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Cyclin-Dependent Kinase-Associated Proteins Cks1 and Cks2 Are Essential during Early Embryogenesis and for Cell Cycle Progression in Somatic Cells[▽]

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Cks proteins associate with cyclin-dependent kinases and have therefore been assumed to play a direct role in cell cycle regulation. Mammals have two paralogs, Cks1 and Cks2, and individually deleting the gene encoding either in the mouse has previously been shown not to impact viability. In this study we show that simultaneously disrupting *CKS1* and *CKS2* leads to embryonic lethality, with embryos dying at or before the morula stage after only two to four cell division cycles. RNA interference (RNAi)-mediated silencing of *CKS* genes in mouse embryonic fibroblasts (MEFs) or HeLa cells causes cessation of proliferation. In MEFs *CKS* silencing leads to cell cycle arrest in G_2, followed by rereplication and polyploidy. This phenotype can be attributed to impaired transcription of the *CCNB1*, *CCNA2*, and *CDK1* genes, encoding cyclin B1, cyclin A, and Cdk1, respectively. Restoration of cyclin B1 expression rescues the cell cycle arrest phenotype conferred by RNAi-mediated Cks protein depletion. Consistent with a direct role in transcription, Cks2 is recruited to chromatin in general and to the promoter regions and open reading frames of genes requiring Cks function with a cell cycle periodicity that correlates with their transcription.

Cks proteins bind a subset of cyclin-dependent kinases (Cdks) with high affinity at a position remote from the ATP and cyclin binding sites (2), but in contrast to cyclins they are not required for the general activation of the Cdk kinase activity (63). Loss of Cks function leads to mitotic defects in the yeast species *Saccharomyces cerevisiae* (63) and *Schizosaccharomyces pombe* (20) as well as in *Xenopus* eggs (48) and *Caenorhabditis elegans* (53). Studies with *Xenopus* egg extracts have shown that the Cks protein is required for the optimal phosphorylation of the mitotic regulators Cdc25, Cdc27, Myt1, and Wee1 by Cdk1/cyclin B without changing the overall Cdk1 kinase activity (49, 50). More recently, it has been shown that Cks proteins are required for optimal preanaphase ubiquitylation and degradation of cyclin A in mammalian cells (69), although this function does not appear to be essential for viability. On the other hand, the critical defect in yeast cells lacking Cks1 has been traced to their inability to induce the transcript encoding Cdc20, an essential positive regulator of mitosis (38). It has been shown that Cks1 is required for the essential recruitment of proteasomes to chromosomal sites of active *CDC20* transcription (38). More recently, this function has been generalized to the expression of a significant fraction of yeast genes (72). Interestingly, Cdk1 is an essential cofactor in this process, although its kinase activity is not required (72).

Cks proteins are evolutionarily conserved in eukaryotes. Yeasts contain only one Cks protein-encoding gene (*CKS1* in budding yeast and *suc1*$^+$ in fission yeast), whereas vertebrates have two paralogs, *CKS1* and *CKS2* (55), whose protein products are 81% identical in both humans and mice. Both genes have previously been disrupted in the mouse, and animals nullizygous for either gene are viable (59, 60). Mice nullizygous for *CKS1* are smaller than wild-type littermates but appear otherwise normal (59). This phenotype has been traced to a direct role for Cks1 as a cofactor for the protein-ubiquitin ligase SCF^{Skp2}, resulting in a deficiency in the degradation of the Cdk inhibitors $p27^{Kip1}$, $p21^{Cip1}$, and p130 in *CKS1*$^{-/-}$ animals (16, 19, 59). This function is specific for Cks1, since SCF^{Skp2} activity is not affected in *CKS2*$^{-/-}$ mice. The only reported phenotype associated with these animals is sterility of both males and females due to failure of germ cells to progress past the first meiotic metaphase (60). Interestingly, the sterility phenotype can be attributed to the germ line exclusion of *CKS1* expression because the metaphase I arrest of *CKS2*$^{-/-}$ oocytes can be rescued by microinjection of *CKS1* mRNA (60). This result suggests that the Cks1 and Cks2 proteins share one or more redundant functions, which is supported by the observation that both human *CKS1* and *CKS2* can complement a disruption of the single *CKS1* gene in budding yeast (55).

We have undertaken two approaches to determine if Cks1 and Cks2 share a redundant function in mammalian cells. We have investigated the phenotype of *CKS1*$^{-/-}$ *CKS2*$^{-/-}$ doubly nullizygous mice and depleted mouse and human cell lines of Cks1 and Cks2 protein by small interfering RNA (siRNA)-mediated gene silencing. Doubly nullizygous mice die before implantation at the morula stage, suggesting a critical role at an extremely early point in embryogenesis. Consistent with an essential and fundamental role for Cks proteins in mammalian

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cells, cell lines depleted of Cks1 and Cks2 by RNA interference (RNAi) cease proliferation.

MATERIALS AND METHODS

Embryo collection. Embryos were isolated from intercrosses between *Cks1*$^{-/-}$ *Cks2*$^{+/-}$ females and males as described previously (23). Embryonic day 13.5 (E13.5) and E8.5 embryos were surgically removed from the uterus, and E8.5 embryos were subsequently dissected manually from the deciduas. For the isolation of blastocysts and morulae, females were superovulated by injection of 12 IU of pregnant mare serum gonadotropin, followed by injection of 7.5 IU of human chorionic gonadotropin 48 h later. The embryos were flushed from the uterus at between 3.5 and 4.5 days postcoitum. Morulae were cultured in EmbryoMax potassium simplex optimized medium plus amino acids with D-glucose (Specialty Media), and blastocysts were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 15% fetal bovine serum (Gemini Bioproducts), 100 μM β-mercaptoethanol, 2 mM glutamine, 100 μM nonessential amino acids, 100 U/ml penicillin, and 100 U/ml streptomycin (Invitrogen) in a humidified incubator with a 5% CO_2-95% air mixture at 37°C. Embryos were photographed at 40× magnification with a Nikon D100 camera connected to a Nikon Diaphot microscope.

Genotyping. Genomic DNA was isolated from tail clips of E13.5 and E8.5 embryos (12), and the *CKS1* and *CKS2* genotypes were determined by PCR as described previously (59, 60). Blastocysts and morulae were washed three times in threefold-concentrated PCR buffer and digested with 45 μg/ml proteinase K for 90 min. After heat inactivation of proteinase K at 94°C for 30 min, the early embryos were genotyped by a nested PCR approach using primers BG47 (CTG TGGTTTCCAAATGTGTCA), BG71 (ATTCAAATCCAGAGCGTTGGGC), and BG72 (CCCGCAACACTACACAAAGCAA) in the first reaction. A fraction (1/25) of the first PCR mixture was added to the second PCR mixture, which was run with either primers BG48 (GTACTCGTAGTGCTCATCGAA) and BG49 (TTGGCCTCATTCGAGTCGTT) to amplify the *CKS2* wild-type sequence or primers BG65 (CAAATGTGTCAGTTTCATAGC) and BG67 (TC ATTCGAGTCGTTGCGTCGG) to amplify the *CKS2* deletion construct.

Cells and culture conditions. *CKS1*$^{-/-}$ *CKS2*$^{+/-}$ and *CKS1*$^{+/-}$ *CKS2*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were cultured from E13.5 embryos and immortalized by infection with an ecotropic retroviral vector expressing the human papillomavirus E6 oncoprotein (6). The retroviral particles were generated using the 293T-derived packaging cell line Phoenix-Eco (30). MEFs, HeLa cells, and HEK293A cells were grown in DMEM supplemented with 10% newborn calf serum (Gemini Bioproducts), 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin (Invitrogen) in a humidified incubator with 5% CO_2-95% air mixture at 37°C.

RNAi and cyclin B1 expression. siRNA duplexes were synthesized by Dharmacon. To silence mouse or human *Cks1* mRNA or *Cks2* mRNA, Dharmacon SMARTpools were used. siRNA designed to target *GFP* mRNA (CAAGCUG ACCCUGAAGUUC) was used as a control. Cells were transfected using Lipofectamine 2000 (Invitrogen Corporation) according to the protocol provided by the manufacturer. A final concentration of 42 nM was used for MEFs and 10.5 nM for HeLa cells. Cells were transfected 3 days and 1 day before being assayed. In some HeLa cell silencing experiments, short hairpin RNA (shRNA)-expressing adenoviruses based on a Mir30 microRNA backbone (46) were used instead of siRNAs. The cyclin B-expressing adenovirus utilized a tetracycline-regulated promoter, and therefore expression required a second adenovirus expressing the tetracycline transactivator (tTA) (27). For cyclin B1 bypass experiments, HeLa cells were split and 60,000 cells per plate were seeded in 6-cm petri dishes. On day 0 one triplicate set of plates was trypsinized and counted. The remaining cells were transduced with the following sets of adenoviruses: (i) empty vector control and tTA-expressing adenoviruses; (ii) empty vector control and tetracycline operator-cyclin B1- and tTA-expressing adenoviruses; (iii) empty vector control and tTA-, shRNA targeting Cks1 (shCks1)-, and shCks2-expressing adenoviruses; or (iv) tetracycline-cyclin B1-, tTA-, shCks1-, and shCks2-expressing adenoviruses. Transductions was carried out in DMEM supplemented with L-glutamine and antibiotics but without serum. After 2 hours DMEM with L-glutamine, antibiotics, and 20% newborn calf serum was added to achieve a final serum concentration of 10%. Total numbers of virus particles were normalized for every transduction using the empty vector. On day 1, one set of triplicates for each virus combination was counted and the remaining cells were transduced a second time as described above. On both day 2 and day 3 one set of triplicates was counted for each of the virus combinations. Relative cell numbers were normalized to the control-shCks population (set iii above), which was set at a value of 1.0 throughout the time course. This adjustment was necessary because, after two consecutive days of adenoviral transduction, it became impossible to trypsinize and count the cells without a systematic loss due their fragility, particularly on day 3, when we could easily observe cell number increases on plates, prior to harvesting, which were not adequately reflected in the postharvest counts. This was reflected in all populations independently of which viruses they received. The same result was obtained in three independent experiments.

Real-time PCR. Total RNA was extracted from cells using RNeasy columns (Qiagen) according to the manufacturer's instructions. Total RNA (5 μg/ml) was reverse transcribed, amplified, and quantified using iQ Sybr green Supermix (Bio-Rad) and a Chromo4 real-time PCR detector (Bio-Rad). Each sample was run at least in triplicate. Primers used for amplification of all mRNAs can be obtained upon request. The expression levels of all analyzed mRNAs were determined relative to that of a control mRNA, using the formula $2^{CT(\text{control mRNA}) - CT(\text{mRNA of interest})} \cdot 10{,}000$, where CT is the threshold cycle. β-Actin, γ-actin, or GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNAs were used as control mRNAs. Standard deviations (SD) of the relative expression values were calculated taking error propagation into account with the formula $\{[2^{-CT(\text{mRNA of interest})} \cdot \ln 2 \cdot 2^{CT(\text{control mRNA})}]^2 \cdot SD_{CT(\text{control mRNA})}{}^2 + [2^{CT(\text{control mRNA})} \cdot \ln 2 \cdot 2^{-CT(\text{mRNA of interest})}]^2 \cdot SD_{CT(\text{mRNA of interest})}{}^2\}^{1/2} \cdot 10{,}000$.

Fluorescence imaging. For semiquantitative analysis of the DAPI (4′,6-diamidino-2-phenylindole) staining, image data of the stained cells were collected using an Axioskop 2 microscope mounted with an AxioCam MRm monochrome charge-coupled device camera (both from Zeiss) coupled to a computer with AxioVision software (Zeiss). A mercury lamp, conventional microscope optics, and selective wavelength filters were used. A Zeiss Plan-Neofluar 63×/1.30-numerical-aperture oil immersion lens was employed to visualize the cells. Images at the DAPI wavelength were captured and analyzed using the AxioVision software. Nuclear segmentation was performed by hand, and the fluorescence intensity and area were measured using the software. For the control sample, cells with normal-size nuclei were imaged and the average of the total fluorescence intensity of the nuclei was normalized. For cells treated with Cks1 or cyclin B1 siRNA, cells judged as having normal-size nuclei and large nuclei, respectively, were imaged and the average intensity of the large nuclei was normalized to the average intensity of the normal-size nuclei (assuming they are equal to the normal-size nuclei of the control sample) to estimate the relative amount of DNA in the large nuclei. Ten to 20 cell nuclei were analyzed for each sample (i.e., 10 to 20 for normal-size nuclei and 10 to 20 for large-size nuclei).

Immunological procedures and reagents. Cell lysates were prepared using a Tris-HCl lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 mM Na PP_i, 50 mM NaF, 50 mM Na_3VO_4, 1 mg/ml leupeptin, 1 mg/ml pepstatin, and 2 mg/ml aprotinin), and protein concentrations were determined using the Bradford protein assay (Bio-Rad). One hundred sixty to 200 μg denatured protein lysates was separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, transferred onto a polyvinylidene difluoride membrane (Immobilon P; Millipore), and immunoblotted with the following antibodies: a monoclonal antibody against cyclin B1 (GNS-1; BD Biosciences Pharmingen), a monoclonal antibody against Cdk sequence PST AIRE (71), a polyclonal antibody against cyclin A2 (22), a polyclonal antibody against cyclin D3 and a monoclonal antibody against cyclin D1 (C-16 and D11; Santa Cruz Biotechnology Inc.), a polyclonal antibody against cyclin E1 (14), and a polyclonal antibody against β-actin (NB 600-503; Novus Biologicals).

Laser-scanning cytometry (LSC). Cells were grown to 50 to 80% confluence on 10-well immunofluorescence slides (Polysciences Inc.), fixed in −20°C methanol overnight, permeabilized in phosphate-buffered saline (PBS) containing 0.05% Triton X-100, and incubated with propidium iodide (PI) staining solution (0.2 mg/ml RNase A, 0.2 mg/ml PI, and 0.1% Triton X-100 in PBS) at 37°C for 45 min. After being mounted in antifading reagent (ProLong Gold; Molecular Probes), the PI-stained cells were analyzed using a CompuCyte laser-scanning cytometer equipped with a 20-mW argon ion air-cooled laser, a 5-mW He-Ne laser, and a DP11 digital camera (Olympus). Scanning was done using the 20× objective, and the PI fluorescence was used as the contouring parameter. DNA histograms were generated based on the DNA content measured in these cells.

Cell fractionation. 293A cells cross-linked for 1 minute in 0.5% formaldehyde were resuspended in 200 μl of ice-cold CSK-TX100 (20 mM HEPES, pH 7.5, 100 mM NaCl, 3 mM $MgCl_2$, 300 mM sucrose, and 0.5% Triton X-100 plus protease and phosphatase inhibitors), incubated on ice for 15 min, and spun at 6,000 rpm for 5 min at 4°C. Supernatant was the soluble fraction. Pellets were washed once in 500 μl of ice-cold CSK-TX100, resuspended in 70 to 100 μl of ice-cold CSK-TX100, sonicated for 10 s using a Braun-Sonic tip sonicator, and centrifuged at 12,000 rpm for 10 min at 4°C. This supernatant was the soluble chromatin fraction.

Chromatin immunoprecipitation. 293A cells expressing Flag-tagged Cks2, as well as nontagged control cells, were grown asynchronously prior to harvest or

TABLE 1. Genotypes and numbers of progeny of *Cks1*$^{-/-}$ *Cks2*$^{+/-}$ intercrosses at different developmental stages

Stage	No. of progeny of genotype:			Total no.	P^b
	Cks1$^{-/-}$ *Cks2*$^{+/+}$	*Cks1*$^{-/-}$ *Cks2*$^{+/-}$	*Cks1*$^{-/-}$ *Cks2*$^{-/-}$		
Weaning	66	122	0	188	2.1E-14
E13.5	4	3	0	7	0.0947
E8.5	9	17	0	26	0.0130
Blastocyst[a]	9	20	0	29	0.0076
Morula[a]	18	18	16	52	0.0790
Egg to 4 cells[a]	3	3	3	9	0.6065

[a] Isolated at E3.5.
[b] Chi-square test.

were treated with thymidine (2 mM) for 24 h. Cells were harvested (S phase) or released from the thymidine block for 8 h and then harvested (G_2). Enrichment in S phase or G_2 was confirmed by fluorescence-activated cell sorter (FACS) analysis. After being harvested, cells were cross-linked by addition of formaldehyde to a final concentration of 0.5% for 10 min at room temperature. Glycine was then added to a final concentration of 125 mM for 5 min at room temperature. Cells were washed twice with ice-cold PBS, and pellets were snap-frozen in liquid nitrogen. Thawed cells were resuspended in 500 μl lysis buffer (50 mM HEPES, pH 7.5, 140 mM NaCl, 1% Triton X-100, 1 mM EDTA, and protease inhibitors), incubated on ice for 15 min, and sonicated 20 times for 30 s each with a Cup-Horn Sonicator 3000, with 2-min intervals between pulses. After centrifugation, lysates were incubated overnight at 4°C with agarose-coupled Flag antibody (Sigma) or RNA polymerase II C-terminal domain repeat antibody (8WG16; Abcam), followed by a 2-h incubation with protein A-coupled Dynabeads. Beads were washed twice with lysis buffer, twice with lysis buffer containing 0.5 M NaCl, twice with wash buffer (10 mM Tris, pH 8.0, 250 mM LiCl, 0.75% NP-40, 0.75% Triton X-100, 1 mM EDTA, and protease inhibitors), and once with TE (10 mM Tris, pH 7.5, 1 mM EDTA). Bound proteins were eluted with 100 μl elution buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 1% sodium dodecyl sulfate) for 30 min at 65°C. DNA-protein cross-links were reversed by overnight incubation at 65°C, and DNA was purified using a QIAquick PCR purification kit (Qiagen), in accordance with the manufacturer's instructions. Real-time PCRs were performed using 1/25 of the immunoprecipitation fraction or 1/500 of the input fraction as the template. Primer sequences are available upon request. The control primers amplify a nontranscribed region on human chromosome 19 (62). The amount of DNA immunoprecipitated as related to the amount of input DNA was calculated for the different genomic regions and then normalized to that for the control region.

RESULTS

Simultaneous disruption of *CKS1* and *CKS2* leads to embryonic lethality. We intercrossed *CKS1*$^{-/-}$ *CKS2*$^{+/-}$ females and males to generate *CKS1*$^{-/-}$ *CKS2*$^{-/-}$ mice. None of the 188 weanlings that were genotyped from this cross lacked both *CKS* genes (Table 1). However, the two other possible *CKS* genotypes obtainable from this cross were recovered at the expected Mendelian ratio (Table 1). To determine whether *CKS1* is haploinsufficient in the CKS2$^{-/-}$ background (not addressable from the cross described above), we intercrossed *CKS1*$^{+/-}$ *CKS2*$^{+/-}$ mice. *CKS1*$^{+/-}$ *CKS2*$^{-/-}$ offspring were easily obtained and viable (data not shown), leading to the conclusion that only the complete absence of both *CKS* genes leads to embryonic death and supporting the conclusion that the Cks1 and Cks2 proteins share a redundant essential function.

***CKS1*$^{-/-}$ *CKS2*$^{-/-}$ mice die at the morula stage.** To determine the time of embryonic death of *CKS* doubly nullizygous mice, we first genotyped postimplantation embryos at E13.5 and E8.5. No *CKS1*$^{-/-}$ *CKS2*$^{-/-}$ embryos could be detected among 7 embryos at E13.5 and 26 embryos at E8.5 (Table 1). However, doubly nullizygous embryos were isolated at E3.5, prior to implantation in the uterine wall. At that time, the embryos were either at the early-blastocyst (32 to 64 cells) or compacted-morula (8 to 32 cells) stage, but we also recovered several two- and four-cell stage embryos as well as what appeared to be a few unfertilized eggs. From this group, 19 *CKS1*$^{-/-}$ *CKS2*$^{-/-}$ embryos were recovered, either as compacted morulae (16 out of 52) or at an even earlier development stage (3 out of 9). No doubly nullizygous embryos were observed among 29 blastocysts that were isolated. When morulae were cultured in vitro, *CKS1*$^{-/-}$ *CKS2*$^{+/+}$ and *CKS1*$^{-/-}$ *CKS2*$^{+/-}$ embryos were able to develop into blastocysts, hatch from the zona pellucida, and attach to the culture dish. *CKS1*$^{-/-}$ *CKS2*$^{-/-}$ embryos, on the other hand, never developed beyond the morula stage and deteriorated rapidly after 1 day in culture. Even at the time of isolation, several doubly nullizygous embryos were already misshapen and contained cells that appeared abnormally large, granular, and in the process of deteriorating (Fig. 1), indicating that these embryos were dying or already dead at this point. Thus, the *CKS1*$^{-/-}$ *CKS2*$^{-/-}$ double mutation leads to early embryonic lethality at or before the morula stage.

Depletion of Cks protein in cell lines impairs proliferation. *CKS* doubly nullizygous embryos might die because of a general defect in proliferation- or cell cycle progression-related functions or because of the failure of an embryogenesis-specific or a general housekeeping function. To determine whether depletion of Cks1 and Cks2 protein leads to impaired proliferation, we investigated the behavior of MEF cell lines after Cks depletion by siRNA-mediated silencing (15). To be able to deplete cells of as much Cks protein as possible, we chose to work initially with MEF lines nullizygous for either *CKS1* or *CKS2* and heterozygous for the remaining paralogous gene. To prevent the MEFs from become senescent, immortalized lines were prepared by transduction with a retrovirus expressing the human papillomavirus oncoprotein E6. In all experiments described, cells were transiently transfected with siRNA oligonucleotide duplexes 3 days and 1 day before beginning the analysis. To assess the efficiency of silencing under these conditions, we determined the levels of Cks1 or Cks2 mRNA in *CKS*-silenced versus nonsilenced cells by real-time PCR. As shown in Fig. 2a, the efficiency of silencing of Cks1 was greater than 90% compared to that for the siRNA control whereas the silencing of Cks2 was approximately 80%. Since the control level in this experiment corresponds to nullizygosity at one locus and heterozygosity at the other, the degree of silencing of total CKS protein in the *CKS2*$^{-/-}$ *CKS1*$^{+/-}$ MEF line silenced for Cks1 is most likely greater than 95% compared to that in a *CKS1*$^{+/+}$ *CKS2*$^{+/+}$ MEF line. Therefore subsequent experiments were carried out with this system.

Depletion of Cks1 in the *CKS2*$^{-/-}$ *CKS1*$^{+/-}$ MEF line by siRNA oligonucleotide transfection arrested proliferation, whereas transfection with a control (green fluorescent protein [GFP]) siRNA had no effect on proliferation (Fig. 2b). Beginning 1 day after the second transfection, no significant increase in cell number could be detected in Cks-silenced populations for several days, after which cells began to resume proliferation. However, resumption of proliferation correlated with recovery of *CKS* mRNAs from silencing (Fig. 2b). Based on

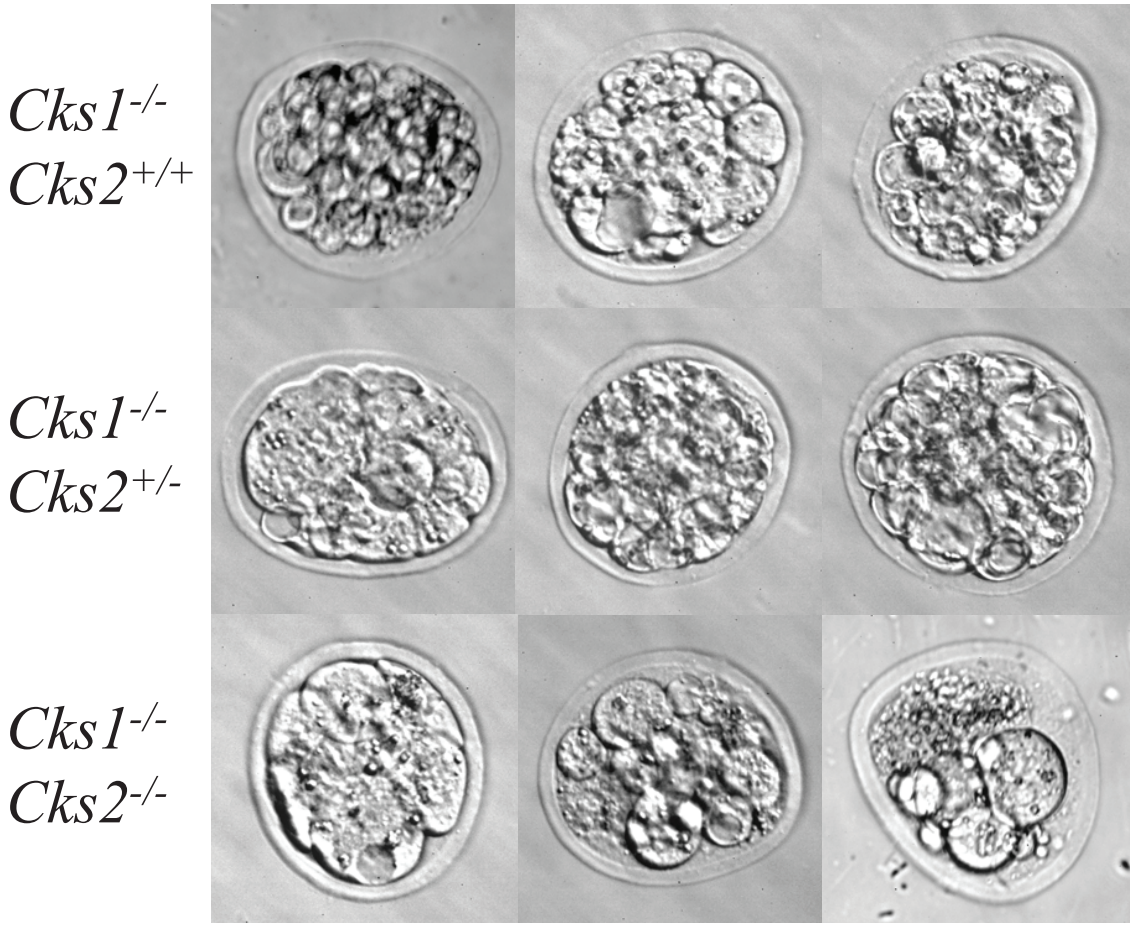

FIG. 1. Micrographs of morulae of each genotype obtained from *CKS1*$^{-/-}$ *CKS2*$^{+/-}$ intercrosses. The genotype represented in each row is shown on the left.

direct observation and terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay, the lack of cell number increase was not due to an increase in cell death (data not shown). To control for off-target effects of the Cks1 siRNA pool, *CKS1*$^{-/-}$ *CKS2*$^{+/-}$ MEFs were transfected with Cks1-specific siRNA. No effect on proliferation was observed (Fig. 2c), confirming that the block to proliferation conferred by transfection with Cks1 siRNA is due Cks protein depletion.

In parallel, simultaneous siRNA-mediated depletion of Cks1 and Cks2 was carried out in HeLa cells (Fig. 2d). As was the case with MEFs, HeLa cells significantly depleted of Cks1 and Cks2 ceased proliferation (Fig. 2e). However, unlike MEFs silenced for Cks1 and Cks2, a significant level of apoptosis was detected based both on TUNEL and FACS analysis (data not shown).

Depletion of Cks protein in MEFs leads to G_2 arrest and polyploidy. Since Cks-depleted cells ceased proliferation, we sought to determine whether this was due to a block at a particular cell cycle phase. *CKS2*$^{-/-}$ *CKS1*$^{+/-}$ MEFs silenced for Cks1 were analyzed for distribution within the cell cycle by LSC. Whereas the control population did not change its cell cycle distribution over the 4-day time course, the Cks-depleted populations showed a consistent and progressive increase in 4C and >4C regions of their distributions (Fig. 3a). Concomitantly, a complementary decrease in the 2C (G_1) region was observed. These data are consistent with depletion of Cks protein conferring arrest in G_2 or M phase followed by rereplication without division, leading to polyploidy. As there was no observed accumulation of rounded mitotic cells (data not shown), it can be inferred that depletion of Cks proteins in MEFs leads to cell cycle arrest in G_2. Over time, these G_2-arrested cells appear to be capable of undergoing an additional round of DNA replication, becoming tetraploid. The LSC data indicating polyploidy are consistent with microscopic observation of increasing numbers of cells with very large nuclei in the Cks-depleted populations (Fig. 3b). When the enlarged nuclei were counted manually, the level was found to be significantly larger (60%) than that determined by LSC (Fig. 3c). To confirm that the scoring of nuclei as "large" corresponded to an increased DNA content, images of nuclei were quantitated by determining the area of the optical section and the average DAPI staining intensity. Compared to typical nuclei in the control population or nuclei scored as "not large" in the Cks-depleted population, the nuclei scored as large had an average increase in cross-sectional DNA content of approximately 5.5-fold (Fig. 3d). This is likely an underestimate of the increase in nuclear DNA content due to the two-dimensional nature of the

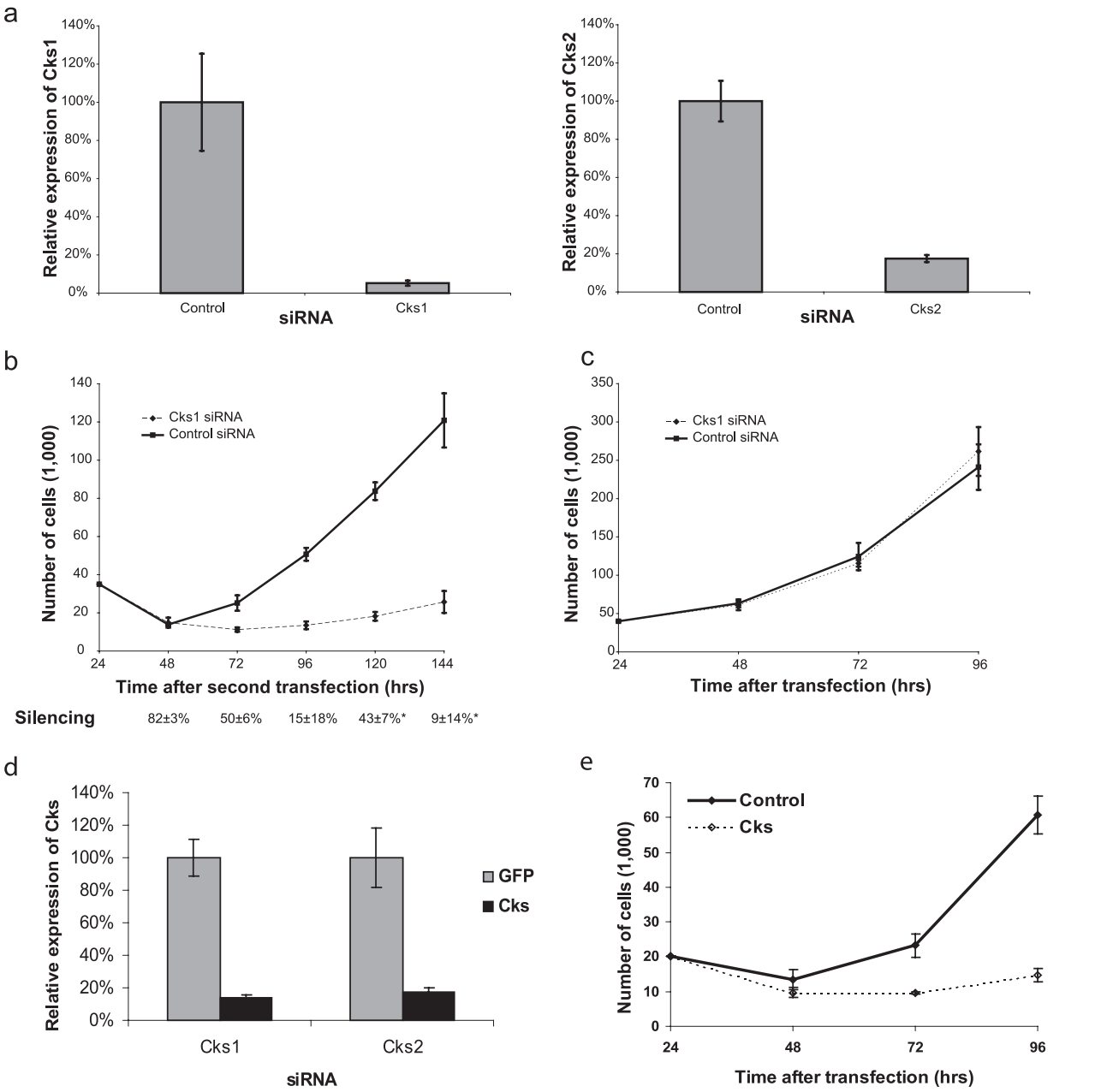

FIG. 2. Depletion of Cks proteins leads to a cessation of proliferation. (a) Cks1 and Cks2 silencing. *CKS2*$^{-/-}$ *CKS1*$^{+/-}$ (left) or *CKS1*$^{-/-}$ *CKS2*$^{+/-}$ (right) MEFs were transfected with either Cks1-specific siRNA or Cks2-specific siRNA, respectively, or control (GFP) siRNA. Cells were transfected twice at 48-hour intervals, and then RNA was prepared for real-time PCR 24 h after the second transfection. Bars indicate the levels of Cks1 mRNA (left) and Cks2 mRNA (right). (b) *CKS2*$^{-/-}$ *CKS1*$^{+/-}$ MEFs transfected as in panel a were counted beginning 24 h after the second transfection. The asterisks mark a third transfection 96 h after the second transfection. (c) Control for off-target effects of Cks1-specific siRNA transfection. *CKS1*$^{-/-}$ *CKS2*$^{+/-}$ MEFs were transfected with Cks1-specific siRNA, and cells were counted as for panel b. (d) Simultaneous Cks1 and Cks2 silencing in HeLa cells. Cells were transfected and real-time PCR analysis was carried out as for panel a. (e) HeLa cells silenced for Cks1 and Cks2 were counted beginning after the second transfection.

analysis. Another indicator of polyploidy occurring by failure to undergo division is increase in centrosome number. When control and Cks-depleted populations were compared for numbers of centrosomes by staining with an antibody to γ-tubulin, a significant increase in cells containing more than two centrosomes in the Cks-depleted population was found (Fig. 3e). Taken together, these data suggest that, whereas control-transfected cells undergo a normal cell cycle, where cell division follows chromosome duplication, Cks-depleted cells arrest transiently in G_2 after chromosome duplication and then undergo another round or multiple rounds of chromosome duplication without division, thereby increasing in ploidy. The reproducible discrepancy between the LSC data and the manual counts with respect to the percentage of polyploid cells

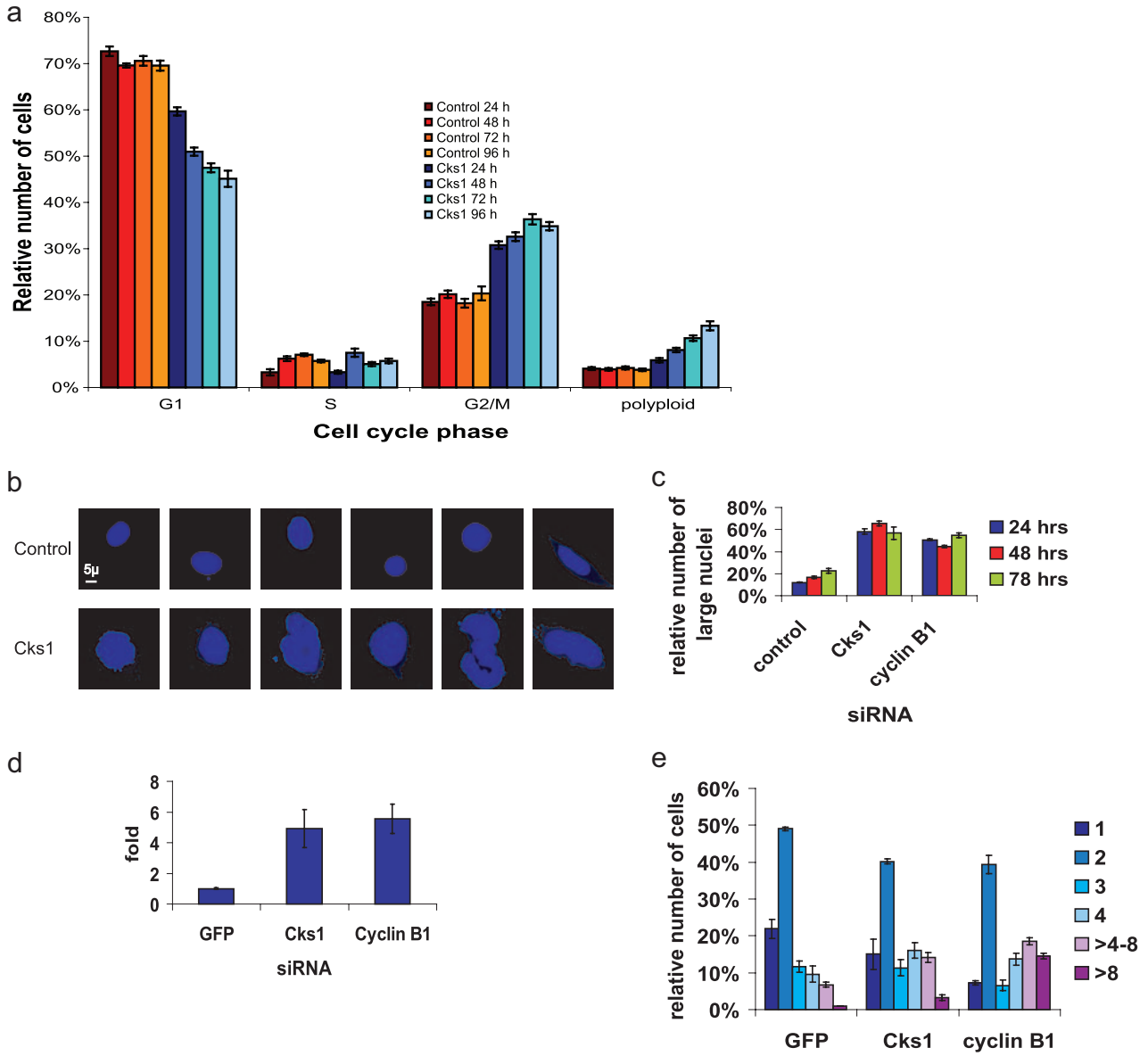

FIG. 3. Cell cycle and ploidy analysis of populations depleted for Cks proteins. *CKS2*$^{-/-}$ *CKS1*$^{+/-}$ MEFs were transfected with Cks1-specific, cyclin B1-specific, or GFP-specific siRNA twice, separated by 48 h. (a) At 24-hour intervals after the second transfection, slides were fixed, stained with PI, and analyzed for DNA content by LSC. The polyploid population was determined as all cells of >4C DNA content. Error bars represent standard errors. (b) Examples of large nuclei that accumulate in the Cks-silenced population. Typical nuclei in the control-transfected population are shown in the top row. Large nuclei apparent at high frequency in the Cks-silenced population are shown in the bottom row. Bar, 5 μm. All micrographs are at the same magnification. (c) Manual scoring of cells with large nuclei based on DAPI staining at the indicated times after the second transfection using GFP, Cks1, or cyclin B1 siRNAs. Three hundred cells were scored for each of three experiments for each siRNA. Error bars represent standard errors. (d) Estimation of relative DNA content of large nuclei 72 h posttransfection. DAPI staining of nuclear optical sections was determined by measuring both the sectional area and the average staining intensity. For the GFP siRNA control, only apparently normal-size cells were scored and the average was set to 1.0. For the Cks1- and cyclin B1-silenced populations, separate determinations were made for cells scored as normal and those scored as large in panel c. The ratios given are the averages of large nuclei divided by the averages of small nuclei. For each determination, 10 to 20 nuclei were analyzed. The nuclei scored as normal in the GFP control population had a DNA content similar to that of nuclei scored as normal in the Cks1- and cyclin B1-silenced populations. Error bars correspond to standard errors. (e) Determination of centrosome number 72 h after siRNA transfection. Cells transfected with the indicated siRNAs were stained with γ-tubulin-specific antibody and, centrosomes in each cell were counted by immunofluorescence microscopy. Two hundred cells were scored for each of three experiments for each siRNA. Error bars correspond to standard errors.

most likely results from the irregular lobular shape of many of the Cks-depleted nuclei (Fig. 3b). The logic circuit in the LSC software for distinguishing closely spaced objects most likely scores these as two individual nuclei.

Cks-depleted cells arrest in G_2 and become polyploid due to a defect in transcribing the *CCNB1*, *CCNA2*, and *CDK1* genes. To determine the underlying mechanism of G_2 arrest under conditions of Cks depletion, we analyzed the levels of a num-

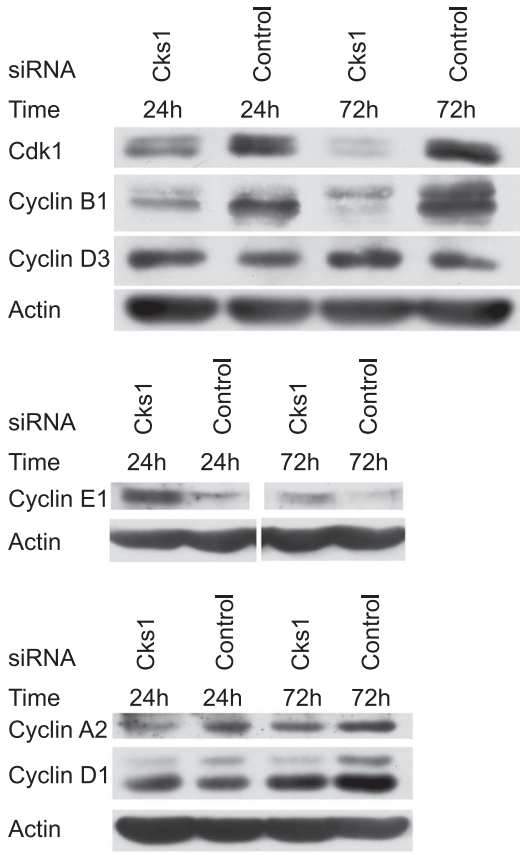

FIG. 4. Analysis of cell cycle-regulatory proteins in Cks-depleted populations. *CKS2*$^{-/-}$ *CKS1*$^{+/-}$ MEFs were silenced for Cks1 twice, separated by 48 h, after which cell extracts were prepared at the indicated times after the second transfection and levels of cell cycle-regulatory proteins were analyzed by immunoblotting. β-Actin was used as a loading control.

ber of known cell cycle-regulatory proteins. Western blots prepared during a time course following siRNA transfection of MEFs revealed a defect in expression of the mitotic cyclin B1 and its cognate kinase Cdk1, as well as cyclin A2, the somatic cell isoform of cyclin A (Fig. 4), consistent with the observed G_2 arrest. Cyclin E1, cyclin D1, and cyclin D3 were expressed at the same or at greater levels than in controls. To determine whether low cyclin B1, cyclin A2, and Cdk1 levels reflect reduced transcript levels, real-time PCR analysis of the respective mRNAs was carried out in Cks-silenced and control populations (Fig. 5a). Depletion of Cks proteins leads to a significant reduction in cyclin B1, cyclin A2, and Cdk1 mRNAs, but not cyclin E1 and cyclin D3 mRNAs, consistent with the Western blot data. Since Cks-depleted populations accumulate in G_2, where cyclin B1, cyclin A2, and Cdk1 mRNA levels are maximal compared to control populations (37, 52), which are asynchronous, the differential observed most likely underestimates the defect in mRNA expression of the three genes. We therefore carried out a second experiment using siRNA to silence cyclin B1 mRNA to levels comparable to those observed in Cks-depleted cells. RNAi-mediated silencing of cyclin B1 conferred a phenotype similar to that characterized for Cks silencing: transient G_2 arrest followed by polyploidy (Fig. 3c, d, and e and 5b), supporting the idea that the phenotype conferred by Cks protein depletion is caused by failure to express cyclin B1 and other mitotic proteins. When cyclin A2 and Cdk1 transcript levels in Cks-silenced MEFs were normalized to those in cyclin B1-silenced MEFs to correct for cell cycle effects, the reduction was even more dramatic (Fig. 5c). Finally, simultaneous silencing of Cks1 and cyclin B1 conferred the same transcriptional phenotype as Cks1 silencing alone (Fig. 5c), confirming that transcriptional downregulation of cyclin A2 and Cdk1 (and presumably cyclin B1) is a direct effect of Cks protein depletion.

To extend these studies, HeLa cells were simultaneously silenced for Cks1 and Cks2 by siRNA transfection (Fig. 5d). A strong reduction in cyclin B1, cyclin A2, and Cdk1 mRNA levels was observed. Therefore, as in MEFs, depletion of Cks proteins in HeLa cells leads to a failure to induce mitotic regulatory genes *CCNB1*, *CCNA2*, and *CDK1*.

Ectopic expression of cyclin B1 can rescue the proliferation phenotype conferred by RNAi-mediated Cks protein depletion. Since RNAi-mediated depletion of cyclin B1 confers a cell cycle phenotype similar to that observed upon Cks protein depletion, we determined whether ectopic expression of cyclin B1 could restore proliferation in Cks-depleted HeLa cells. HeLa cells were transduced with recombinant shRNA-expressing adenoviruses simultaneously targeting *CKS1* and *CKS2*. One population was also transduced with a recombinant adenovirus expressing human cyclin B1 (27), while the other was transduced with a control adenovirus. Cks protein-depleted HeLa cells continued to proliferate if cyclin B1 was ectopically expressed, but not when transduced with control virus (Fig. 6). In addition, it was observed that both populations ectopically expressing cyclin B1 proliferated more slowly than control populations without cyclin B1 expression. This is most likely due to inhibitory effects of cyclin B1 deregulation on mitotic progression (18, 26, 29). The level of cyclin B1 mRNA elevation was approximately 10-fold, as determined by real-time PCR. However, the relative level in mitotic cells was probably significantly lower, since endogenous cyclin B1 mRNA is highly regulated and peaks at mitosis whereas the ectopic cyclin B1 mRNA is not regulated and is therefore expressed throughout the cell cycle. Nevertheless, because of the toxic effect of unregulated overexpression of cyclin B1, it was not possible to determine whether a complete rescue of Cks silencing was achieved. This result was obtained in three independent experiments. Therefore, cyclin B1 expression is most likely the limiting Cks protein-dependent function required for proliferation of MEFs.

Cks proteins are chromatin bound and associate directly with the *CCNB1* and *CDK1* promoters and ORF. In budding yeast, Cks1-depleted cells are impaired for progression through mitosis because of a defect in transcribing the periodically expressed gene *CDC20*, which encodes an essential cofactor for the mitotic ubiquitin ligase APC^{Cdc20} (68). Efficient transcription of *CDC20* involves direct recruitment of Cks1 and Cdk1 to chromatin of the *CDC20* open reading frame (ORF) and promoter (38) (V. Yu and S. I. Reed, unpublished data). It was therefore interesting to observe that, in MEFs, the defect in cell cycle progression mediated by Cks depletion could be attributed to impairment of the transcription of similarly periodically expressed genes *CCNB1*, *CCNA2*, and *CDK1*. To determine whether Cks proteins and Cdk1 act di-

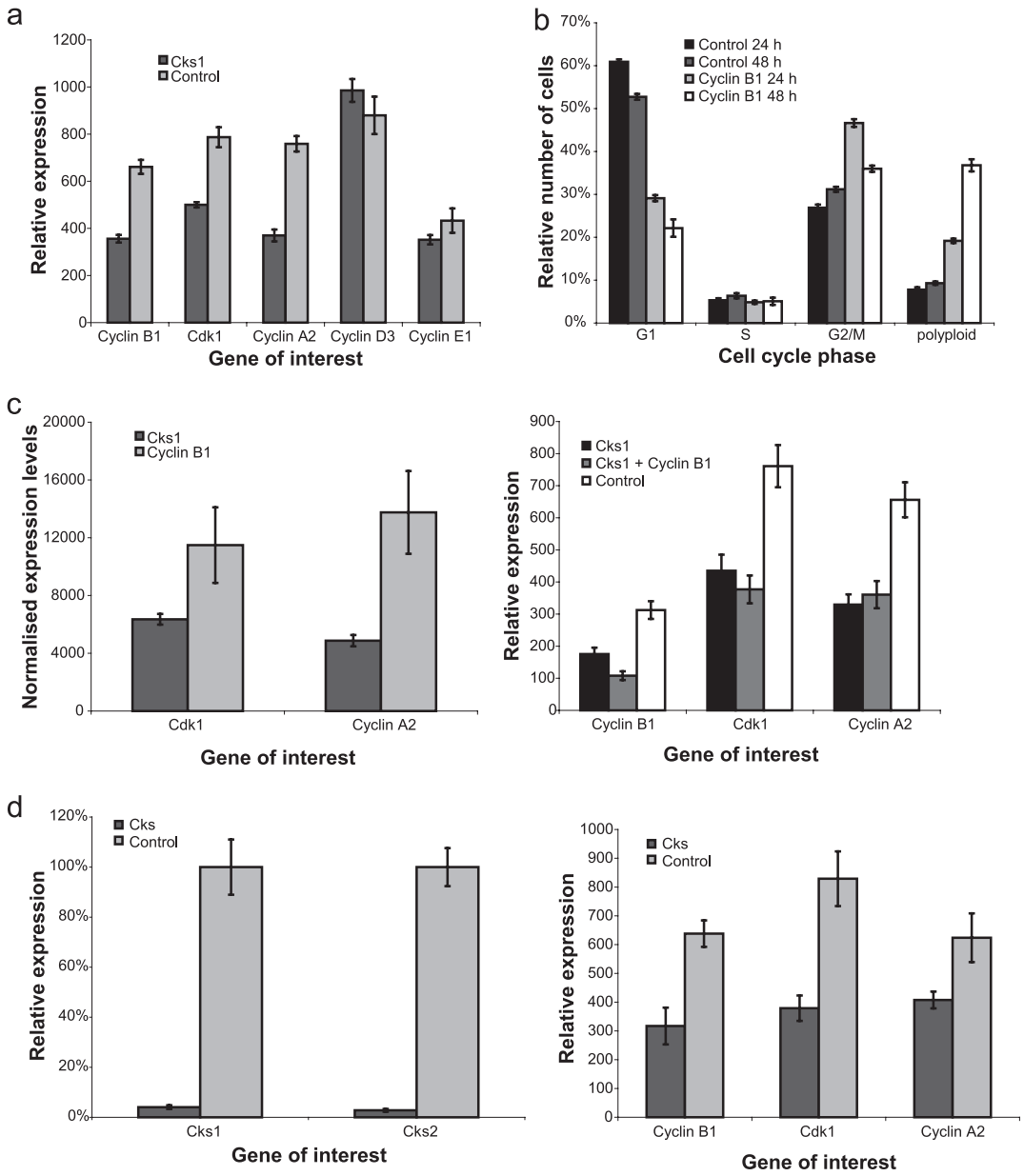

FIG. 5. Analysis of cyclin B1, Cdk1, and cyclin A2 transcript levels in Cks-depleted cells. (a) *CKS2*$^{-/-}$ *CKS1*$^{+/-}$ MEFs were silenced for Cks1 twice at 48-hour intervals, and 24 h later the indicated transcript levels relative to actin transcript levels were analyzed by real-time PCR. (b) Cell cycle analysis of populations depleted for cyclin B1. *CKS2*$^{-/-}$ *CKS1*$^{+/-}$ MEFs were transfected with cyclin B1-specific and GFP-specific siRNA twice, separated by 48 h. At 24-hour intervals after the second transfection, slides were fixed, stained with PI, and analyzed for DNA content by LSC. The polyploid population was determined as all cells of >4C DNA content. (c, left) Normalization for cell cycle effects was carried out by comparing transcript levels in Cks-depleted MEFs to transcript levels in cyclin B1-depleted MEFs. (Right) Comparison of cyclin B1, Cdk1, and cyclin A2 transcript levels in *CKS2*$^{-/-}$ *CKS1*$^{+/-}$ MEFs silenced for Cks1 alone or Cks1 and cyclin B1. (d, left) HeLa cells were silenced twice for Cks1 and Cks2, separated by 48 h, and 24 h later Cks1 and Cks2 levels were determined by real-time PCR. (Right) The same experiment, but the indicated transcript levels relative to actin levels were analyzed by real-time PCR.

rectly at the level of chromatin, we first carried out cellular-fractionation experiments. HEK293A cells were transduced with a retrovirus expressing Flag-Cks2. Under these conditions, Cks2 was only modestly overexpressed (data not shown). Lysates from lightly formalin-fixed cells were separated into a soluble fraction and an insoluble chromatin fraction solubilized by sonic disruption. Western blots revealed that a significant fraction of the Flag-Cks2 protein copurified with the chromatin fraction, as did Cdk1 (Fig. 7a). Histone H3 purified mostly with the chromatin fraction, as expected, whereas tubulin partitioned almost exclusively with the soluble fraction. Next, chromatin immunoprecipitation (ChIP) experiments were carried out on the chromatin fraction to determine if Cks2 is specifically associated with the *CCNB1* promoter and ORF regions and if so whether occupancy changed with the cell cycle. Untagged HEK293A cells were used as controls. In

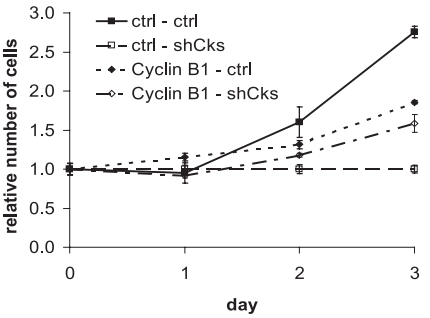

FIG. 6. Ectopic expression of cyclin B1 can rescue the proliferation defect associated with RNAi-mediated depletion of Cks proteins. HeLa cells were transduced simultaneously with Cks1- and Cks2-specific shRNA adenovirus or control (ctrl; empty vector) as well as a second set of recombinant adenoviruses (human cyclin B1 or empty vector). Cells were transfected both at day 0 and day 1. Cell counts were carried out beginning on day 0. Data are presented as relative increases in cell number. Error bars represent standard errors of the means. Levels of silencing of Cks1 and Cks2 mRNAs on day 3 were 92% and 75%, respectively. Cells transduced with cyclin B1-expressing adenovirus exhibited a 10-fold elevation of cyclin B1 mRNA throughout the time course.

order to determine whether transcription of *CCNB1* and *CDK1* correlates with increased association of Cks2 with the *CCNB1* promoter and/or ORF, early-S-phase and G_2-enriched populations were prepared by carrying out a thymidine block for 24 h (early S phase) and a release for 8 h (G_2 enriched; 70% 4C DNA content based on FACS analysis). *CCNB1* and *CDK1* are preferentially transcribed in G_2 (37, 52). Under the synchronization conditions employed, the cyclin B1 mRNA was expressed at a 3.5-fold-higher level in the G_2-enriched population than in the thymidine-arrested population, based on real-time PCR (data not shown). ChIP analysis revealed that in both S-phase (data not shown) and G_2-enriched cells (Fig. 7b) there was clear association of Cks2 directly with the *CCNB1* and *CDK1* genes, as a significantly lower signal was obtained using an untagged strain. Furthermore, there was a significant increase in Cks2 association with the *CCNB1* and *CDK1* promoters and ORFs in the G_2-enriched population that paralleled an increase in RNA polymerase II (Fig. 7c). Although this increase was small, it was statistically significant for *CCNB1* and reproducible in several independent experiments. Note that the observed cell cycle-dependent differences between Cks2 and RNA polymerase II association with the *CDK1* promoter, and particularly the *CDK1* ORF, were consistently small. On the other hand, based on the same ChIPs, there was no cell cycle-dependent difference in recruitment of Cks2 to the *CCND3* and *GAPDH* promoters (Fig. 7d). Thus, Cks proteins may contribute to the expression of genes such as *CCNB1* and *CDK1* through direct interactions with chromatin and/or the transcriptional machinery.

DISCUSSION

The early embryonic lethality associated with *CKS* nullizygosity is consistent with a defect in cyclin B1 expression. In this report we show that mice nullizygous for both genes encoding Cks orthologs die as morulae after only three or four embryonic division cycles, a dramatic difference relative to the singly nullizygous mice, both of which are viable. Although no detailed analysis of mice nullizygous for *CCNB1* has been reported (only lethality prior to E10) (3), the absolutely essential function of cyclin B1 is consistent with the early-embryonic-lethal phenotype of *CKS* doubly nullizygous mice, if the principal defect is expression of cyclin B1 and other proteins required for mitosis. To our knowledge, this is the earliest time point of embryonic death for any disruption of a gene encoding a Cdk or Cdk-associated protein in the mouse reported to date. Mice with all cyclins and Cdks that are known to be involved in cell cycle control, except for Cdk1, knocked out have been generated. Mice lacking all three cyclin D orthologs die around E15.5 (32), whereas ablation of both genes encoding cyclin E orthologs leads to embryonic death at E11 due to placental failure rather than an intrinsic defect in embryonic development (17, 47). Mice lacking the somatically expressed cyclin A isoform, cyclin A2, die after implantation around E7 (39). Ablation of cyclin B1, as stated above, leads to death at an undetermined time point before E10 (3). Surprisingly, mice lacking Cdk2, which is activated by cyclin A and cyclin E, are viable (1, 43) and mice lacking both Cdk4 and Cdk6, which are activated by D-type cyclins, die just before birth (43). No disruption has been described so far for Cdk1, although cell lines with a temperature-sensitive allele of the gene encoding Cdk1 arrest in G_2 phase and die at a restrictive temperature (64). The observation that embryos lacking complete cyclin families or Cdks seem to die of specific developmental defects rather than a general proliferation deficiency suggests that cyclins and Cdks are highly functionally redundant and that the mammalian cell cycle has a high level of regulatory plasticity in most tissues and organs. The result that mice lacking both Cks-encoding genes die much earlier than any of the described Cdk and cyclin knockout mice suggests that cyclin B1 and presumably Cdk1 are exceptions in that they provide a unique activity and perform a nonredundant essential function.

The link between cyclin B1 downregulation, G_2 arrest, and polyploidy. The cyclin B1-Cdk1 protein kinase, also known as mitosis promoting factor (MPF), comprises the activity that drives eukaryotic cells into mitosis (13). Genetic studies of both yeast and mammalian cells, as well as studies with chemical inhibitors (67), indicate that this activity is essential for mitosis and that without it cells cannot progress past the G_2 phase of the cell cycle (13, 42, 54, 64, 67). The G_2 arrest conferred by siRNA-mediated silencing of *CKS* genes and concomitant failure to express cyclin B1, therefore, is consistent with the requirement of cyclin B1 for entry into mitosis. Indeed, we could show that ectopic expression of cyclin B1 could rescue the proliferation defect conferred by *CKS* gene silencing, supporting this interpretation. It has also been shown both in yeast and mammalian cells that inhibition or inactivation of cyclin B-Cdk1 results in the accumulation of polyploid cells (4, 8, 21, 25, 41). Presumably this phenomenon is a direct consequence of the requirement for high levels of Cdk activity for preventing prereplication complex assembly (also known as replication origin licensing) (11). Under normal circumstances, this mechanism restricts origin licensing and replication to one round per cell cycle, as Cdk activities remain high from the beginning of S phase until the end of mitosis. It has been shown in yeast, however, that if cyclin B-Cdk1 activity is inhibited after one round of replication but before cell division, origins

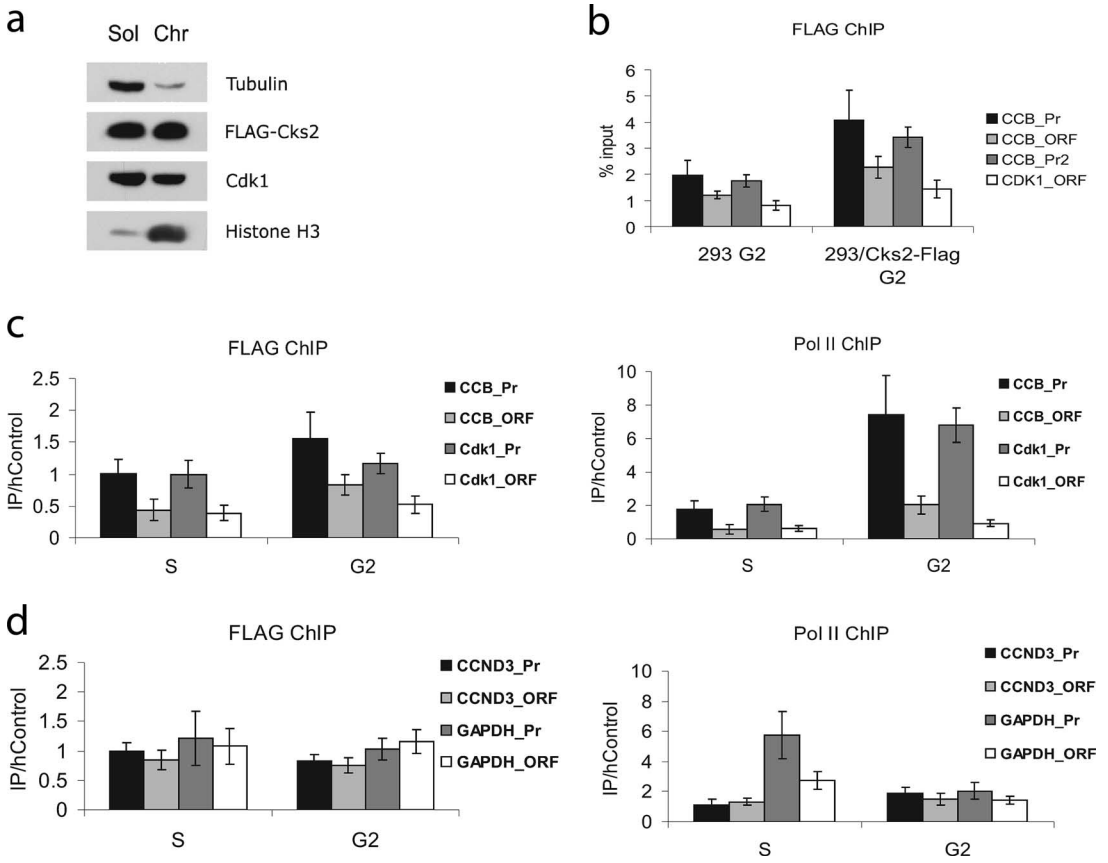

FIG. 7. Cks2 associates with the *CCNB1* and *CDK1* promoters and ORFs in a cell cycle-regulated manner. (a) Chromatin fractionation of HEK293A cells stably expressing Flag-Cks2 by retroviral transduction. Immunoblotting was carried on the soluble fraction (lane Sol) and insoluble chromatin-enriched fraction solubilized by sonic disruption (lane Chr). Histone H3 is a marker for chromatin proteins; α-tubulin is a marker for soluble proteins. (b) Chromatin immunoprecipitation of Flag-Cks2 at the *CCNB1* promoter (CCB_Pr) and ORF and the *CDK1* ORF in G_2-enriched populations of HEK293A cells arrested by thymidine block and released for 8 h (G_2 enriched). The cells stably transduced with a retrovirus expressing Flag-Cks2 were compared to untransduced controls. Real-time PCR values are normalized to input. In this experiment, two different primer sets (Pr and Pr2) corresponding to the *CCNB1* promoter region were used. (c) Chromatin immunoprecipitation of Flag-Cks2 and RNA polymerase II at the *CCNB1* and *CDK1* promoters and ORFs. Populations of Flag-Cks2 HEK293A cells arrested by thymidine block (S enriched) and released for 8 h (G_2 enriched), respectively, were analyzed by ChIP and real-time PCR. Values given are normalized to the signal corresponding to a nontranscribed region on chromosome 19 from the same immunoprecipitates. Error bars correspond to 1 standard deviation. (d) ChIP of Flag-Cks2 and RNA polymerase II at the *CCND3* and GAPDH promoters and ORFs. The same chromatin immunoprecipitates as in panel c were analyzed using primers corresponding to the *CCND3* and *GAPDH* promoters and ORFs.

are relicensed, leading to another round of replication and polyploidy (4, 8, 21). Therefore the induction of polyploidy as a result of *CKS* gene silencing, like the observed G_2 arrest, is likely a direct consequence of impairment of cyclin B1 and Cdk1 expression. This conclusion is consistent with our observation of G_2 arrest and polyploidy in cells silenced for cyclin B1 by RNAi. Although we did not carry out cell cycle or ploidy analysis of failing *CKS1*$^{-/-}$ *CKS2*$^{-/-}$ embryos, the abnormally large size of many of the embryonic cells suggests that they exhibited a phenotype similar to that of Cks-depleted somatic cells in culture, namely, G_2 arrest and polyploidy.

Impairment of *CCNB1*, *CCNA2*, and *CDK1* transcription by *CKS* gene silencing. Analysis of yeast has revealed that Cks1 and Cdk1 have a direct role in transcription of a significant number of genes (72). This function does not require the kinase activity of Cdk1 but instead involves the recruitment of proteasomes to chromatin of actively transcribed genes (72). Although the precise function of proteasomal recruitment in the context of transcriptional elongation is not well understood, preliminary evidence suggests that at least in yeast the function of the Cks1-Cdk1-proteasome complex is to reduce nucleosome density in order to facilitate transcriptional elongation of induced genes (S. R. Chaves, V. Yu, and S. I. Reed, unpublished data). In the context of *CCNB1* and *CDK1* transcription in mammalian cells, we have shown that Cks2 (Cks1 was not analyzed) and Cdk1 generally associate with chromatin and that Cks2 is recruited to the *CCNB1* promoter in a cell cycle-specific fashion that correlates with active transcription (Fig. 7). Although we do not yet know whether Cdk1 and the proteasome are similarly recruited, it is tempting to speculate that the Cks dependency of *CCNB1*, *CCNA2*, and *CDK1* transcription represents conservation of a fundamental proteasome-mediated chromatin-remodeling pathway. Indeed, all three genes share a common regulatory mechanism in that transcription is primarily controlled by a cell cycle-dependent repression element known as CHR (34, 51, 73, 74). Relief of

repression at the appropriate time in the cell cycle allows the binding of a constitutive positive transcription factor, NF-Y (5, 35). It is possible the Cks proteins are involved in establishing an open chromatin environment permissive for NF-Y binding.

Cks proteins and cancer. Although the functions of Cks proteins have remained elusive, tumor profiling has revealed that both Cks1 and Cks2 are frequently overexpressed, at least at the mRNA level, in a variety of human malignancies (7, 9, 10, 24, 28, 31, 33, 36, 40, 44, 45, 56–58, 61, 65, 70). Furthermore, in one large breast cancer study, overexpression of Cks2 was particularly associated with aggressive disease and poor patient outcome (66). Although the link between Cks protein overexpression and cancer is unknown at this point, it is tempting to speculate, based on the data presented in this report, that it involves perturbations to normal cellular gene expression.

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