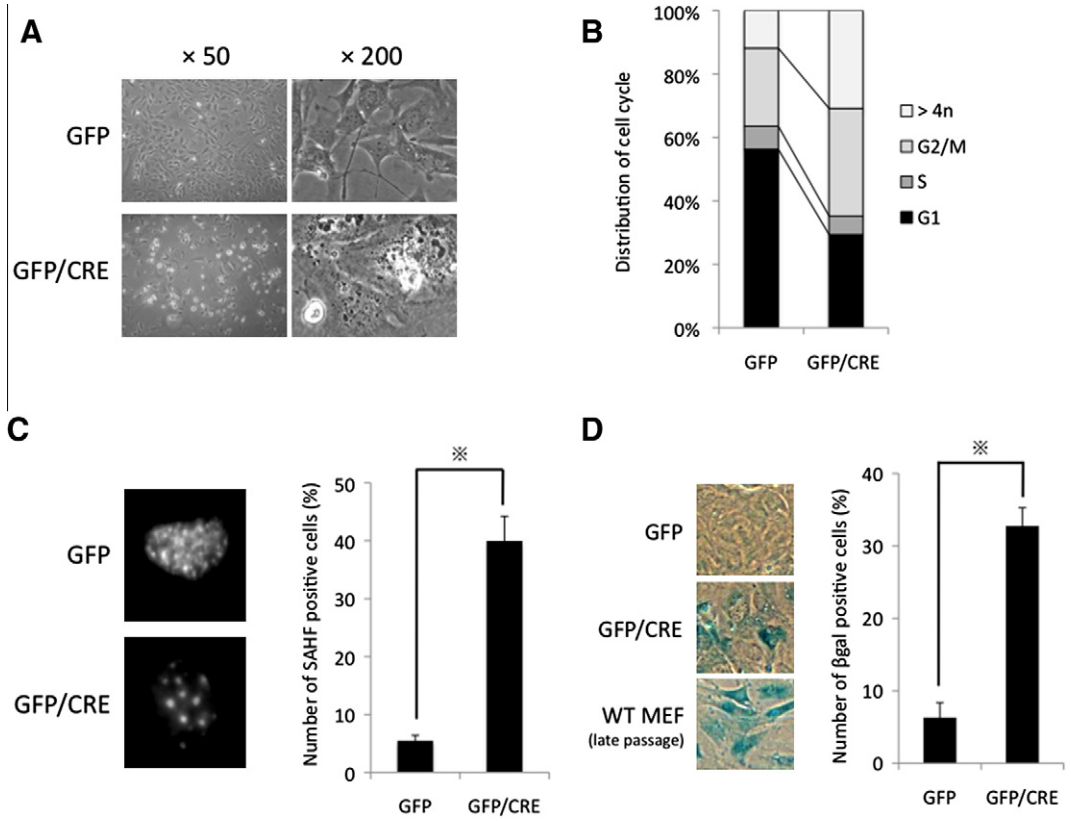

Fig. 5. Effects of CSN5/Jab1-depletion were independent of p53 function. CSN5/Jab1$^{-/f}$ p53$^{-/-}$ MEFs were infected with viral vectors encoding GFP and CRE (GFP/CRE). (A, C and D) At 6 days post-infection, cells were stained with Hoechst 33342 (C) and for β-galactosidase (D, for the positive control, wild-type MEFs in the late passage were used) and observed under phase-contrast (A and D) and fluorescent (C) microscopes. The cells exhibiting a SAHF-like structure (C, right panel, $^{*}P < 0.0005$) and β-gal-positive cells (D, right panel, $^{*}P < 0.0001$) were enumerated. (B) Alternatively, cells were subjected to a cell cycle analysis as in Fig. 3B.

3.5. Proliferative defects induced by CSN5/Jab1-depletion was independent of the function of p53

We observed an upregulation of the expression of the tumor suppressor p53 together with its target gene product p21 in CSN5/Jab1-depleted MEFs (Fig. 4). This is the anticipated result because CSN5/Jab1-null embryos contained high levels of p53 [15]. To examine whether the defect of cell cycle progression induced by CSN5/Jab1-depletin is mediated by the p53 pathway, we crossed CSN5/Jab1$^{-/f}$ mice with p53 knockout mice and eventually isolated MEFs exhibiting the CSN5/Jab1$^{-/f}$ p53$^{-/-}$ genotype. We infected CSN5/Jab1$^{-/f}$ p53$^{-/-}$ MEFs with the CRE virus and analyzed the cell proliferation phenotype. At 2 weeks post infection of the CRE/GFP virus, we found few colonies of MEFs with GFP signals. Microscopic observation of cells at an earlier stage showed that they were flat with little sign of proliferation, and, later, all died. We frequently found multi-nuclear cells in the CRE-infected culture but not in the control culture (Fig. 5A). Flow cytometric analysis showed that G1 and S phase cells were decreased, G2/M phase cells were slightly increased, and the population of cells with higher ploidy was markedly increased (Fig. 5B). In addition, nuclear staining with the Hoechst dye revealed that a significant population of CSN5/Jab1-depleted cells exhibited a senescence-associated heterochromatin foci (SAHF)-like structure in the absence (Fig. 5C) and presence (Fig. 3C, middle panel) of p53, suggesting that CSN5/Jab1-depletion initiated a senescence program without the functioning of p53 (other senescence-associated markers are an increase in p21 and p16 proteins (Fig. 4) for p53$^{+/+}$ cells, and induction of the β-gal activity (Fig. 5D) for p53$^{-/-}$ cells).

4. Discussion

It has been shown that the activity of the CSN holo-complex or function of the CSN5/Jab1 subunit is critical to the proliferation of mammalian cells. CSN5/Jab1 knockout mice ceased to develop at a very early stage of embryogenesis [15]. CSN5/Jab1 knockdown in cancer cells inhibited proliferation in culture [11,12]. Conditional knockout of CSN5/Jab1 in T cells prevented cell proliferation [16]. However, it remained to be clarified precisely how the loss of CSN5/Jab1 affects the mammalian cell cycle. In this study, we took advantage of the conditional allele of the CSN5/Jab1 gene and simultaneously deleted the gene from cultured MEFs in vitro by introducing a CRE recombinase. We first confirmed that CSN5/Jab1 is indispensable for mammalian cell proliferation, and found that progression of multiple phases of the cell cycle was inhibited, implying that CSN5/Jab1 functions at multiple points during the cycle. To preclude the influence of cellular senescence in an in vitro culture, we used naturally immortalized MEFs and p53$^{-/-}$ MEFs, which never enter senescence, as well as the primary MEF culture, and obtained the same results. This finding is consistent with reports that (1) CSN5/Jab1$^{+/-}$ MEFs exhibited a delay in progression from the G0 to S phase [15], (2) CSN5/Jab1$^{-/-}$ T cells showed a defect in S phase progression [16], and (3) CSN5/Jab1 targets multiple cell cycle regulators (from the G1 to M phase) such as p27, cyclin E, p53, DNA topoisomerase II, and APC/C (see [4] for review). Thus, we conclude that CSN5/Jab1 controls multiple cell cycle events from the G1 to M phase.

In addition, we found that a significant population of CSN5/Jab1-depleted MEFs showed an increase in ploidy. DNA content ranged from 4n to 8n and microscopic observation revealed that the culture contained many multi-nuclear cells, suggesting that CSN5/Jab1 regulates cytokinesis and/or endoreplication. In fact, it was reported that the CSN activity is connected with the endocycle in plants [3,23], and we previously observed that, in CSN5/Jab1-knocked out embryos, trophoblasts, which undergo endoreplication, remained intact, whereas the proliferation and survival of ICM (Inner Cell Mass), which go through the somatic cell cycle, were more severely inhibited [15].

The machinery regulating endoreplication in the mammalian system remains poorly understood though the activity associated with cyclin E [24] and APC/C [25] may play a role. It is worth noting that in CSN5/Jab1-depleted cells, cyclin E expression is upregulated and this could be partly responsible for the aberrant entry into the endocycle. However, this could not be the sole reason because (1) overexpression of cyclin E alone did not induce endoreplication [26], and (2) knockdown of Cdk2 did not reverse the increase in ploidy in CSN5/Jab1-depleted cells (unpublished observation, AY, NYK, and JYK). Thus, the mechanism regulating entry into the endocycle in mammals largely remains a mystery, and the CSN5/Jab1-depletein system may be a good tool to investigate this phenomenon.

Finally, DNA staining revealed that a significant population of cells exhibited a SAHF-like structure, suggesting that CSN5/Jab1 may regulate cellular senescence. This functioned in the absence of p53 and occurred with no significant change in the level of Skp2, which was recently implicated in the regulation of senescence [27], suggestive of a novel mechanism inducing proliferative senescence. In conclusion, we propose that CSN5/Jab1 is a modulator of the mammalian cell cycle (somatic cell cycle/endocycle/senescence).

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