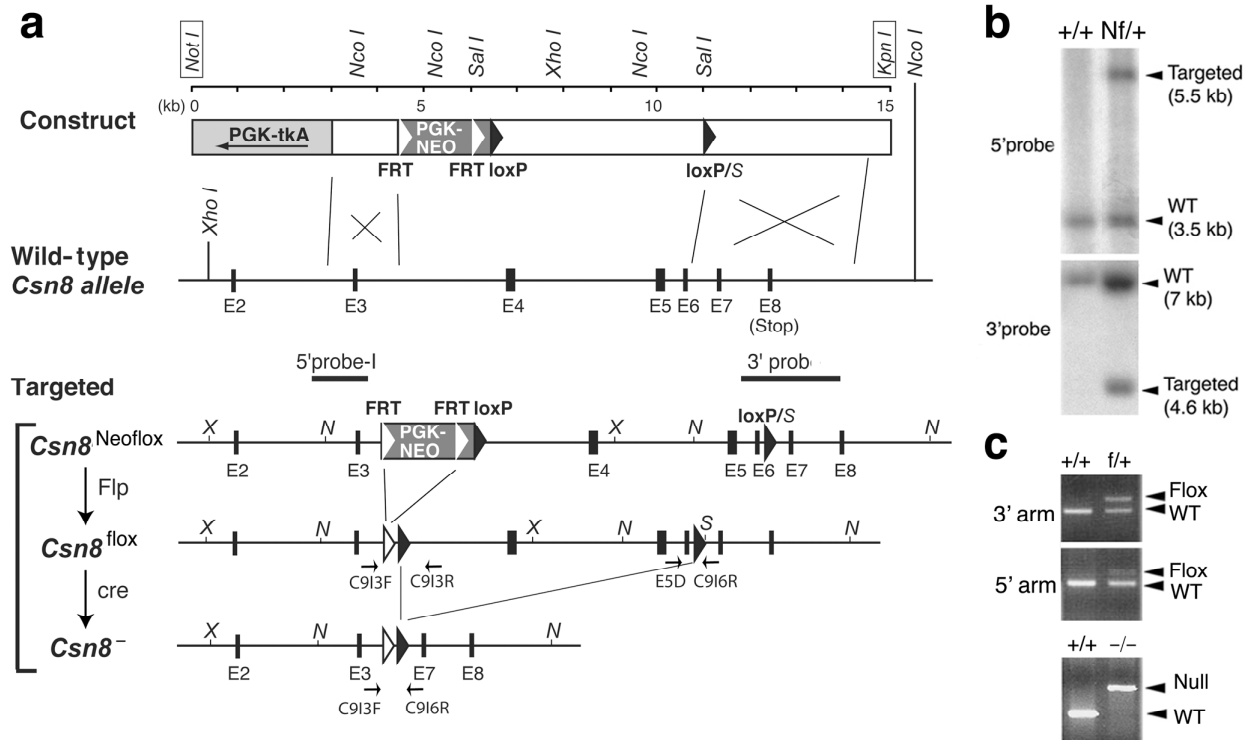
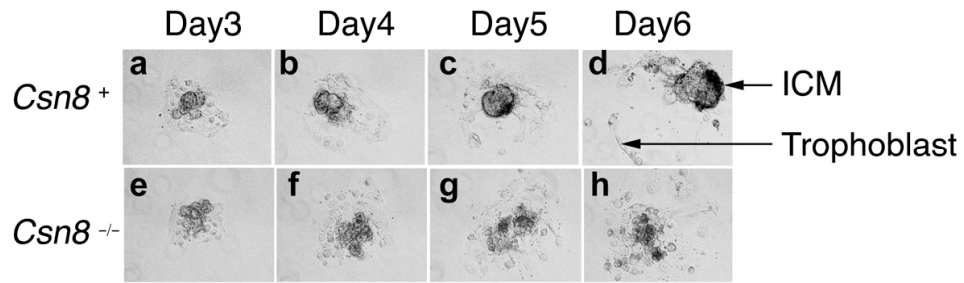


SUPPLEMENTARY FIGURES AND LEGENDS



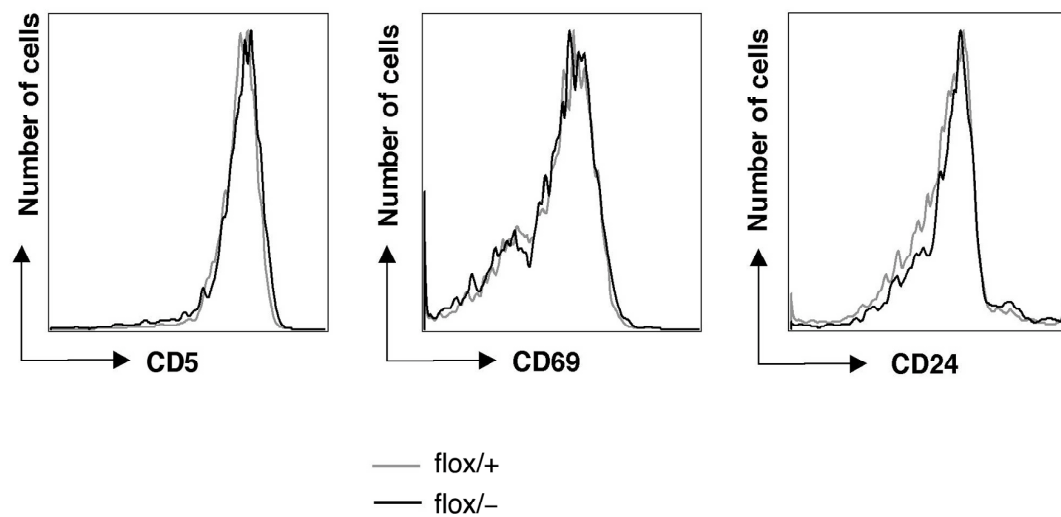
Supplementary Fig. 1

Strategy and verification of conditional disruption of the mouse *Csn8* locus (a) Strategy for targeted deletion of exons 4 to 6 of the *Csn8* gene. The targeting vector was designed to contain loxP sites flanking exons 4 to 6, a PGK-Neo cassette flanked by FRT sites as a positive selection marker, and a PGK-Thymidine Kinase A (PGK-tkA) cassette as a negative selection marker. After homologous recombination in ES cells, *Csn8*^{Neoflox} allele was generated. PGK-Neo cassette was removed by Flp mediated recombination after mating with *CMV-Flpe* deleter mice, generating *Csn8*^{flox} allele. *Csn8*⁻ allele was generated by Cre mediated deletion of exons 4 to 6 after mating with β -actin-Cre mice. (b) Identification of homologous recombinants. Genomic DNA from the ES cells (*Nf*) were digested with *Xho*I and analyzed by Southern blot using the 5' probe to identify clones carrying a 5.5 kb fragment specific for the targeted allele (targeted). The positive clones were further confirmed using the 3' probe in an *Nco*I and *Sal*I digest to verify the presence of a 4.6 kb targeted fragment. (c) PCR genotyping. Primer pairs C913F-C913R and E5D-C916R were used to detect the *Csn8*⁺ allele (WT) and the floxed allele, *Csn8*^{flox} (Flox) in the 5' arm and 3' arm, respectively. Primer pair C913F-C916R was used to detect the deleted allele (*Csn8*⁻). The location of the various primers is indicated in (a).



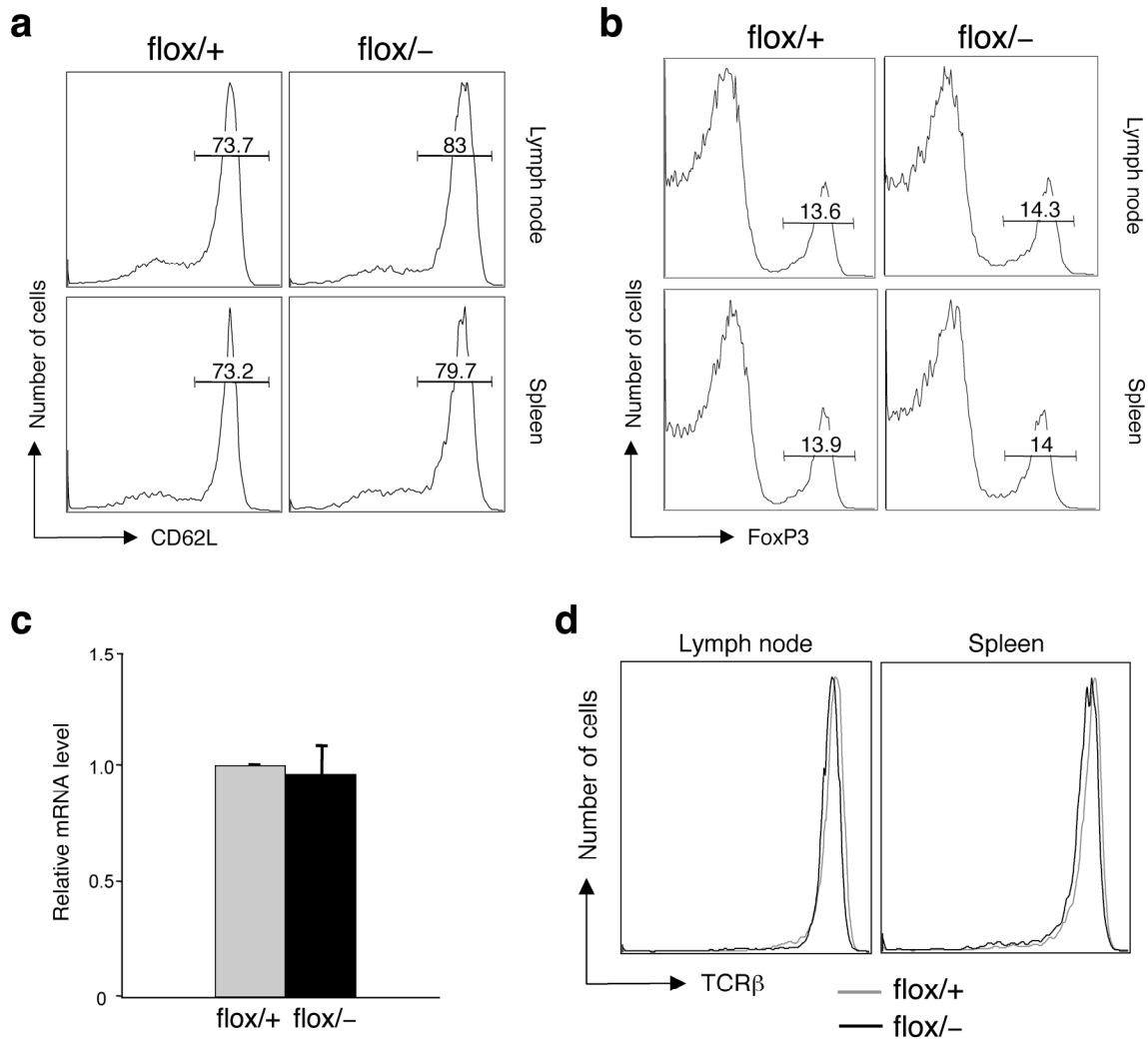
Supplementary Fig. 2

Premature arrest of *Csn8*^{-/-} blastocysts in the outgrowth assay. E3.5 embryos isolated from *Csn8*^{+/-} intercrosses were cultured *in vitro* to allow outgrowth. Photographs were taken each day from day 3 to day 6 after isolation. Inner cell mass (ICM) and trophoblast cells are marked. Panels **a-d** are wild-type and **e-h** are *Csn8*⁻ deficient blastocysts.



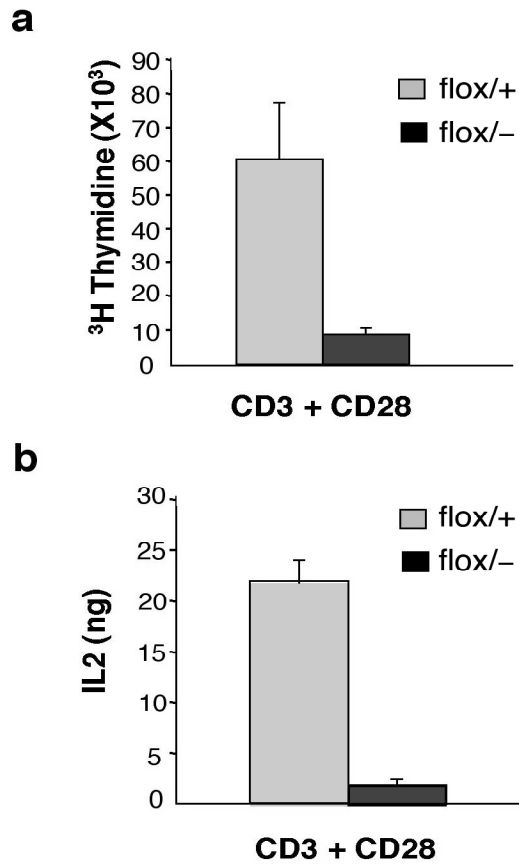
Supplementary Fig. 3

Normal expression of surface markers in flox/- thymocytes. Flow cytometric analysis of CD5, CD69 and CD24 expression by CD4 single-positive thymocytes from flox/+ and flox/- mice.



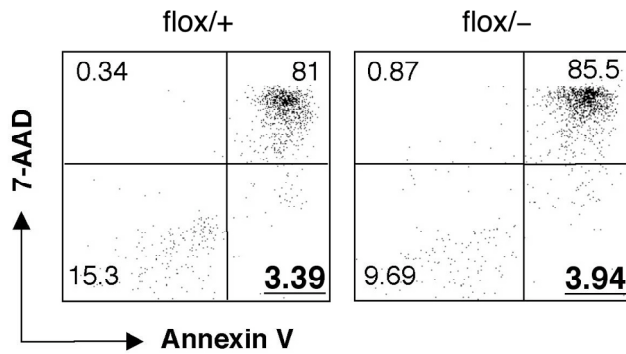
Supplementary Fig. 4

Normal distribution of naive and regulatory cell populations and normal expression of TCR β and IL-7R α mRNA in peripheral CD4⁺ T cells from flox/- mice. Flow cytometric analyses of CD4⁺ T cells from flox/+ and flox/- mice were performed on (a) CD62L expression, (b) FoxP3 expression and (d) TCR β expression. (c) Expression of IL-7R α mRNA in flox/+ and flox/- CD4⁺ T cells analyzed by real time PCR.



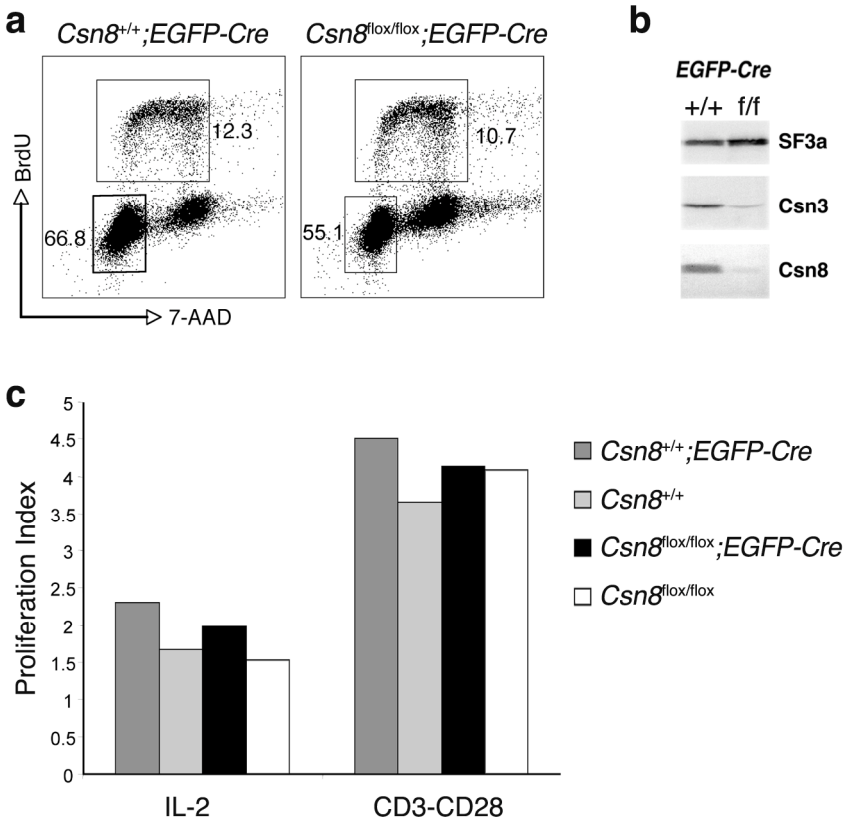
Supplementary Fig. 5

Proliferation and IL-2 production of naive CD4^+ T cells after TCR activation. **(a)** Proliferation of naive CD4^+ T cells. Sorted naive CD4^+ T cells ($\text{CD4}^+ \text{CD25}^- \text{CD62L}^{\text{hi}} \text{CD44}^{\text{lo}}$) from flox/+ and flox/- mice were stimulated with anti-CD3 and anti-CD28 for 40 hrs and pulsed with ^3H -thymidine for an additional 8 hrs. Amount of incorporated radioactivity indicates the level of proliferation. **(b)** IL-2 secretion by naive CD4^+ T cells. ELISA for IL-2 after 40 hrs of stimulation in the experiment described in (a) is presented.



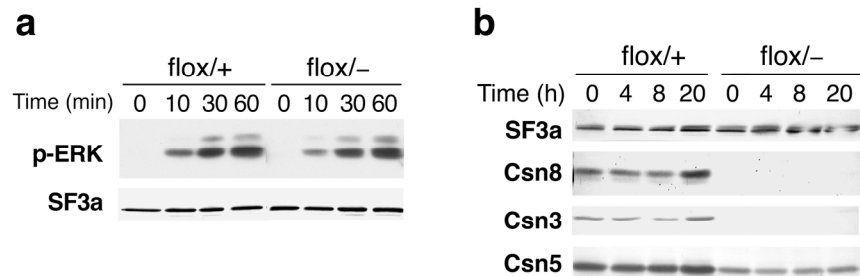
Supplementary Fig. 6

Csn8-deficient CD4⁺ T cells do not exhibit altered apoptosis after TCR-induction. Sorted CD4⁺ T cells from flox/+ and flox/- mice were stimulated with anti-CD3 and anti-CD28. Twenty hours after stimulation the cells were stained with Annexin V and 7-AAD and analyzed by flow cytometry. The percentages of apoptotic cells (Annexin V^{hi}7-AAD^{lo}) are underlined.



Supplementary Fig. 7

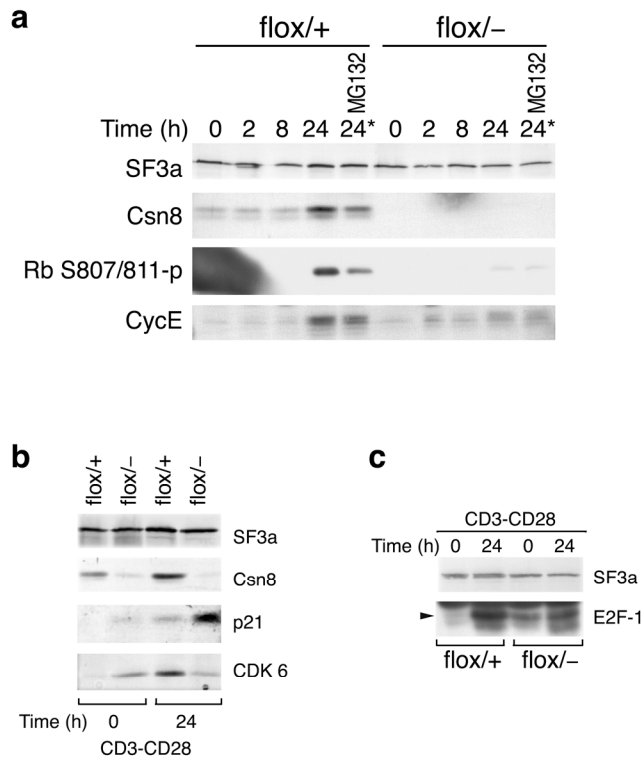
Csn8 is not required for proliferation of effector CD4⁺ T cells. Purified *Csn8*^{+/+} and *Csn8*^{lox/lox} CD4⁺ T cells (Cre negative) were activated with 5 μg/ml anti-CD3, 2 μg/ml anti-CD28, and 100 U/ml IL-2, and were transduced with the MSCV retrovirus encoding *EGFP-Cre* for 5 days. **(a)** Cell cycle analysis of sorted *EGFP-Cre* positive *Csn8*^{+/+} and *Csn8*^{lox/lox} CD4⁺ T cells. The cells were pulsed with BrdU for 4 hrs and analyzed by flow cytometry after staining for BrdU and 7-AAD. Numbers indicate the percentages of cells in the G1 phase (BrdU⁻, bottom gate) and those active in DNA synthesis (BrdU⁺, top gate). **(b)** Immunoblot analysis of *EGFP-Cre* positive *Csn8*^{+/+} and *Csn8*^{lox/lox} effector CD4⁺ T cells with anti-*Csn8*, anti-*Csn3* and SF3a (loading control). **(c)** Comparison of cell proliferation between sorted *EGFP-Cre* positive *Csn8*^{+/+} and *Csn8*^{lox/lox} CD4⁺ T cells. *EGFP-Cre* negative *Csn8*^{+/+} and *Csn8*^{lox/lox} CD4⁺ T cells were used as a further control. Cells were stimulated with IL-2 (500 U/ml) or anti-CD3/CD28 in the presence of ³H-thymidine for 24 hrs. ³H-thymidine incorporation in activated cells was normalized against that in un-stimulated cells to obtain the proliferation index.



Supplementary Fig. 8

Phosphorylation of ERK and protein expression of Csn subunits following TCR activation.

(a) TCR stimulated phosphorylation of ERK1/2 is normal in the absence of *Csn8*. CD4⁺ T cells from flox/+ and flox/- mice were stimulated with anti-CD3 and anti-CD28 for the indicated time points and analyzed by immunoblot using phosphor-specific antibodies against ERK. **(b)** Protein expression of Csn subunits following TCR activation. CD4⁺ T cells from flox/+ and flox/- mice were stimulated with anti-CD3 and anti-CD28 for the indicated time points. Total proteins from each sample were immunoblotted with the indicated antibodies. SF3a was used as a loading control.



Supplementary Fig. 9

Immunoblot analyses of cell cycle regulators in *Csn8* deficient T cells. CD4⁺ T cells from flox/+ and flox/- mice were stimulated with anti-CD3 and anti-CD28 for the indicated time points. Total proteins from the lysates were separated by SDS-PAGE and immunoblotted with indicated antibodies. **(a)** Absence of phosphorylated Rb^{S807/811} and defective Cyclin E expression were not due to proteasome-dependent protein degradation. The Proteasome inhibitor, MG132 (10 μM) was added 19 hrs after stimulation and was incubated for an additional 5 hrs. Rb^{S807/811} phosphorylation specific antibody was used. **(b)** CDK inhibitor p21 is induced in *Csn8*-deficient T cells. At 24 hrs post stimulation, p21 was detected in normal CD4⁺ T cells, and its expression was drastically increased in *Csn8*-deficient cells. In addition, *Csn8*-deficient CD4⁺ T cells had elevated level of Cdk6 before stimulation compared to wild-type cells, and the level was not induced at 24 hr post stimulation. **(c)** Abnormal expression of E2F1 in *Csn8*-deficient CD4⁺ T cells was shown by anti-E2f1 immunoblot. **(a-c)** SF3a was used as a loading control.

SUPPLEMENTARY TABLES

Table 1. PCR primers used for genotyping.

Name	Sequence (5' to 3')	Note
C9I3F	AACAGCTCAGCTGATAAGAGTGG	<i>Csn8</i> ^{Neoflox} allele: ~400bp
C9Neo	ACGTGCTACTTCCATTTGTCACG	
E5D	GACTCCACCACAAGAATGGTT	<i>Csn8</i> ^{flox} allele: ~190 bp. <i>Csn8</i> ⁺ allele (WT): ~130 bp.
C9I6R	GTAGGTGACCTTCAATGTCAC	
C9I3F	AACAGCTCAGCTGATAAGAGTGG	<i>Csn8</i> ⁻ allele: ~270 bp
C9I6R	GTAGGTGACCTTCAATGTCAC	
Cre fwd	CCAGCTAAACATGCTTCATCGTC	<i>CD4-cre</i> transgene: ~100bp
Cre rvs	CCTGATCCTGGCAATTTCCGG	

Table 2. PCR primers used for ChIP analyses.

Name	Sequence (5' to 3')	Note
CycD2 fwd	AGCAGGAAGG AGGTGAGGAA ACG	~160bp. Approximate Position: -1200bp to mRNA start site.
CycD2 rvs	TCTAGGAAAT GGCTCGGGAG G	
CDK4 fwd	GCCCACTCAG AGACCCATAG	~190 bp. Position: -345bp to <i>Cdk4</i> mRNA start site*.
CDK4 rvs	GGGAAGCAGA GGTGTGGTTA	
p21 fwd	CGCTGCGTGAC AAGAGAATA	203bp. Encompassing <i>Cdkn1a</i> transcription start site*.
p21 rvs	TCGAGCTGCC TCCTTATAGC	
rDNA fwd	TGGTGTCCAA GTGTTTCATGC	~150bp. Position: +500bp of <i>45S pre-rRNA</i> start site.
rDNA rvs	AATCGGGAGA AACAAAGCGAG	

* Primers for *Cdk4* and *Cdkn1a* promoter regions were according to the following reference: Cairo S, De Falco F, Pizzo M, Salomoni P, Pandolfi PP, Meroni G. (2005) PML interacts with Myc, and Myc target gene expression is altered in PML-null fibroblasts. *Oncogene* 24(13):2195-203.

SUPPLEMENTARY METHODS

Targeting vector

A mouse *Csn8* 12 kb genomic DNA clone was isolated from a lambda phage genomic library of 129 mouse strain^{S1}. To construct the targeting vector, a fragment of *Csn8* gene extending from *EcoRI* site in intron 3 to a *HindIII* site in exon 4 was subcloned into pPCRscript (Stratagene) and is referred to as pEH. A *SalI* - *SacII* fragment (blunt ended) containing the positive selection marker, PGK-Neo flanked by Flp recombination (FRT) sites with an adjacent loxP site was inserted at the *NdeI* (blunt ended) site of pEH, creating pENEOLH. The negative selection marker, thymidine kinase A was inserted into the pENEOLH vector at the *SalI* site (ptkENEOLH). A *HindIII* digested *Csn8* genomic DNA fragment containing exons 4 to 8 and the 3' UTR was subcloned into pPCRscript vector (pHH), which was then linearized with *PpuMI* and an oligonucleotide containing a loxP site was inserted (pHLH). Finally, the *Csn8* gene-targeting vector was constructed by inserting the *HindIII* fragment from pHLH into *HindIII* digested ptkENEOLH. The targeting vector has a right flanking arm of 1.9 kb and left arm of 4.5 kb (**Supplementary Fig. 1a**). A *NotI* site was used to linearize the construct prior to electroporation.

Generating *Csn8*-deficient mice, Southern analysis and PCR genotyping

Standard procedures for ES cell gene targeting were followed. Putative homologous recombinant ES clones were identified by Southern blot analysis of *XhoI* digested genomic DNA. The *EcoRI* - *NdeI* fragment from pEH vector (1.2 kb) was used as the 5' probe. This probe detects a 3.5 kb band in the wild-type allele and a 5.5 kb band in the targeted allele. The positive clones were further verified by digesting the genomic DNA with *NcoI* and *SalI* and Southern analysis with the 3' probe, an *EcoRV* - *XbaI* fragment from pHH vector (1 kb). This probe detects a 4.6 kb band in the targeted allele and a 7 kb band in the wild-type allele.

All mouse experiments were approved by Institutional Animal Care and Use Committee of Yale University. One confirmed ES clone was karyotyped and injected into C57BL/6J (B6) blastocysts to generate chimeras. Germline transmission was confirmed by Southern blot. The *Csn8*^{Neoflox/+} founders were backcrossed with B6 mice for four to five generations. The PGK-Neo cassette was removed by mating *Csn8*^{Neoflox/+} mice with *CMV-Flpe* mice to generate *Csn8*^{fllox/+} mice. *Csn8*^{+/-} mice were generated by crossing *Csn8*^{fllox/+} mice with *β-actin-Cre* mice. Both *CMV-Flpe* and *β-actin-Cre* mice were obtained from the Yale Transgenic Mouse facility. *Csn8*^{fllox/+} and *Csn8*^{+/-} mice were backcrossed to the B6 mice to remove the *CMV-Flpe* and *β-actin-Cre* transgenes. To generate T cell specific *Csn8*-deficient mice, *CD4-Cre* transgenic mice²⁷ were first crossed with *Csn8*^{+/-} mice and the resulting *CD4-Cre*⁺*Csn8*^{+/-} mice were mated with *Csn8*^{fllox/fllox} mice to obtain *CD4-Cre*⁺*Csn8*^{fllox/+} and *CD4-Cre*⁺*Csn8*^{fllox/-} mice. PCR primers used for genotyping are listed in **Supplementary Table 1**.

Blastocyst outgrowth assay and immunohistochemistry

Blastocyst outgrowth assay, paraffin sectioning and immunohistochemical staining were performed as previously described²⁴. After recording the image of blastocysts, they were genotyped individually by PCR using the primers E5D and C9I6R to detect the wild-type *Csn8* allele and the primers C9I3F and C9I6R to detect the null allele. The antibodies used for immunohistochemical staining include anti-Csn8 and anti-Csn2^{24,44}.

***In vivo* BrdU labeling**

In vivo proliferation of peripheral T cells was assessed by administering BrdU (0.8 mg/ml) in drinking water to mice for 2 weeks. Cells from spleen and lymph nodes were isolated and stained for CD4, followed by BrdU staining according to manufacturer's protocol (BD Pharmingen).

Chromatin immunoprecipitation

Stimulated or unstimulated CD4⁺ T cells (2 million per sample) were fixed with 1% formaldehyde for 10 min at 37°C, washed with cold PBS and lysed in 200 μ l SDS lysis buffer (50mM Tris-HCl (pH 8.0), 10mM EDTA, 1% SDS) and sonicated to shear the DNA. After centrifugation, input control was taken from the supernatant before diluting 10 volumes with ChIP dilution buffer [1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl (pH 8.0), 167mM NaCl, 1x Protease inhibitor cocktail (Roche)]. Samples were equally divided and mixed with indicated antibody or a pre-immune serum control and incubated overnight at 4°C. Protein A-agarose was added in the last hour. Beads were washed once each with dilution buffer containing 150 mM NaCl, then the buffer containing 500 mM NaCl, LiCl buffer (0.25M LiCl, 1% IGEPAL CA630, 1% deoxycholic acid (sodium salt), 1mM EDTA, 10mM Tris-HCl (pH 8.1)), and finally twice with TE buffer. Protein-DNA complexes were eluted twice by incubating with 250 μ l elution buffer (0.1M NaHCO₃, 1% SDS) for 15 min at room temperature. Reverse crosslinking was performed in 0.2M NaCl at 65 °C for 4 h. After treatment with 2 μ l Proteinase K (10mg/ml) for 1 h at 45 °C, the DNA was phenol-chloroform extracted and ethanol-precipitated in the presence of 20 μ g glycogen. PCR conditions were 95°C for 40 sec, 60°C for 40 sec, 72°C for 30 sec, 35 cycles. PCR primers are provided in **Supplementary Table 2**, online.

Supplementary reference

S1. Lykke-Andersen, K. & Wei, N. Gene structure and embryonic expression of mouse COP9 Signalosome subunit 8 (*Csn8*). *Genes*. **321**, 65-72 (2003).