Cks1 Promotion of S Phase Entry and Proliferation Is Independent of p27^{Kip1} Suppression

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Cks1 is an activator of the SCF^{Skp2} ubiquitin ligase complex that targets the cell cycle inhibitor p27^{Kip1} for degradation. The loss of *Cks1* results in p27^{Kip1} accumulation and decreased proliferation and inhibits tumorigenesis. We identify here a function of Cks1 in mammalian cell cycle regulation that is independent of p27^{Kip1}. Specifically, *Cks1*^{-/-}; p27^{Kip1}^{-/-} mouse embryonic fibroblasts retain defects in the G₁-S phase transition that are coupled with decreased Cdk2-associated kinase activity and defects in proliferation that are associated with *Cks1* loss. Furthermore, concomitant loss of *Cks1* does not rescue the tumor suppressor function of p27^{Kip1} that is manifest in various organs of p27^{Kip1}^{-/-} mice. In contrast, defects in mitotic entry and premature senescence manifest in *Cks1*^{-/-} cells are p27^{Kip1} dependent. Collectively, these findings establish p27^{Kip1}-independent functions of Cks1 in regulating the G₁-S transition.

Cell cycle progression is a highly ordered process that is regulated by the oscillating expression of positive and negative factors (42–44). One central regulatory protein that exerts functions at the G₁-S and G₂-M transition is the cyclin-dependent kinase (Cdk) inhibitor p27^{Kip1} (44, 45). Overexpression of p27^{Kip1} arrests cells in G₁ (10), while complete loss of p27^{Kip1} leads to increased cell proliferation as shown in knockout mice *in vivo* (13, 22, 33). The fundamental role of p27^{Kip1} for controlled cell proliferation is further established through its function in tumor suppression and as a prognostic factor in various malignancies (9, 45).

The regulation of p27^{Kip1} expression occurs mainly at the post-translational level. The established model involves phosphorylation of p27^{Kip1} at threonine 187 (T187) during G₁ by cyclin E/A-Cdk2 complexes, which marks p27^{Kip1} for recognition by the E3 ubiquitin ligase SCF^{Skp2} (8, 24, 40, 45). SCF^{Skp2} requires the presence of a small protein, cyclin-dependent kinase subunit 1 (Cks1), that constitutes a part of the substrate binding surface for efficient ubiquitylation of T187-phosphorylated p27^{Kip1} (14, 16). Tissues from mice lacking Cks1 accumulate p27^{Kip1} exhibit proliferative defects and, accordingly, *Cks1*^{-/-} mice are abnormally small (46). This phenotype thus resembles the one seen in *Skp2*-deficient mice with regard to body size but lacks additional defects such as enlarged nuclei with polyploidy and multiple centrosomes in various epithelial tissues (34).

Cks1 proteins (Cks1 and Cks2) interact tightly with Cdk complexes (7). Based on structural considerations Cks1 is involved in Cdk activation and acts as a targeting protein for Cdks (7, 31). Several lines of evidence suggest a function of Cks1 in mitosis. First, the loss of Cks1 function results in mitotic defects in yeast (47). Second, in mammalian cells, Cks proteins are required for ubiquitylation and degradation of cyclin A complexed with Cdc20 in pre-anaphase, which is required for mitotic progression (52). Third, transcriptional regulation of essential mitotic factors has been demonstrated in yeast and mammalian cells (29, 32, 51, 53). RNA interference-mediated suppression of both Cks family members in mouse embryonic fibroblasts (MEFs) results in impaired transcription of *Cdk1*, *Ccna2*, and, most critically, *Ccnb1*, and, *in*

in vivo, simultaneous *Cks1* and *Cks2* deficiency leads to embryonic lethality (29).

The common understanding of the *Cks1*^{-/-} phenotype involves a crucial role for p27^{Kip1} accumulation that results in a severe proliferative defect *in vitro* (MEFs) and *in vivo* (body size). Here, we use a genetic approach to dissect Cks1 functions that are dependent or independent of p27^{Kip1} ubiquitylation. We provide genetic evidence that the function of Cks1 at the G₁/S phase transition is predominantly independent of the SCF^{Skp2} complex and p27^{Kip1} and, in fact, is associated with Cdk2 activity. In contrast, defects in mitotic entry and premature senescence manifest in *Cks1*^{-/-} cells are p27^{Kip1} dependent. Collectively, our findings establish p27^{Kip1}-independent functions of Cks1 in regulating S phase entry.

MATERIALS AND METHODS

Animals. *Cks1*-null mice (C57BL/6-129 mixed background) (46) were bred to p27^{Kip1}-null mice (C57BL/6) (13) for >6 generations to generate *Cks1*^{-/-}; p27^{Kip1}^{-/-} and control animals on a mixed C57BL/6-129 background. Genotyping was performed as described previously (13, 46). All animal experiments were performed in accordance with the regional animal ethics committee approvals.

Cell culture and cell cycle analysis. Primary MEFs were obtained from embryonic day 13.5 (E13.5) to E14.5 embryos and cultured as described earlier (21). Primary mouse fibroblasts were cultured in Dulbecco modified Eagle medium (DMEM; Gibco) with 10% fetal calf serum (FCS), 1% penicillin-streptomycin (Pen-Strep), 1% nonessential amino acids, and 50 μM 2-mercaptoethanol (all from Invitrogen) with 5% CO₂. Early-passage MEFs were used for all experiments. For proliferation assays, 5 × 10⁴ cells were plated into six-well plates. Early passage

Received 28 December 2011 Returned for modification 21 January 2012

Accepted 2 April 2012

Published ahead of print 16 April 2012

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doi:10.1128/MCB.06771-11

$p21^{Cip1-/-}$ and $p21^{Cip1-/-}$; $p27^{Kip1-/-}$ MEFs were kindly provided by Martine Roussel (St. Jude Children's Research Hospital, Memphis, TN) and cultured under identical conditions. For some experiments, MEFs were cultured in the presence of 3% oxygen to reduce oxidative stress (41). NIH 3T3 cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and cultured in DMEM (Gibco) supplemented with 10% FCS and 1% Pen-Strep.

Immortalized fibroblasts were generated from the indicated genotype MEFs according to the 3T3 protocol (49). In brief, cells were cultured and split twice a week until a complete growth arrest was noted. Cells were then treated with trypsin and reseeded at the same density twice a week until growth was noted, which occurred at approximately passage 40.

For bromodeoxyuridine (BrdU) staining, 3×10^5 cells were plated in 6-cm dishes on the day before analysis. The cells were labeled with 10 μ M BrdU (BD Biosciences) for 45 min, collected, and stained according to the manufacturer's protocol. For propidium iodide (PI) cell cycle analysis, the cells were fixed in 70% ice-cold ethanol and stained in PI staining solution (50 μ g of propidium iodide/ml, 100 μ g of RNase/ml, phosphate-buffered saline). To block cell cycle progression at the G_1 -S transition, the cells were incubated for 48 h with medium containing 0.1% FCS, followed by incubation with 1 μ g of aphidicolin (Sigma-Aldrich)/ml in medium containing 15% FCS for 16 h. The aphidicolin was washed out, and the cells were released from the block by the addition of normal growth medium for the indicated times. To synchronize cells with double thymidine treatment, 2 nM thymidine was added to culture medium for 18 h following a first release for 8 h and a second block with 2 nM thymidine for an additional 18 h. The thymidine was washed out, and the cells were released from the block by the addition of normal growth medium. To inhibit proteasomal degradation, MG132 was added to the culture medium in a final concentration of 5 μ M for 5 h.

Viral infection. 293T cells stably transfected with helper virus plasmids were transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen) (21). Supernatant of virus producing cells was harvested, filtered, and used to infect primary MEFs in the presence of 8 μ g of Polybrene (Sigma-Aldrich)/ml. At 24 h postinfection, medium was changed, and the cells were selected in 2 μ g of puromycin (Sigma-Aldrich)/ml for 48 h. For stable *Cks1* knockdown experiments, lentiviral sh-RNA plasmids (Open Biosystems) were cotransfected with lentiviral helper plasmids for virus production into 293T cells as described above. For some experiments, the puromycin resistance gene was replaced by a green fluorescent protein (GFP) reporter gene. Details of cloning procedures can be obtained upon request. shRNA constructs for stable knockdown of p130 were obtained from Mission shRNA (Sigma-Aldrich). For double-knockdown experiments, two supernatants containing different viral particles were combined for the infection of NIH 3T3 or MEFs as indicated. GFP-positive cells were sorted on a Dako Cytomation MoFlo fluorescence-activated cell sorter.

RNA extraction and real-time PCR. RNA extraction was performed using an RNeasy minikit (Qiagen). cDNA synthesis was performed according to the manufacturer's protocol (Qiagen). Real-time PCR was performed using Platinum SYBR green Q PCR SuperMix-UDG (Invitrogen) on an ABI Prism 7700 (Applied Biosystems). The data analysis was done by comparing threshold cycle (C_T) values with a control sample set as 1. Sequences for primers are available upon request.

Plasmids and mutagenesis. $p27^{Kip1}$ and *Cks1* cDNA was amplified from primary MEFs after RNA preparation and reverse transcription (Qiagen). cDNA was cloned into the retroviral expression vector pBabe-Puro. A point mutation of $p27^{Kip1}$ cDNA was introduced using a QuikChange site-directed mutagenesis kit (Stratagene). Details on cloning and primer selection can be obtained from the authors upon request.

Immunoblotting, immunoprecipitation, kinase assays, and antibodies. Cell lysis for immunoblotting was performed with lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5

mM EGTA, and 0.1% Tween 20 (all from Sigma-Aldrich) and protease inhibitors (Roche), followed by sonification. After SDS-PAGE, the proteins were transferred to nitrocellulose membranes and probed with antibody.

To assess Cdk-associated kinase activity primary MEFs were resuspended in lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA [pH 8.0], 2.5 mM EGTA [pH 8.0], 0.1% Tween 20, 10% glycerol, 1 mM dithiothreitol [DTT], 5 mM NaF, 0.1 mM Na_3VO_4 , and 10 mM β -glycerophosphate [all from Sigma-Aldrich]) containing a protease inhibitor cocktail (Roche) and incubated for 30 min. The lysates were cleared by centrifugation at $10^4 \times g$ for 20 min. Then, 200 μ g of total lysates was incubated with antibody for 12 h at 4°C with gentle agitation, followed by incubation for 1 h with Protein A/G Plus-agarose beads (Santa Cruz Biotechnology). Immunocomplexes bound to agarose beads were collected by centrifugation and washed three times. To determine the kinase activity, pelleted bead-protein complexes were incubated for 30 min at 30°C in kinase reaction buffer (50 mM HEPES [pH 7.5], 10 mM MgCl_2 , 1 mM DTT, 2 mM EGTA, 1 mM NaF, 0.1 mM Na_3VO_4 , and 10 mM β -glycerophosphate [all from Sigma-Aldrich]) with 20 μ M cold ATP, 5 μ g of histone H1 (Upstate)/ μ l as the substrate, and 10 μ Ci of [γ - 32 P]ATP (Hartmann Analytik) and then separated by SDS-PAGE. Phosphorylation was visualized by autoradiography.

The antibodies against Cdk1, Cdk2, Cdk4, cyclin A, cyclin B1, cyclin E, p130, and $p21^{Cip1}$ were purchased from Santa Cruz Biotechnology. Anti-cyclin E for NIH 3T3 and MEF lysates were used as previously described (3). Anti-Cks1 was purchased from Zymed Laboratories, anti-phospho-Cdk1(Y15) was obtained from Cell Signaling, anti-phospho-Cdk2(Y15) was obtained from Abcam, anti-Cdk6 was obtained from BD Biosciences, anti- $p27^{Kip1}$ was obtained from Transduction Laboratories, anti- $p57^{Kip2}$, and anti- β -actin was obtained from Sigma-Aldrich.

Histological analyses and assessment of senescence. Organ tissue samples were prepared from 10- to 12-week-old or 1-year-old (only analysis of pituitary glands for proliferation) mice and placed in formalin for 24 to 48 h. Slides of 5- to 6- μ m sections cut from formalin-fixed paraffin-embedded tissues were deparaffinized and stained with hematoxylin and eosin (Dako), dehydrated, and then covered with a coverslip. Ki67 staining was done with a specific monoclonal Ki67 antibody (Lab Vision Corp.). Assessment of senescence was performed by using a β -galactosidase staining kit according to the manufacturer's protocol (Cell Signaling).

Statistical methods. Statistical significance was determined by a Student *t* test.

RESULTS

Cks1 loss results in $p27^{Kip1}$ -independent cell cycle phenotypes.

The role of Cks1 (Cks1b in humans) has thus far been recognized as the major rate-limiting component of the SCF^{Skp2} complex that mediates binding to T187-phosphorylated $p27^{Kip1}$ and its subsequent proteasomal degradation (8, 14, 46). Asynchronously growing primary early-passage *Cks1*^{-/-} MEFs show a significant increase of cells in G_1 and G_2 /M phase and a reduction of cells in S phase as assessed by BrdU labeling (Fig. 1A). These cell cycle alterations were associated with an accumulation of $p27^{Kip1}$ (Fig. 1B) (46), which could be only marginally enhanced by treatment with the proteasome inhibitor MG132 in *Cks1*^{-/-} MEFs (Fig. 1B). To test whether high levels of $p27^{Kip1}$ are the cause for the reduced proliferation of *Cks1*^{-/-} MEFs, we ectopically expressed $p27^{Kip1}$ or the T187A- $p27^{Kip1}$ mutant (which is not targeted by SCF^{Skp2} ubiquitin ligase [30]) in wild-type (wt) and *Cks1*^{-/-} primary early-passage MEFs (Fig. 1C). Enforced $p27^{Kip1}$ (or T187A- $p27^{Kip1}$) expression did not alter the percentage of *Cks1*^{-/-} MEFs in S phase, while wt MEFs were clearly affected (Fig. 1D). Surprisingly, the extent of S phase reduction caused by $p27^{Kip1}$ in *Cks1*^{+/+}

FIG 1 The effects of *Cks1* loss differ from ectopic p27^{Kip1} expression. (A) The percentage of primary asynchronously growing MEFs of the indicated genotype in the G₁, S, and G₂/M phases was assessed by flow cytometry (BrdU incorporation, 7-AAD staining). Bars represent the mean of three independent experiments $\pm$ the standard error of the mean (SEM). (B) Immunoblot analysis of MEFs of the indicated *Cks1* genotype for p27^{Kip1} expression with or without 5 h of incubation with proteasome inhibitor MG132. (C) Ectopic expression of p27^{Kip1} and the mutant T187A-p27^{Kip1} (T187) in *Cks1*^{+/+} and *Cks1*^{-/-} MEFs as assessed by immunoblotting. (D) Cell cycle assessed by BrdU incorporation. A representative histogram of three independently performed experiments is shown.

MEFs was far lower than expected and did not reach the effect of *Cks1* loss (Fig. 1A).

Concomitant loss of *Cks1* partly rescues the p27^{Kip1}-null phenotype. Loss of *Cks1* results in decreased proliferation and body size (21, 46). To evaluate the functional relevance of p27^{Kip1} accumulation in *Cks1*-null mice *in vivo*, mice lacking both p27^{Kip1} and *Cks1* (129S1/Svlm;C57BL/6 mixed background) were generated. The rate of mice born alive was reduced in the absence of p27^{Kip1}, irrespective of the *Cks1* genotype (Fig. 2A). Furthermore, the infertility of female p27^{Kip1}-null mice (13, 33) was not rescued upon *Cks1* loss (data not shown). Next, male mice were monitored for growth, as determined by an increase in body weight. The body size of p27^{Kip1}-/- mice was significantly increased at all time points (Fig. 2B and C) (13). *Cks1*-null mice showed the expected decrease in body size (Fig. 2B and C) (46). The *Cks1*; p27^{Kip1} double-knockout mice were significantly smaller than their p27^{Kip1}-null littermates and differed only moderately from the wt siblings (Fig. 2B and C). Both spleen and thymus of *Cks1*^{-/-}; p27^{Kip1}-/- mice showed a partial rescue in organ size and did not differ significantly from that of wt mice (Fig. 2D). Mice lacking both p27^{Kip1} and *Skp2* show a comparable body size phenotype (35). Thus, *Cks1*'s role in SCF^{Skp2} function is at least in part responsible for decreased *Cks1*^{-/-} body size. The observation that the additional loss of *Cks1* does not completely recapitulate the p27^{Kip1}-null phenotype may point at *Cks1* functions independent of a role in p27^{Kip1} ubiquitylation and degradation.

***Cks1* is dispensable for p27^{Kip1} tumor suppressor function.** Histological analysis was performed on littermate mice of all genotypes to assess the full extent of changes in the p27^{Kip1} mice upon additional *Cks1* loss. The loss of p27^{Kip1} led to disorganization of the retinal nuclear layers, as described earlier (13, 33),

whereas no abnormalities were observed in *Cks1*-null mice (Fig. 3A). In mice lacking both p27^{Kip1} and *Cks1*, the degree of disorganization of the retinal nuclear layers was clearly attenuated but still evident (Fig. 3A). p27^{Kip1} is a haploinsufficient tumor suppressor and p27^{Kip1}-null animals develop pituitary tumors originating from the intermediate lobe starting at 12 weeks of age (33). We therefore evaluated *Cks1* dependency of the p27^{Kip1} tumor suppressor function. The pituitary glands of 1-year-old wt and *Cks1*^{-/-} mice were macroscopically (Fig. 3B) and histologically (Fig. 3C) normal. The development of pituitary gland hyperplasia was observed in p27^{Kip1}-/- mice and to a comparable extent in *Cks1*^{-/-}; p27^{Kip1}-/- mice. Immunohistochemical analysis applying the proliferation marker Ki67 showed a significantly increased proliferative activity in the pars intermedia of p27^{Kip1}-/- and *Cks1*^{-/-}; p27^{Kip1}-/- mice (Fig. 3D). Thus, there is no evidence that additional loss of *Cks1* results in gross effects on the tumor suppressor function of p27^{Kip1}, at least in a steady-state environment.

***Cks1* prevents p27^{Kip1} accumulation at G₂-M but regulates G₁-S independent of p27^{Kip1}.** To test the hypothesis that *Cks1* has intrinsic functions that are independent of its role in p27^{Kip1} ubiquitylation, we obtained early-passage p27^{Kip1}-/-; *Cks1*^{-/-} and control MEFs and analyzed their proliferative capacity. The growth kinetics of p27^{Kip1}-/- cells were comparable to wt cells, whereas *Cks1*^{-/-} cells showed profoundly impaired proliferation. Unexpectedly, the proliferation of *Cks1*^{-/-}; p27^{Kip1}-/- cells resembled that of *Cks1*^{-/-} cells (Fig. 4A). To further assess the consequences of p27^{Kip1} regulation by *Cks1* in different stages of the cell cycle, we performed two-dimensional flow cytometric analyses of BrdU-pulsed MEFs (Fig. 4B and C). There was no significant difference between wt and p27^{Kip1}-/- cells, and loss of *Cks1* led to a profound reduction of S phase cells and an increased percentage

FIG 2 Partial rescue of the *p27*^{Kip1}-null size phenotype by concomitant *Cks1* loss. (A) Rates for mice of the indicated genotypes born alive compared to the expected Mendelian distribution. (B) Representative *p27*^{Kip1}^{-/-}, wild-type, *p27*^{Kip1}^{-/-}; *Cks1*^{-/-}, and *Cks1*^{-/-} mice depicting size differences. (C) Development of body weight in *p27*^{Kip1}^{-/-}; *Cks1*^{+/+} (blue line), wild-type (*p27*^{Kip1}^{+/+}; *Cks1*^{+/+}, black line), *p27*^{Kip1}^{-/-}; *Cks1*^{-/-} (red line) and *p27*^{Kip1}^{+/+}; *Cks1*^{-/-} (green line) mice. Each time point indicates the mean body weight $\pm$ the SEM assessed in 4 male mice of each genotype. (D) Mean maximum diameter $\pm$ the SEM of the spleen and thymus from 10- to 12-week-old mice of the indicated genotype (*n* = 6 each). Asterisks indicate significant differences.

of cells in G₂/M. The reduction of S phase observed in *Cks1*^{-/-} cells was not reversible upon simultaneous loss of *p27*^{Kip1}, while G₂/M accumulation was clearly absent in *Cks1*^{-/-}; *p27*^{Kip1}^{-/-} MEFs (Fig. 4B and C). Since MEFs are very sensitive to oxidative stress, the experiments were repeated under low atmospheric oxygen conditions when most MEFs are immortal and can be cultivated to a high passage number (41). Under these conditions, the same phenotype was observed, thus largely excluding premature senescence due to oxidative stress as a cause of the observed phenotype (Fig. 4D and E). Importantly, ectopic reconstitution of *Cks1* expression (Fig. 4F) rescued the decreased proliferation rate as measured by BrdU incorporation (Fig. 4G), thus indicating that *Cks1*^{-/-} MEFs have no additional inherent proliferative deficiency.

To further exclude compensatory effects evolving in primary *Cks1*-null and *Cks1*^{-/-}; *p27*^{Kip1}^{-/-} MEFs, we used shRNA-mediated stable knockdown of Cks1 in wt and *p27*^{Kip1}^{-/-} MEFs, resulting in acute loss of Cks1. Cks1 knockdown using two different shRNA constructs resulted in strong and reproducible protein suppression in early-passage *Cks1* wt *p27*^{Kip1}^{+/+} MEFs (Fig. 5A, left panel) and in *Cks1* wt *p27*^{Kip1}^{-/-} MEFs (Fig. 5B, left panel). The same shRNA constructs produced a strong reduction of cells in S phase in both *p27*^{Kip1}^{+/+} (Fig. 5A, right panel) and, to a slightly lesser extent, in *p27*^{Kip1}^{-/-} MEFs (Fig. 5B, right panel).

Previously, *Cks1*^{-/-} MEFs have been reported to show a senescence phenotype (46). To address whether the reduced proliferation of *Cks1*-null cells is associated with premature senescence we first investigated NIH 3T3 fibroblasts. Acute reduction of Cks1 expression using the established shRNA plasmids resulted in the same proliferative defects in immortalized fibroblasts (Fig. 5C). Moreover, Cks1 depletion caused this effect irrespective of accumulation of *p27*^{Kip1}, since it was also present in immortalized fibroblasts we generated from *Cks1* wt *p27*^{Kip1}^{-/-} MEFs (Fig. 5D). To further test in-depth whether premature cellular senescence caused by *p27*^{Kip1} accumulation (11) accounts for the impaired proliferation in *Cks1*^{-/-}; *p27*^{Kip1}^{-/-} MEFs, we performed β -galactosidase staining in passage 6 MEFs (Fig. 6). Only *Cks1*^{-/-}; *p27*^{Kip1}^{+/+} MEFs revealed a highly elevated fraction of senescent cells, whereas concomitant loss of *p27*^{Kip1} completely reversed that phenotype, suggesting that the *p27*^{Kip1} accumulation is causative for the senescence phenotype.

To analyze the kinetics of cell cycle progression and the percentage of cycling cells in synchronized cell populations, we used NIH 3T3 cells that stably expressed *Cks1* shRNA (see Fig. 5C) and released cells from a G₁/S block. Notably, the percentage of cells progressing through the cell cycle was reduced in the absence of Cks1 (Fig. 7A). A clearly higher percentage of control cells entered S phase and reached the G₂-M transition, while a significant proportion of cells lacking Cks1 remained in G₁ phase of the cell cycle

FIG 3 Cks1 is dispensable for p27^{Kip1} tumor suppressor function. (A) Representative histological sections of hematoxylin-and-eosin (HE)-stained retinæ derived from 10- to 12-week-old mice of the indicated genotypes. Arrows display disorganization of nuclear layers. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. The mean number of cells per infiltrate was 25 in *p27^{Kip1}-/-* retinæ versus 5 cells per infiltrate in *p27^{Kip1}-/-*; *Cks1^{-/-}* retinæ. (B) Representative photographs of pituitary glands from 1-year-old mice show enlargement upon loss of p27^{Kip1}. (C) HE staining of representative histological sections from pituitary glands of 1-year-old mice showing hyperplastic nodules in *p27^{Kip1}-/-*; *Cks1^{+/+}* mice and *p27^{Kip1}-/-*; *Cks1^{-/-}* mice. (D) Ki67 staining of the Pars intermedia of the pituitary gland shows increased proliferative activity upon p27^{Kip1} loss independent of the *Cks1* status. The lower panel shows the quantification of Ki67 positivity in the pituitary glands of mice of the indicated genotype. The differences between wt and *p27^{Kip1}-/-* mice, and as well as *p27^{Kip1}-/-*; *Cks1^{-/-}* mice, are statistically significant. *, *P* < 0.05.

(Fig. 7B). This phenotype was associated with a reduced turnover of cyclin A and cyclin E in the absence of Cks1. Importantly, the defective S phase entry was not caused by early accumulation of the SCF^{Skp2}-regulated cell cycle regulators p27^{Kip1} or p57^{Kip2}, whereas an increase in p21^{Cip1} and p130 was observed (Fig. 7C). To further establish that the observed proliferative defect is associated with the entry into S phase, we analyzed BrdU uptake after release from a double thymidine block and confirmed the above results (Fig. 7D). Taken together, our data imply a role for Cks1 in S phase entry and show that the proliferative defect manifest in *Cks1^{-/-}* MEFs is not simply caused by p27^{Kip1} accumulation or premature senescence.

Cks1 drives cell proliferation independent of SCF^{Skp2} targets. In addition to p27^{Kip1}, other cyclin-CDK inhibitors, including p21^{Cip1} (6, 54), p57^{Kip2} (20), and p130 (5, 48), have been shown to be targeted to the proteasome via SCF^{Skp2}. We therefore analyzed whether accumulation of these cell cycle inhibitors could be responsible for the herein reported proliferation defects caused by *Cks1* deficiency. Immunoblot analysis of asynchronously growing early-passage MEFs showed elevation of the p130 protein level in cells lacking Cks1, whereas p21^{Cip1} accumulation was abolished in the absence of p27^{Kip1} and p57^{Kip2} was not affected (Fig. 8A). p130 negatively regulates proliferation by controlling the G₁-S transition and by inhibiting the activity of Cdk2-cyclin E and A com-

FIG 4 Cks1 prevents *p27*^{Kip1} accumulation at G₂-M but regulates G₁-S independent of *p27*^{Kip1}. (A) *Cks1*^{-/-} MEFs show defective proliferation irrespective of *p27*^{Kip1} status. A growth curve of primary early-passage MEFs was determined. The cells were counted by trypan blue exclusion at the indicated times. Shown is the mean cell number $\pm$ the SEM ($n = 4$ for each genotype). (B) Cell cycle assessment of asynchronously growing MEFs of the indicated genotypes by flow cytometry. Shown are the results of a representative analysis of BrdU incorporation and DNA content. (C) Quantification of cells in each phase of the cell cycle (mean $\pm$ the SEM; $n = 2$ for each genotype). Asterisks indicate significant differences at $P < 0.05$. (D) MEFs of the indicated genotype were cultivated in 3% atmospheric oxygen. The results of a cell cycle assessment of asynchronously growing MEFs by flow cytometry are shown. (E) Quantification of cells in each phase of the cell cycle (mean $\pm$ the SEM; $n = 3$ each genotype) that were grown as described in panel D. *, $P < 0.05$. (F and G) Immunoblotting (F) and flow cytometry (G) upon reexpression of Cks1 show rescued proliferation assessed by BrdU incorporation.

plexes (12, 25). To investigate whether Cks1 controls proliferation independent of the inhibitory function of p130, NIH 3T3 cells were infected with shRNA viruses targeting *Cks1* and *p130* (Fig. 8B). There was no complete rescue of proliferation upon simultaneous knockdown (Fig. 8B, right panel). Moreover, significantly reduced proliferation was observed in primary early-passage wt and *p27*^{Kip1}^{-/-} MEFs upon acute suppression of both *Cks1* and *p130* (Fig. 8C). *p21*^{Cip1} is a universal inhibitor of Cdk activity (17). Although *p21*^{Cip1} did not strongly accumulate in *Cks1*^{-/-}; *p27*^{Kip1}^{-/-} MEFs, a change in expression of

Cdks or their respective active complexes could shift the *p21*^{Cip1}/CDK ratio above the inhibitory threshold of *p21*^{Cip1} (44). We therefore addressed the moderate accumulation of *p21*^{Cip1} (Fig. 8A) to further substantiate a role for Cks1 independent of CKI degradation. Indeed, *Cks1* depletion resulted in decreased proliferation in both *p21*^{Cip1}^{-/-} MEFs and *p21*^{Cip1}^{-/-}; *p27*^{Kip1}^{-/-} MEFs (Fig. 8D), suggesting that neither *p27*^{Kip1} nor *p21*^{Cip1} accumulation is responsible for the observed *Cks1* phenotype. Taken together, our results indicate that the defect in S phase entry and proliferation established

FIG 5 Acute loss of *Cks1* leads to defective growth in primary MEFs and in immortalized fibroblasts. (A) Acute *Cks1* depletion with two different shRNA constructs in *Cks1*^{+/+}; *p27*^{+/+} MEFs. Immunoblotting is shown in the left panel; quantification of the BrdU-positive cell fraction is shown in the right panel ($n = 4$ independent experiments; bars represent the mean $\pm$ the SEM). Single asterisks indicate significant differences at $P < 0.05$; **, $P < 0.01$. (B) *Cks1* knockdown in *p27*^{-/-} MEFs. Immunoblotting is shown in the left panel; quantification of the BrdU-positive cell fraction is shown in the right panel ($n = 4$ independent experiments). (C) NIH 3T3 murine fibroblasts were infected with shRNA for *Cks1*. Shown is the protein expression (left panel) and the fraction of BrdU-positive cells (right panel) for three independent experiments; *, $P < 0.01$. (D) *p27*^{-/-} MEFs were immortalized according to the 3T3 protocol, and passage 50 cells were infected with shRNA for *Cks1*. Shown is the protein expression (left panel) and the quantification of BrdU-positive immortal *p27*^{-/-} passage 50 fibroblasts (right panel, $n = 2$).

in *Cks1*^{-/-} MEFs may at least partly be independent of the SCF^{Skp2}-regulated cell cycle inhibitors *p21*^{Cip1}, *p27*^{Kip1}, *p57*^{Kip2}, and *p130*.

Consequences of *Cks1* loss on Cdk expression and activity. To further assess the function of *Cks1* at the G₁-S transition we analyzed RNA and protein levels of different cyclins and Cdks in asynchronously growing MEFs. For most of the analyzed candidate genes, there was no significant difference in mRNA (Fig. 9A) or protein expression (Fig. 9B) among the four genotypes. Of all proteins analyzed, Cdk1 showed the most significant alterations in expression upon *Cks1* loss. *Cdk1* (as well as *cyclin A* and *cyclin B*)

mRNA levels were reversed to near normal in *Cks1*^{-/-}; *p27*^{Kip1}^{-/-} MEFs, suggesting that *Cks1* mediated effects on transcription are indirect and caused by *p27*^{Kip1} accumulation (Fig. 9A). In contrast, Cdk1 protein levels were reduced in *Cks1*^{-/-} cells irrespective of *p27*^{Kip1} status (Fig. 9B). *Cks1* is a binding partner of Cdk1 and Cdk2 (2, 7, 15, 18). We therefore additionally evaluated the impact of *Cks1* on Cdk- and cyclin-associated kinase activities. Although Cdk1 protein levels were reduced in *Cks1*^{-/-} MEFs irrespective of the *p27*^{Kip1} status (Fig. 9B), the ability of Cdk1 complexes to phosphorylate histone H1 was unaltered in *Cks1*^{-/-} compared to *Cks1*^{+/+} MEFs (Fig. 9C and D). In striking contrast,

FIG 6 Senescence in *Cks1*^{-/-} MEFs is caused by *p27*^{Kip1} accumulation. (A) β -Galactosidase staining of passage 6 MEFs. Various senescent cells are labeled by arrows. Representative pictures are shown. (B) Quantification of senescent cells. Bars indicate the mean $\pm$ the SEM from three independent experiments. ***, $P < 0.001$.

FIG 7 Loss of Cks1 reduces the fraction of cells entering the S phase of the cell cycle. NIH 3T3 cells were infected with virus bearing shRNA constructs directed against *Cks1* (shCks1) or empty plasmid (control). (A) The cells were then released from a G₁/S block (serum starvation and aphidicholin treatment). Analysis of DNA content at the indicated time points following the release was performed. Shown are the results of a representative experiment. (B) Percentage of cells in the G₁, S, or G₂/M phase at the indicated time points. Shown are the results (mean $\pm$ the SEM) from $n =$ three independent experiments. *, $P < 0.05$. (C) Immunoblot analysis of the indicated proteins at each given time point. (D) The cells described above were released from a double thymidine block. BrdU uptake was detected by flow cytometry at the indicated time points. Shown is the quantification of two independently performed experiments.

Cdk2 immunoprecipitated from *Cks1*^{-/-} MEFs showed significantly decreased Histone H1 phosphorylation activity. The decrease in kinase activity was rather cyclin A- than cyclin E-associated and not rescued in *p27*^{Kip1}-deficient MEFs (Fig. 9C and D). To analyze whether the reduced Cdk2 activity observed was associated with changes in inhibitory phosphorylation, we next evaluated the inhibitory threonine 15 (Y15) phosphorylation of Cdk1 and Cdk2. In *Cks1*^{-/-} cells the Cdk1 and Cdk2 phosphorylation at Y15 was not increased (Fig. 9E). In immortalized NIH 3T3 fibroblasts acute loss of Cks1 results in decreased Cdk1 protein level and Cdk2 kinase activity, comparable to primary MEFs (Fig. 9F). Again, inhibitory Y15 phosphorylation was not increased upon loss of Cks1. The observed defect in S phase entry of cells lacking Cks1 may thus be attributable to the combined loss of Cdk1 protein and Cdk2 activity.

DISCUSSION

Accumulation of p27^{Kip1} at the end of G₁ has been shown to inhibit G₁-S transition (23), and *Skp2* loss was associated with minor

proliferative defects that are rescued upon *p27*^{Kip1} deletion (35). If the main role of Cks1 was to regulate p27^{Kip1} levels at the end of G₁, then additional loss of *p27*^{Kip1} should have rescued defective proliferation of *Cks1*^{-/-} MEFs, as has been observed in cells that lack SCF^{Skp2} activity and *p27*^{Kip1} expression (35). Our data therefore provide evidence that an additional level of Cks1-dependent regulation of the G₁-S transition must exist. p27^{Kip1} is the principal downstream effector of SCF^{Skp2} that inhibits Cdk function at the G₁-S and G₂-M transitions. Consequently, disruption of p27^{Kip1} has been associated with an endoreduplication phenotype (36, 39). A similar phenotype has recently been reported in *p27*^{Kip1}^{-/-}; *Skp2*^{-/-} cells (23, 35). Therefore, Cks1 appears to collaborate with Skp2 in mediating p27^{Kip1} degradation during G₂-M. This suggests an essential function of the SCF^{Skp2}/Cks1-p27^{Kip1} axis to promote cyclin-Cdk1 activity. Consistent with this observation, and supporting the role of SCF^{Skp2}-mediated p27^{Kip1} control, we show that simultaneous loss of *p27*^{Kip1} reverses *Cks1* deficiency at G₂-M.

In contrast to its role at the G₂-M transition, our data and the

FIG 8 Cks1 promotes cell proliferation independent of p21^{Cip1}, p27^{Kip1}, p57^{Kip2}, and p130. (A) Protein expression of p21^{Cip1}, p27^{Kip1}, p57^{Kip2}, and p130 in asynchronously growing MEFs of the indicated genotypes assessed by immunoblotting. (B) Combined shRNA-mediated knockdown (*p130* and *Cks1*) in NIH 3T3 cells. Immunoblot analysis for protein expression (left panel) was carried out. The percentage of BrdU-positive cells was determined (right panel, $n = 3$ independent experiments; *, $P < 0.05$). (C) shRNA-mediated knockdown of the indicated genes in early-passage p27^{Kip1}+/+ and p27^{Kip1}-/- MEFs. Immunoblot analysis for protein expression (left panel) was performed. The percentages of BrdU-positive cells (right panel, $n = 2$ independent experiments; **, $P < 0.01$) were determined. (D) *Cks1* shRNA knockdown in early-passage p21^{-/-} and p21^{-/-}; p27^{-/-} MEFs. Protein expression (left panel) and BrdU flow cytometry analysis (right panel) were performed.

results of others indicate that the function of Cks1 in the regulation of the G₁-S transition is multilayered (1, 4, 19). Considering that the effect of *Cks1* loss in MEFs, in contrast to *Cdk2* or *Skp2* deficiency (1, 27, 34), was not reverted at G₁-S in the absence of p27^{Kip1}, crucial targets and/or functions of Cks1 were likely unmasked in our genetic approach. Although we experimentally addressed the most likely candidate cell cycle inhibitors that are established targets of SCF^{Skp2} next to p27^{Kip1} (5, 6, 20, 48, 54), our analyses cannot fully exclude an important compound effect of Cks1 in SCF^{Skp2} function with regard to p21^{Cip1}, p27^{Kip1}, p57^{Kip2}, and p130 degradation, since redundant functions of cell cycle inhibitory proteins are a known phenomenon. For example, p130 can inhibit cyclin E/Cdk2 activity in serum-starved p21^{Cip1}-/-; p27^{Kip1}-/- double-knockout MEFs (10). Nevertheless, we provide data suggesting that Cks1 promotes proliferation by other means in single p21^{Cip1}-, p27^{Kip1}-, and p130-deficient or knockdown cells and even in cells doubly deficient for p21^{Cip1}/p27^{Kip1} and p27^{Kip1}/p130. Importantly, the senescence phenotype associated with *Cks1* loss observed in the present study and a previous report (46) is reverted upon p27^{Kip1} deficiency, but this cannot rescue defective proliferation. Also, the low proliferative phenotype of *Cks1*^{-/-} MEFs is preserved when cultured in low atmospheric

oxygen conditions where most MEFs are immortal and can be cultivated to a high passage number (41), irrespective of the p27^{Kip1} status.

Cks1 is a binding partner of Cdk1 and Cdk2 (2, 7, 15, 18). The ability of Cks1 complexes to phosphorylate Histone H1 was unaltered in *Cks1*^{-/-} MEFs, but *Cks1*-null cells showed significantly reduced Cdk2-associated kinase activity that was also not rescued in p27^{Kip1}-deficient MEFs. These data are in accord with recently published results by Liberal et al. (26), who show that Cks1 binding can activate Cdk2 activity and can override inhibitory Y15 phosphorylation. Moreover, others have shown that Cdk2 activity at least in some part is required for normal MEF proliferation (4, 38) and that the expression of a dominant-negative kinase-dead Cdk2 mutant causes G₁ arrest (50). *Cdk2*-deficient cells are most likely rescued by compensatory binding of Cdk1 to cyclin E, which can promote G₁-S transition in the absence of *Cdk2* (1, 38). The observed defect in S phase entry of cells lacking *Cks1* in our study could thus possibly be attributed to the combined reduction of Cdk1 protein and Cdk2 activity. One surprising earlier finding was that loss of Cks1, but not Skp2, significantly delays Myc-induced lymphomagenesis in the same disease model (21, 37), although both genetic events lead to comparable p27^{Kip1} accumu-

FIG 9 Cks1 effects on transcript and protein levels of cell cycle regulators unmasked in *p27*-null cells. (A) Real-time PCR analysis of transcript levels in asynchronously growing MEFs. Shown is the mean relative expression $\pm$ the SEM. Values are normalized to the expression of ubiquitin (*ub*), with the wt control set as 1. At least three embryos per genotype were used to generate MEFs and analyzed separately. (B) Immunoblot analysis of the indicated proteins from asynchronously growing MEFs. Protein expression of two different embryos of each genotype is shown. (C) Kinase activities of Cdk1, Cdk2, cyclin A, and cyclin E complexes. Immunoprecipitates from asynchronously growing MEFs were assessed for their histone H1 phosphorylation activity. Shown are the results of one representative experiment. (D) Quantification of kinase activity. Shown are the means $\pm$ the SEM of three independently performed experiments. Asterisks indicate $P < 0.05$. (E) The inhibitory phosphorylation of Cdk1 and Cdk2 at tyrosin 15 (P-Y15-Cdk1 and P-Y15-Cdk2) was assessed by immunoblotting with specific antibodies. (F) Assessment of Cdk1 and Cdk2 protein level, inhibitory phosphorylation, and kinase activity in immortal NIH 3T3 cells following shCks1 knockdown.

lation in the premalignant cells. This finding may indirectly indicate oncogenic functions of Cks1 besides suppression of $p27^{Kip1}$. $p27^{Kip1}$ is an established tumor suppressor (9, 28, 45), and $p27^{Kip1}$ -deficient mice develop pituitary gland tumors (13, 33). The $Cks1^{-/-}; p27^{Kip1}/-$ pituitary gland phenotype of older mice shown in our study resembles the one observed upon simultaneous $p27^{Kip1}$ and *Skp2* loss (35), as well as the one seen in

$p27^{Kip1}/-; Cdk2^{-/-}$ pituitary glands (27). Our data thus support the conclusions that $p27^{Kip1}$ is the target of SCF^{Skp2} that hinders pituitary gland hyperplasia, although a moderate reduction of proliferative activity in the pituitary gland of $p27^{Kip1}$ -null mice that concomitantly lack *Cks1* was evident.

In summary, our data favor a model where, at least in some tissues, Cks1 is important for regulating Cdk1 expression levels

and Cdk2 kinase activity. Our genetic approach shows that the function of Cks1 at the G₁/S phase transition is predominantly independent of the SCF^{Skp2} complex and p27^{Kip1}. On the contrary, mitotic entry defects and premature senescence manifest in *Cks1*^{-/-} cells are p27^{Kip1} dependent. Collectively, our findings establish p27^{Kip1}-independent functions of Cks1 in regulating S phase entry and proliferation.

ACKNOWLEDGMENTS

We thank Sabine Stritzke and Claudia Kloss for expert technical assistance and the Zentrum Präklinische Forschung (TU München) for animal care. We acknowledge Matthias Schiemann (Department of Microbiology, Technische Universität München) for help with flow cytometric cell sorting. We are indebted to Martine Roussel (St. Jude Children's Research Hospital, Memphis, TN) for providing *p21*^{-/-} and *p27*^{-/-}; *p21*^{-/-} MEFs.

This study was supported by the Deutsche Forschungsgemeinschaft (SFB TRR54, project C5 to U.K.) and by an Emmy Noether program grant to F.B.

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