

Cdk2 and Cdk4 Regulate the Centrosome Cycle and Are Critical Mediators of Centrosome Amplification in p53-Null Cells[∇]

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The two mitotic centrosomes direct spindle bipolarity to maintain euploidy. Centrosome amplification—the acquisition of ≥ 3 centrosomes—generates multipolar mitoses, aneuploidy, and chromosome instability to promote cancer biogenesis. While much evidence suggests that Cdk2 is the major conductor of the centrosome cycle and that it mediates centrosome amplification induced by various altered tumor suppressors, the role played by Cdk4 in a normal or deregulated centrosome cycle is unknown. Using a gene knockout approach, we report that Cdk2 and Cdk4 are critical to the centrosome cycle, since centrosome separation and duplication are premature in *Cdk2*^{-/-} mouse embryonic fibroblasts (MEFs) and are compromised in *Cdk4*^{-/-} MEFs. Additionally, ablation of *Cdk4* or *Cdk2* abrogates centrosome amplification and chromosome instability in p53-null MEFs. Absence of *Cdk2* or *Cdk4* prevents centrosome amplification by abrogating excessive centriole duplication. Furthermore, hyperactive Cdk2 and Cdk4 deregulate the licensing of the centrosome duplication cycle in p53-null cells by hyperphosphorylating nucleophosmin (NPM) at Thr199, as evidenced by observations that ablation of *Cdk2*, *Cdk4*, or both *Cdk2* and *Cdk4* abrogates that excessive phosphorylation. Since a mutant form of NPM lacking the G₁ Cdk phosphorylation site (NPM^{T199A}) prevents centrosome amplification to the same extent as ablation of *Cdk2* or *Cdk4*, we conclude that the Cdk2/Cdk4/NPM pathway is a major guardian of centrosome dysfunction and genomic integrity.

The centrosome maintains genomic integrity by enforcing euploidy (20). A centrosome consists of two centrioles, containing proteins such as α -tubulin; structural proteins including pericentrin, γ -tubulin, and centrin-2; and cell cycle-regulatory proteins, which include p53 and cyclin E/Cdk2. Normal cells have one mature centrosome during early G₁ (20). At late G₁, each of the centrioles composing the mature centrosome separates and duplicates to form a new (or daughter) centriole between late G₁ and late S phase, culminating in two fully mature centrosomes at G₂. The two mitotic centrosomes associate with spindle fibers and migrate toward opposite sides of the spindle pole to establish bipolarity. This ensures that sister chromatids segregate toward each spindle pole. Following cytokinesis, each daughter cell receives one centrosome and an equal complement of chromatids. Normal centrosome duplication must be strictly controlled and strictly coordinated with S-phase initiation and progression (68). When this control fails, centrosome amplification occurs, leading to aberrant and multipolar mitotic spindles, increased frequency of chromosome segregation errors, aneuploidy, and chromosome instability (12, 20). Centrosome amplification, aneuploidy, and chromosome instability contribute to cancer biogenesis and

progression by triggering reduced expression of tumor suppressors and overexpression of proto-oncogenes.

One of the pathways contributing to centrosome amplification is deregulated centrosome duplication triggered by the G₁ cyclin-dependent kinases (Cdks) (27, 51, 59). The Cdks, a family of serine/threonine protein kinases, control the onset of the major cell cycle events, such as DNA synthesis and mitosis (65). Cdk activities are positively regulated by association with different cyclins, which are temporally expressed at specific phases of the cell cycle; they are negatively regulated by a variety of Cdk inhibitors (CKIs) (65). Individual and combinatorial gene knockouts of the cyclins and Cdks have uncovered redundancy in the regulation of DNA synthesis and specificity in their abilities to control development and tumorigenesis (2, 5, 6, 24, 25, 38, 52, 55, 61, 72, 77). However, how the cyclins and Cdks individually or cooperatively impinge on centrosome duplication is poorly understood. Biochemical and pharmacological evidence pointed to Cdk2 as the only Cdk coordinating the centrosome duplication and cell cycles (32, 33, 36, 40, 44). Cdk2 was proposed to coordinate the cell and centrosome duplication cycles by phosphorylating Rb to promote S phase (28) and by phosphorylating various centrosomal proteins to regulate the centrosome duplication cycle (10, 19, 51). Cyclin E/Cdk2 phosphorylates nucleophosmin (NPM)/B23 at T199 to regulate centrosome licensing; this phosphorylation allows centrioles to separate and centriole duplication to commence (70). Cdk2 directly promotes centrosome duplication by phosphorylating Mps-1 and CP110 and by modulating the activity of Plk4 (10, 19, 26). However, gene knockout approaches de-

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throne Cdk2 as the sole Cdk coordinating the cell and centrosome duplication cycles, since mouse embryonic fibroblasts (MEFs) from which *Cdk2* (14) or *cyclins E1* and *E2* (25) had been deleted showed only a minor deviation from normal centrosome ratios and proliferated. These results implied that, as with the cell cycle, there is redundancy among the Cdks regulating the centrosome duplication cycle. These results were unexpected, given the involvement of Cdk2 in the regulation of two central steps in the centrosome duplication cycle: licensing and duplication. To date, the identity of the Cdks supporting Cdk2 in regulating normal centrosome duplication is unknown.

As the cyclins, Cdks, and CKIs control centrosome duplication, altered tumor suppressors and oncogenes deregulate those cell cycle-regulatory molecules, leading to centrosome amplification (12, 21). Ablated genes that result in elevated Cdk2 activity and elevated frequencies of centrosome amplification include *E2F3*, *p53*, *Skp2*, and *p21^{Waf1}*; similarly, ectopically expressed cyclins E and A result in elevated Cdk2 activity and centrosome amplification in *p53^{-/-}* MEFs (13, 27, 44, 49, 59, 68). Likewise, oncogenes and altered tumor suppressors that hyperactivate Cdk4 and result in high frequencies of centrosome amplification include ectopically expressed *Her2* (47), *H-Ras^{V12}*, *v-Mos* (57), *MEK1^{Glu217/Glu221}* (58), *cyclin D1* (50), and silenced *MEK2* (73). Conversely, *p16* restricts excessive centriole reduplication (42, 44). However, the relationships between altered genes, ectopic activities of specific Cdks, and centrosome amplification are correlational, as they deregulate cyclin/Cdk activities as well as complex signal transduction cascades that control a plethora of transcripts.

The abilities of the cell cycle and centrosomal checkpoints—signaling pathways that monitor the integrity and replication status of the genome and the centrosome—to inhibit entry into S phase and centrosome duplication are closely associated with the function of the *p53* tumor suppressor (45, 74). The *p53* transcription factor is inactivated in approximately 50% of human cancers (71). *p53* regulates the transcription of a large number of genes to prevent entry into S phase in the presence of DNA or centrosome damage (45, 74). Indeed, ablation of *p53* allows centrosome amplification, aneuploidy, and chromosome instability (22). A gene product central to centrosome duplication control is *p21^{Waf1}*, expressed at low levels in a *p53*-dependent manner (48) to inhibit the cyclin E/Cdk2 complex (65). In addition, *p21^{Waf1}* has been implicated in the assembly of the cyclin D1/Cdk4 complex, and its overexpression inhibits the activity of Cdk4 at higher concentrations (30, 35, 76). The continual presence of *p21^{Waf1}* guards against premature activation of cyclin E/Cdk2 and perhaps against that of cyclin D/Cdk4, ensuring the coordinated initiation of centrosome and DNA duplication. In *p21^{Waf1}* MEFs, initiation of centrosome and DNA duplication is uncoupled, much like that in cells with constitutively active cyclin E/Cdk2 (13, 48, 68). Importantly, observations that the reintroduction of wild-type *p53* into *p53^{-/-}* cells resulted in nearly complete restoration of the centrosome duplication cycle while ectopic expression of *p21^{Waf1}* in *p53^{-/-}* cells only partially restored that cycle (68) suggest that *p53* controls centrosome duplication through multiple pathways, one of which is mediated by the negative regulation of Cdk2 by *p21^{Waf1}*.

Since direct evidence linking Cdk2 or Cdk4 to centrosome amplification in *p53^{-/-}* MEFs was lacking, we used a genetic

approach to test whether Cdk4 and Cdk2 mediate that abnormal process. Our results revealed that *p53* knockout does not signal centrosome amplification and chromosome instability exclusively through Cdk2, as suggested previously (21). We propose a new paradigm: ablation of *p53* requires the presence of both Cdk2 and Cdk4 activities in order to induce high frequencies of centrosome amplification and chromosome instability.

MATERIALS AND METHODS

Generation of mouse embryonic fibroblasts. Mice were crossed as *Cdk2^{+/-} × Cdk2^{+/-}, Cdk4^{+/-} × Cdk4^{+/-}, Cdk2^{+/-} Cdk4^{+/-} × Cdk2^{+/-} Cdk4^{+/-}* (meiotically recombined [6]), *p53^{+/-} Cdk2^{+/-} × p53^{+/-} Cdk2^{+/-}, p53^{+/-} Cdk4^{+/-} × p53^{+/-} Cdk4^{+/-}*, and *p53^{+/-} Cdk2^{+/-} Cdk4^{+/-} × p53^{+/-} Cdk2^{+/-} Cdk4^{+/-}*. After mating, embryos were isolated from females 13.5 days after detection of seminal plugs. Embryos were collected under sterile conditions and their livers extirpated for extraction of DNA and PCR genotyping. MEFs were generated using established methods (59). The individual genotypes were assessed by PCR genotyping with primers specific for the wild-type and knockout *p53*, *Cdk2*, and *Cdk4* alleles (6). All experiments were performed on passage 2 (p2) MEFs.

Cell culture. MEFs were maintained under proliferating conditions with 10% fetal bovine serum (FBS)—Dulbecco's modified Eagle medium (DMEM). For serum arrest experiments, cells were cultured in 0.2% FBS-DMEM for 60 h.

Centriole reduplication assay. Three independent, proliferating MEFs of the genotypes indicated in Fig. 4E and F, plated in two-well chamber slides, were either left untreated or treated with 2 mM hydroxyurea (HU) for 48 h. For coimmunostaining of α - and γ -tubulins in order to examine centrioles, cells were first incubated on ice for 30 min, to destabilize microtubules nucleated at the centrosomes, and then briefly extracted (~1 min) with cold extraction buffer [0.75% Triton X-100, 5 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 2 mM EGTA (pH 6.7)]. Cells were then briefly washed in cold phosphate-buffered saline (PBS) and were fixed as previously described (59). Cells were immunostained with anti- α -tubulin monoclonal (DMA1; Sigma) and anti- γ -tubulin polyclonal (ab11317; Abcam) antibodies. The antibody-antigen complexes were detected with the appropriate Alexa Fluor-conjugated antibodies (Molecular Probes), and the frequencies of centrosome amplification were calculated by counting 200 cells per group.

Serum starvation and BrdU incorporation assay. MEFs of the indicated genotypes plated in 60-mm-diameter dishes were first grown to confluence in 10% FBS-DMEM and then split into three groups. The cells in groups 1 and 2 were plated onto two-well tissue culture chamber slides, and those in the third group were plated onto 60-mm-diameter petri dishes. MEFs of different groups were first plated at high densities, then starved for 60 h by culturing in medium supplemented with 0.1% FBS, and finally released by the addition of 10% FBS for various times. The cells in group 1 were immediately fixed for centrosome staining at the time points indicated in Fig. 2. To measure the S-phase entry of the indicated genotypes, MEFs in group 2 were pulse-labeled with 20 μ M bromodeoxyuridine (BrdU) (51-7581KZ; BD Pharmingen) and incubated for 30 min as described previously (64, 75). BrdU-positive cells were detected using primary antibodies against BrdU (NA61; Calbiochem) and an Alexa Fluor 555-conjugated secondary antibody (Molecular Probes). We counted 200 cells per group for the BrdU and centrosome assays. Lysates from cells in group 3 were obtained for Western blotting at the time points indicated in Fig. 2.

Immunofluorescence. Immunofluorescence was performed by following our published protocols (59). MEFs were plated at 4×10^4 per well into two-well tissue culture chamber slides and were grown for 2 to 3 days. Cells were fixed in cold 4% paraformaldehyde, washed in PBS, permeabilized in a 1% NP-40-PBS solution, and blocked in 5% bovine serum albumin (BSA) in PBS. Centrosomes were stained overnight at 4°C with monoclonal antibodies against pericentrin (611814; BD Biosciences) and/or γ -tubulin (ab11317; ABCAM). Chromosome breaks were detected using phosphorylated histone 2A variant X (γ -H2AX) (07-164; Upstate) with the appropriate Alexa Fluor-conjugated antibodies (Molecular Probes). Cells were also counterstained with 4',6-diamidino-2-phenylindole (DAPI). For each experiment involving calculations of the frequencies of centrosomes, at least 200 cells from each chamber were counted per group.

Western blotting. Western blotting was performed according to published protocols (59). Protein lysates were obtained by incubating cells in lysis buffer (50 mM HEPES [pH 7.9], 250 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% NP-40, 10% glycerol, 0.5 mM NaF, 0.1 mM NaVO₄, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM β -glycerophosphate, 0.1 mM dithiothreitol, 0.1 mg/ml

aprotinin, 0.1 mg/ml leupeptin) for 30 min at 4°C. Samples were denatured at 95°C for 5 min in sodium dodecyl sulfate (SDS) sample buffer, resolved by SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). The blots were incubated in blocking buffer (5% [wt/vol] nonfat dry milk or BSA in Tris-buffered saline plus 0.1% Tween 20 [TBS-T]) for 1 h and were then probed overnight at 4°C with the primary antibodies. The blots were then rinsed in TBS-T and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies at room temperature. The blots were then rinsed in TBS-T, and the antibody-antigen complex was visualized with the chemiluminescent HRP Antibody Detection reagent (Denville Scientific Inc., Metuchen, NJ). Western blotting for the detection of phosphorylated nucleophosmin was performed similarly, except that the serine/threonine phosphatase inhibitor calyculin A (Upstate, CA) was included in the culture medium 10 min prior to harvest at a concentration of 100 nM. The antibodies used in the various Western blotting experiments were as follows: anti-Cdk2 (sc-163; Santa Cruz), anti-Cdk4 (2906; Cell Signaling), anti-p53 (sc-6243; Santa Cruz), anti-p57^{Kip2} (sc-8298; Santa Cruz), anti-p16^{INK4A} (sc-1207; Santa Cruz), anti-p21^{Waf1} (sc-397; Santa Cruz), anti-p27^{Kip1} (sc-528; Santa Cruz), anti- β -actin (4970; Cell Signaling), anti-cyclin A (ab38; Abcam), anti-cyclin D1 (2922; Cell Signaling), and anti-cyclin E (sc-481; Santa Cruz).

The micronucleus assay. The micronucleus assay was performed as described previously (59). Briefly, 4×10^4 cells were plated into each well of a two-well chamber slide (177380; Nalge Nunc International). After 2 to 3 days in culture, cells were fixed in 4% paraformaldehyde and were stained with DAPI. Micronuclei appear as spherical structures with a morphology similar to that of the nucleus, except that their sizes range from 1/10 to 1/100 the size of a nucleus; 1,000 cells were counted for each genotype analyzed.

Transfections. For transient transfection of wild-type and mutant *NPM/B23*, three independent *p53*^{-/-} MEFs were cotransfected with a plasmid carrying either FLAG-tagged wild-type *NPM/B23* or a FLAG-tagged substitution mutant (Thr¹⁹⁹ → Ala) with a neomycin resistance gene (pcDNA3.1) by using Lipofectamine (Invitrogen, Carlsbad, CA). As a negative control, the empty vector was transfected. After transfection at 37°C overnight in a 5% CO₂ incubator, cells were fed with fresh complete medium for 24 h. The cells were then treated with complete medium containing 2.5 mg/ml neomycin (Sigma, St. Louis, MO) for 7 days. G418-resistant cells were maintained in complete medium containing neomycin (1 mg/ml) for an additional 2 days and were replated for further culture in fresh complete medium for an additional 24 h.

Three independent *Cdk4*^{-/-} MEFs were also transfected with a plasmid carrying an empty vector (pBABE-*hygro*) or encoding Cdk2 (pBABE-*Hygro*-Cdk2) by using Lipofectamine (Invitrogen) according to the manufacturer's protocol. After transfection (37°C overnight), cells were fed with fresh complete medium for 24 h and were then switched to selective medium (150 μ g/ml hygromycin) for 4 days. Selected cells were directly plated onto two-chamber slides, fixed as previously described (59), and stained for centrosome analysis.

RNA interference. Cdk2 and Cdk4 small interfering RNAs (siRNAs) (catalogue no. SC-29260 and SC-29262) were purchased from Santa Cruz Biotechnology, Inc. Three independent MEFs of each genotype were grown in a six-well plate until they became 60 to 80% confluent. The cells were then transfected with each siRNA according to the manufacturer's protocol, and 72 h after the transfection, the cells were used to prepare cell lysates and were plated onto a two-chamber slide for centrosome analysis.

We also knocked down p53 with two synthesized siRNA duplex sequences by transfecting three independent wild-type MEFs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The two siRNA duplex sequences targeting the p53 mRNA are ACCAF161020_1 (sense, 5'-rCrArCrArUrGrUrArCrUrUrGrUrArGrUrGrArUrGrUrGrUrGrUrA-3'; antisense, 5'-rCrCrArCrArUrCrCrArCrUrArCrArGrUrArCrArUrGTG-3') and ACCAF161020_2 (sense, 5'-rGrGrUrGrArArUrArCrUrCrUrCrArUrCrArArGrUrGrUrUrU-3'; antisense, 5'-rArCrCrArCrUrUrGrArUrGrGrArArUrArUrUrU-3').

All the siRNAs were prepared using a transcription-based method with the Silencer siRNA construction kit (Integrated DNA Technologies) according to the manufacturer's instructions. Three days after the addition of complete medium, cell lysates were prepared for appropriate assays.

Cyclin-dependent kinase 4 activity assay. The cyclin-dependent kinase 4 assay was performed according to published methods (67). Briefly, the NPM peptide is phosphorylated because of Cdk4 activity, which is measured using luminometric estimation of ATP depletion. Immunoprecipitated (Ip) Cdk4, obtained from 150 μ g of total-protein extract from wild-type and *Cdk2*^{-/-} MEFs at different time points, was washed three times in cell lysis buffer and twice in kinase buffer and was then resuspended in 30 μ l of kinase buffer, optimized to preserve the Cdk4 activity. A kinase reaction was performed by adding 20 μ l of a mixture containing

0.5 μ M ATP and 2 μ g of NPM peptide (catalog no. ab39518; Abcam) as a substrate in kinase buffer. The reaction mixture was incubated for 30 min at 30°C, and then an equal volume of Kinase-Glo reagent was added. As reaction controls, the kinase reaction was performed with samples in the absence of the peptide substrate (no NPM), with no Ip-Cdk4, and with Ip-Cdk4 obtained from total-protein extracts from *Cdk4*^{-/-} MEFs. Samples were then incubated for 20 min at room temperature, and the developed luminescence was recorded using the SpectraMax Gemini XS luminometer and expressed as relative light units.

Image acquisition and manipulation. Slides were analyzed using a Zeiss Axioplan II microscope with a Plan-Apochromat 100 \times (numerical aperture, 1.4) oil immersion objective. Images were taken using a color digital camera (Axio-cam HRC) and Zeiss Axiovision software. Confocal images were acquired with a Zeiss LSM 510 META point scanning laser confocal microscope mounted on a Zeiss Axioplan II upright microscope equipped with a Plan-Apochromat 63 \times (numerical aperture, 1.4) oil immersion objective. Images were captured by Zeiss Image Browser. All the samples were mounted in Fluoromount-G mounting medium (Southern Biotech) and were analyzed at room temperature.

RESULTS

Mouse embryonic fibroblasts proliferate despite the absence of Cdk2 and Cdk4. To investigate how Cdk2 and Cdk4 individually or synergistically contribute to the regulation of normal centrosome duplication and how they mediate centrosome amplification, wild-type, *p53*^{-/-}, *Cdk2*^{-/-}, *Cdk4*^{-/-}, *Cdk2*^{-/-} *Cdk4*^{-/-}, *p53*^{-/-} *Cdk2*^{-/-}, *p53*^{-/-} *Cdk4*^{-/-}, and *p53*^{-/-} *Cdk2*^{-/-} *Cdk4*^{-/-} embryonic day 13.5 (E13.5) mouse embryonic fibroblasts (MEFs) were generated. Genotypes were determined by allele-specific PCR (Fig. 1A). Western blotting confirmed the presence or absence of p53, Cdk2, and Cdk4 (Fig. 1B). To establish whether Cdk2 and Cdk4 were upregulated as compensatory mechanisms for their loss, expression levels of Cdk2 in *Cdk4*^{-/-} cells and of Cdk4 in *Cdk2*^{-/-} cells were examined using wild-type cells as controls (Fig. 1C). There is no compensatory upregulation of Cdk2 or Cdk4 in *Cdk4*^{-/-} or *Cdk2*^{-/-} MEFs; their levels are the same as those in wild-type controls.

Published observations indicate that early-passage *Cdk2*^{-/-} and *Cdk4*^{-/-} MEFs proliferate and that the kinetics of entry into S phase in *Cdk2*^{-/-} or *Cdk4*^{-/-} MEFs are moderately delayed relative to those of wild-type MEFs (5, 72). Additionally, *Cdk2*^{-/-} *Cdk4*^{-/-} MEFs senesce at earlier passages than the individual knockouts (5, 6, 52, 72). Ablation of *p53* abrogates senescence associated with the single or combined loss of Cdk2 and Cdk4 at late passages (53). To rule out the possibility that any reductions in the frequency of centrosome amplification in *p53*^{-/-} MEFs lacking Cdks are associated with major changes in the frequencies of proliferation, the G₁/S transition in each genotype was investigated. The MEFs indicated in Fig. 2 were plated, serum starved, stimulated with serum, and harvested at the indicated time points for measurement of S-phase entry by BrdU incorporation (data available on request). Most MEFs peaked with 30 to 50% of cells in S phase at 12 h (Fig. 2A). In contrast, *Cdk2*^{-/-} *Cdk4*^{-/-} MEFs entered S phase with delayed kinetics, and fewer than 10% of the cells were actively proliferating at 16 h. Likewise, all MEFs lacking p53 and the Cdks reached their peak frequency of cells in S phase at 12 h (Fig. 2B).

Our goal was to identify deregulated cell cycle-regulatory molecules that may trigger the centrosome amplification observed in *p53*^{-/-} MEFs. Various defects in the cell cycle-regulatory machinery result in deregulated Cdk activities and centrosome amplification. Those include overexpression of cy-

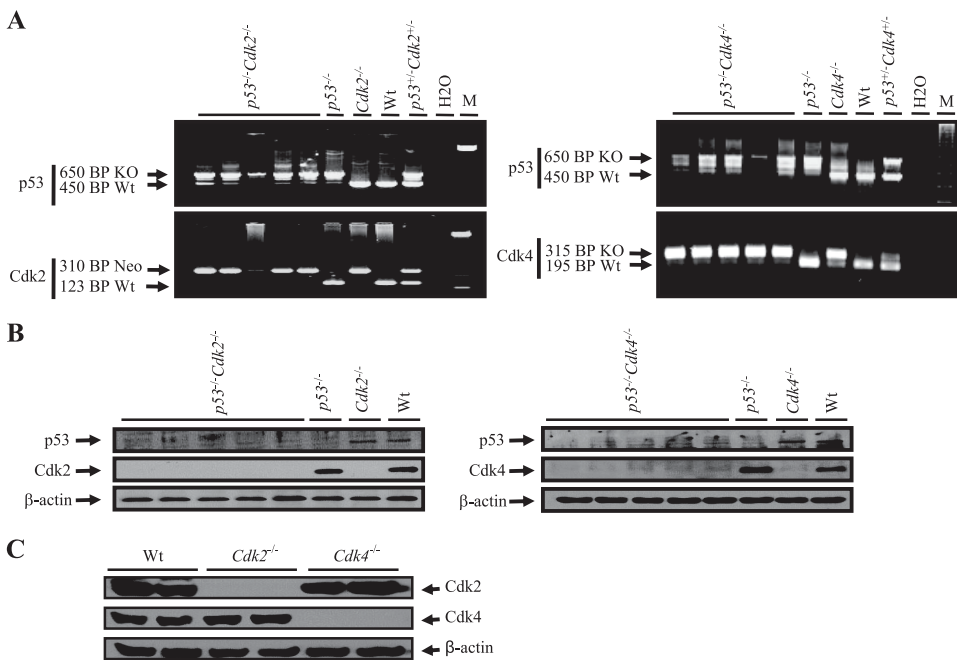


FIG. 1. Genetic ablation of *p53*, *Cdk2*, or *Cdk4* leads to absence of the respective protein expression. (A) PCR-based genotyping. Results of PCR analysis of genomic liver DNA from E.13.5 embryos generated by crossing $p53^{+/-} Cdk2^{+/-}$ or $p53^{+/-} Cdk4^{+/-}$ mice are shown. These gels included five double mutants ($p53^{-/-} Cdk2^{-/-}$ [left panel] or $p53^{-/-} Cdk4^{-/-}$ [right panel]), one $Cdk2^{-/-}$ mutant, one $Cdk4^{-/-}$ mutant, wild-type (Wt) embryos, and a control lacking DNA (H₂O). M, molecular size marker; KO, knockout. (B) To confirm the genotyping data generated in panel A, Western blotting was performed using antibodies specific to p53, Cdk2, and Cdk4. β -Actin was used as a loading control (bottom). (C) Western blotting was conducted to determine the expression levels of Cdk4 in $Cdk2^{-/-}$ MEFs and of Cdk2 in $Cdk4^{-/-}$ MEFs. To ensure that equal amounts of proteins were loaded, β -actin was used to probe the same membrane.

clins A, E, and D (27, 48–50, 59). To further investigate the molecular consequences of Cdk loss for regulatory molecules governing the G₁/S transition, and whether deregulation of various cyclins accounted for centrosome amplification in p53-null cells, Western blotting was performed to analyze the expression of cyclin A, cyclin D1, and cyclin E at the indicated time points (Fig. 2C). Levels of cyclin E were robust throughout the cell cycle, but its accumulation was decreased in $Cdk2^{-/-}$ and $Cdk2^{-/-} Cdk4^{-/-}$ MEFs from 4 to 12 h post-serum addition. Cyclin D1 levels reached maximal accumulation at 12 and 16 h. No major changes in cyclin D1 levels were observed, except for moderately diminished levels in $Cdk4^{-/-}$, $Cdk2^{-/-} Cdk4^{-/-}$, $p53^{-/-} Cdk4^{-/-}$, and $p53^{-/-} Cdk2^{-/-} Cdk4^{-/-}$ MEFs relative to those in MEFs of other genetic groups at 12 and 16 h. Cyclin A expression was low in all the MEFs before 8 h post-serum addition; its expression levels peaked at 16 h post-serum addition, with similar expression in all MEFs. We conclude that except for minor changes in the expression of cyclins E and D1 when their respective catalytic partners, Cdk2 and Cdk4, are ablated, the temporal cyclin expression patterns are what we would expect of a normal cell cycle, consistent with the similar kinetics of entry into S phase for all genetic groups.

Reduced expression of certain CKIs can also result in elevated frequencies of centrosome amplification, and their overexpression can suppress centrosome amplification (42, 66, 68). For example, ectopic expression of p16^{INK4A}, a Cdk4-specific inhibitor, prevents centriole duplication and centrosome amplification (42, 44). On the other hand, p27^{Kip1}, recently re-

ported to be a dual Cdk2 and Cdk4 inhibitor (54), prevents centrosome amplification triggered by gamma-irradiation (36, 66). In addition, ablation of p21^{Waf1}, a Cdk2-specific inhibitor at physiological levels and a Cdk4 inhibitor at higher levels (29, 30, 35), results in centrosome amplification; its ectopic expression partly suppresses centrosome amplification in $p53^{-/-}$ MEFs (13, 39, 68). To determine whether CKIs may deregulate Cdk2 and Cdk4 in $p53^{-/-}$ MEFs, leading to centrosome amplification, we probed Western blots with antibodies against p21^{Waf1}, p27^{Kip1}, p57^{Kip2}, and p16^{INK4A} (Fig. 2D). To detect constitutive signaling triggered by the absence of p53, Western blotting was performed on serum-starved cells. Those analyses showed that in $p53^{-/-}$ MEFs, endogenous p21^{Waf1} was undetectable. In contrast, p27^{Kip1} levels in $p53^{-/-}$ MEFs were unchanged from those in wild-type and other MEFs. p57^{Kip2} and p16^{INK4A} were overexpressed in $p53^{-/-}$ MEFs.

We also examined the expression level of p57^{Kip2} and p16^{INK4A} by siRNA-mediated silencing of p53 in wild-type MEFs (Fig. 2E). p53-specific siRNA duplex sequences were synthesized and used to knock down the p53 gene in three independent wild-type MEFs. Western blot analysis revealed that depletion of p53 in wild-type cells did not lead to a major elevation of p57^{Kip2} levels but that steady-state p16^{INK4A} levels were moderately increased. We conclude that the only major alteration in a cell cycle-regulatory molecule associated with centrosome amplification in $p53^{-/-}$ MEFs is the absence of p21^{Waf1}, consistent with published results (68). We also conclude that the reported high frequencies of centrosome amplification in $p53^{-/-}$ MEFs (22) occurred despite robust levels of

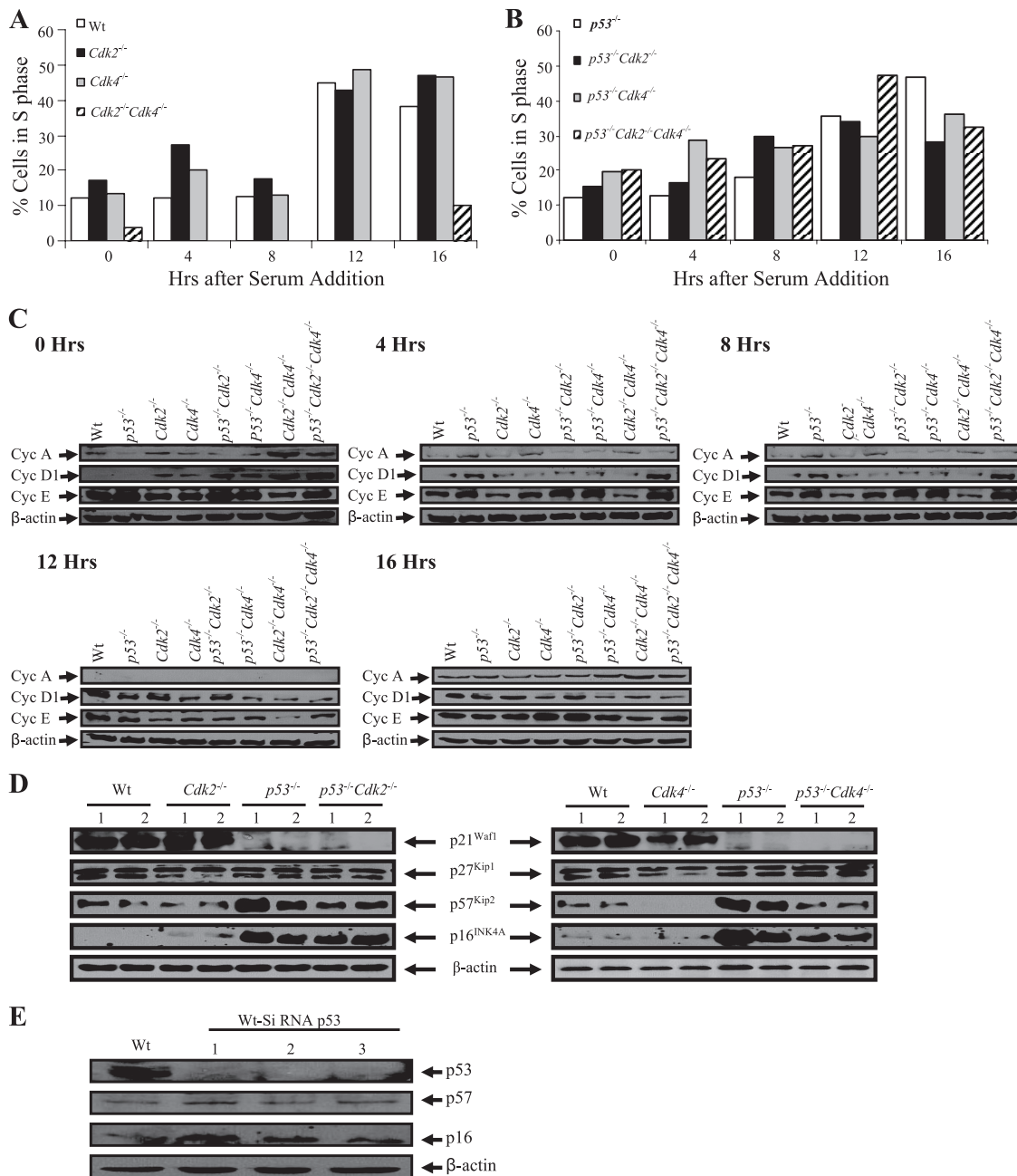


FIG. 2. Ablation of *Cdk2* or *Cdk4* does not significantly alter the cell cycle. (A and B) Cells of the indicated genotypes were arrested in G_0 and subsequently stimulated by addition of serum. Cells were pulse-labeled with BrdU 30 min prior to harvest and were harvested at the indicated time points after serum stimulation. Cells were stained with anti-BrdU antibodies and the appropriate secondary antibodies and were visualized using confocal microscopy and a $63\times$ objective. Nuclei were counterstained with DAPI. This experiment was repeated twice; results of a representative experiment are presented. Frequencies represent BrdU-positive cells in a population of at least 200 cells per group. Wt, wild type. (C) Whole-cell extracts were prepared from MEFs collected at the indicated time points following serum addition and were analyzed by Western blotting using antibodies against cyclin A, cyclin D1, cyclin E, and β -actin as a control. Cyc, cyclin. (D) Western blotting was performed with MEFs cultured in DMEM containing 0.2% FBS for 48 h. The numbers 1 and 2 above the lanes represent the loading of the protein lysates of two independent MEFs of the indicated genotypes. Western blots were probed with antibodies against p21^{Waf1}, p27^{Kip1}, p57^{Kip2}, and p16^{INK4A}. β -Actin served as a loading control. (E) Western blots of proteins extracted from controls (wild-type MEFs) or of wild-type MEFs transfected with siRNAs specific to p53 were probed with p53, p57, p16, and β -actin (control).

CKIs controlling *Cdk2* and *Cdk4* activities, including p16^{INK4A} and p27^{Kip1}, potent inhibitors of centrosome amplification and centriole duplication (36, 42, 44, 66).

Cells lacking *Cdk2* and *Cdk4* display abnormal centrosome cycles. MEFs devoid of *Cdk2* undergo minor defects in cen-

trirole duplication (14). This suggested to us that either the Cdks regulating the centrosome duplication cycle are redundant, as is the case with the Cdks regulating S-phase entry (2, 5, 6, 38, 52, 61, 72), or other Cdks or centrosomal kinases are solely responsible for orchestrating normal centrosome dupli-

cation. Since centrosome duplication licensing must be coordinated with entry into S phase (the latter initiated by cyclin D/Cdk4 and continued by cyclin E/Cdk2), and based on the brief expression overlap between cyclin D/Cdk4 and cyclin E/Cdk2 activities at the G₁/S transition (37), we hypothesized that cyclin D1/Cdk4 is involved in that coordination. To assess the involvement of Cdk4 in normal centrosome duplication, and to explore whether Cdk2 and Cdk4 cooperate to affect centrosome duplication, we measured the frequencies of cells with 1, 2, or ≥ 3 centrosomes in early-passage (passage 2) MEFs devoid of Cdk2 and/or Cdk4 by using immunohistochemistry with antibodies against pericentrin and γ -tubulin, core components of the centrosome (Fig. 3A). Previous studies showed that normal ratios of centrosomes in wild-type MEFs are 60% cells with one centrosome to 40% cells with two centrosomes (68). Any deviation in the ratios of centrosomes within a population is indicative of defects in the various steps driving the centrosome duplication cycle: licensing, separation of centrioles, and duplication of centrioles (59, 68). A centrosome ratio favoring cells with one centrosome is indicative of defective licensing of the centrosome cycle, or centriole separation, while a centrosome ratio favoring cells with two centrosomes is indicative of premature centriole separation and duplication. Consistent with published results, *Cdk2*^{-/-} MEFs did not display a statistically significant deviation in centrosome ratios from wild-type MEFs (48:46% versus 58:38%, respectively). In contrast, *Cdk4*^{-/-} MEFs showed a significant divergence from wild-type MEFs in the ratio of cells with one centrosome to cells with two centrosomes (77:20% versus 58:38%). This accumulation of cells with one centrosome is not due to a longer G₁ phase, since *Cdk4*^{-/-} MEFs entered S phase with kinetics similar to those of wild-type MEFs (as presented in Fig. 2A). In addition, *Cdk2*^{-/-} *Cdk4*^{-/-} MEFs also displayed a severe deviation from wild-type MEFs in centrosome ratios (35:55% versus 58:38%).

To assess the phase in the cell cycle at which the various centrosome defects occurred, we dissected the centrosome duplication kinetics in cells by comparative analysis of synchronized wild-type, *Cdk2*^{-/-}, *Cdk4*^{-/-}, and *Cdk2*^{-/-} *Cdk4*^{-/-} cells (Fig. 3B). MEFs of the indicated genotypes were grown in duplicate, followed by serum starvation for 60 h. Quiescent cells were stimulated with serum, and BrdU was included in the medium to monitor S-phase entry. Every 4 h for a period of 16 h, BrdU incorporation and the number of cells within the population with one or two centrosomes were scored. In accordance with the kinetics of entry into S phase, the percentage of wild-type MEFs with two centrosomes peaked at 12 h post-serum stimulation. *Cdk2*^{-/-} MEFs reached maximal ratios of cells with two centrosomes earlier than wild-type MEFs, at 8 h poststimulation. In contrast to wild-type or *Cdk2*^{-/-} MEFs, the percentages of *Cdk4*^{-/-} MEFs with two centrosomes decreased throughout the cell cycle, as the cells steadily accumulated a centrosome content of one. Another intriguing defect was that in *Cdk2*^{-/-} *Cdk4*^{-/-} MEFs, in which ablation of *Cdk2* overrode the defect in centrosome ratios in *Cdk4*^{-/-} MEFs. Even though *Cdk2*^{-/-} *Cdk4*^{-/-} MEFs were unable to replicate their DNA efficiently, their centrosome duplication peaked at 8 h post-serum stimulation; thus, the centrosome and cell cycles were uncoupled in those cells.

We then explored whether transient downregulation of

Cdk2 and Cdk4 with siRNAs in wild-type MEFs recapitulated the centrosome cycle defects in *Cdk2*^{-/-} or *Cdk4*^{-/-} MEFs (Fig. 3C and D). Wild-type MEFs were transfected with siRNA duplexes against Cdk2 or Cdk4. Seventy-two hours after transfection, cell lysates were obtained, and centrosome analyses were performed. Western blot analysis of the extracts from the transfected cells showed significant depletion of Cdk2 and Cdk4 (Fig. 3C). In contrast to the *Cdk2*^{-/-} centrosome profile, which showed normal ratios of centrosomes, siRNA-mediated downregulation of Cdk2 led to the accumulation of cells with two centrosomes; however, as with *Cdk4*^{-/-} MEFs, depletion of Cdk4 promoted more cells with one centrosome (Fig. 3D). Our results have identified one of the major triggers for normal centrosome duplication, which involves Cdk4, as well as the cooperation of Cdk2 and Cdk4 activities. Experiments that knocked out and knocked down Cdk2 or Cdk4 suggested that the functions of those Cdks are unique. To address whether ectopic expression of Cdk2 rescued the accumulation of cells with one centrosome observed for *Cdk4*^{-/-} MEFs, we overexpressed Cdk2 in *Cdk4*^{-/-} cells. As shown in Fig. 3E, Cdk2 significantly rescued the centrosome defects imposed by ablation of *Cdk4*, demonstrating that the centrosome defect imparted by ablated *Cdk4* is reversed by overexpression of Cdk2; in this scenario, Cdk2 and Cdk4 are redundant.

Individual ablation of *Cdk2* or *Cdk4* abolishes centrosome amplification in *p53*^{-/-} MEFs by preventing excessive centriole duplication. The current models attempting to explain how the absence of *p53* allows centrosome amplification propose that elevated Cdk2 activity is primarily responsible for centrosome amplification in those MEFs (21, 68). This was suggested by observations that *p21*^{Waf1}^{-/-} cells have elevated frequencies of centrosome amplification or that ectopic expression of *p21*^{Waf1} partly restored normal centrosome frequencies in *p53*^{-/-} MEFs (13, 68). Since *p21*^{Waf1} influences cyclinD/Cdk4 positively by promoting its assembly but inhibits it at higher concentrations (3, 29, 30, 35), we speculated that Cdk4 may also mediate centrosome amplification in *p53*^{-/-} MEFs. Therefore, we set out to explore the relative contributions of Cdk2 and/or Cdk4 to centrosome amplification in *p53*^{-/-} MEFs. While wild-type cells did not display elevated frequencies of centrosome amplification, loss of *p53* resulted in 40% of the cells displaying centrosome amplification (Fig. 4A and B). As predicted, ablation of *Cdk2* in *p53*^{-/-} MEFs prevented centrosome amplification (by approximately 70%). To our surprise, since no one has reported elevated Cdk4 activity in *p53*^{-/-} MEFs, and since our Western blots presented in Fig. 2 did not reveal any changes in any cyclins or CKIs that might promote the deregulation of Cdk4, ablation of *Cdk4* suppressed centrosome amplification in *p53*^{-/-} MEFs to the same extent as ablation of *Cdk2*. To establish whether Cdk2 and Cdk4 cooperated to further decrease centrosome amplification, we calculated the frequencies of centrosome amplification in *p53*^{-/-} *Cdk2*^{-/-} *Cdk4*^{-/-} MEFs. Indeed, *p53*^{-/-} *Cdk2*^{-/-} *Cdk4*^{-/-} MEFs displayed frequencies of centrosome amplification similar to those of *p53*^{-/-} *Cdk2*^{-/-} or *p53*^{-/-} *Cdk4*^{-/-} MEFs. We next used siRNAs to silence the expression of Cdk2 or Cdk4 in *p53*^{-/-} MEFs (Fig. 4C and D). Western blotting indicated that most of the Cdk2 or Cdk4 was depleted relative to the levels in the controls (Fig. 4C). Indeed,

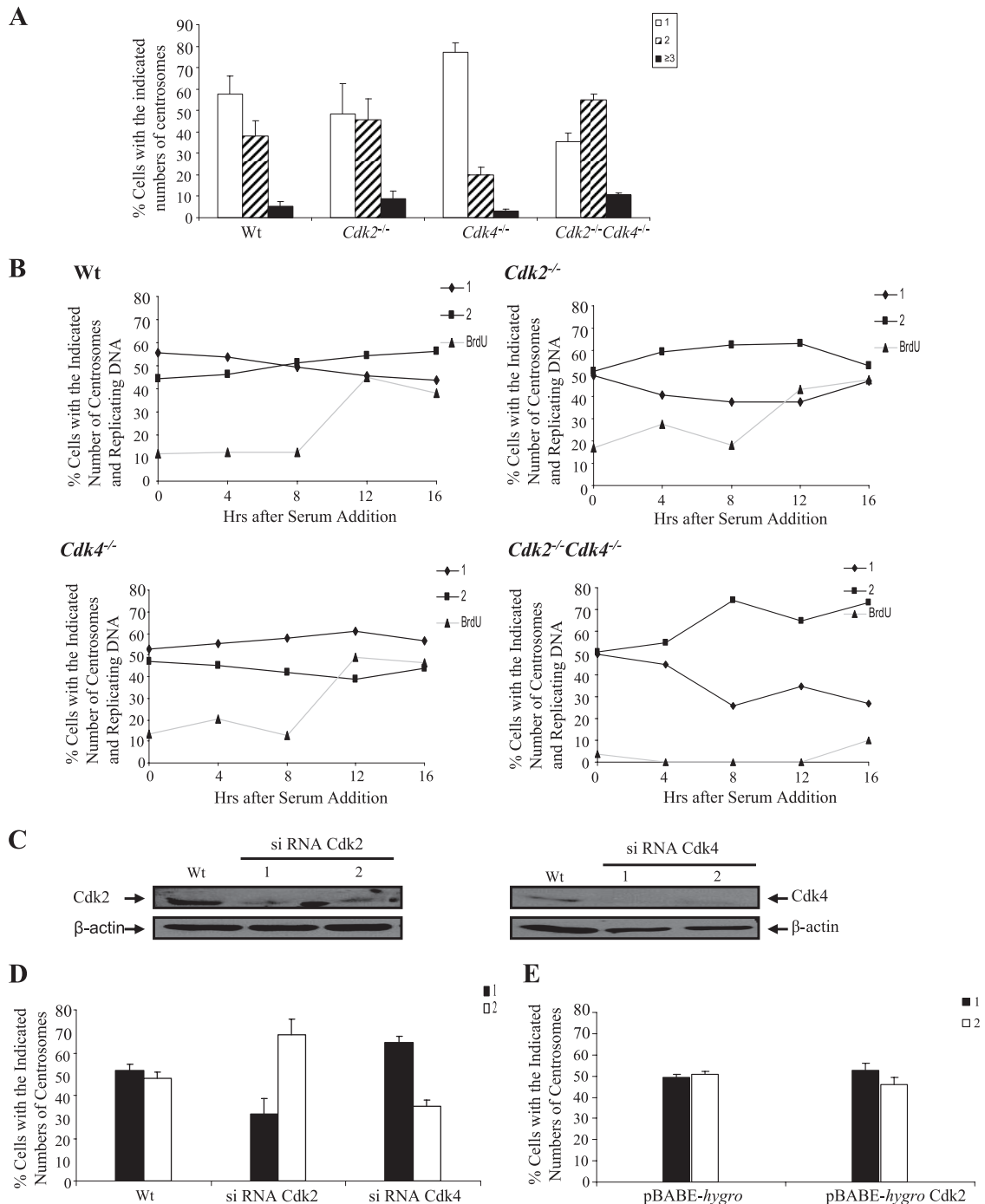


FIG. 3. Ablation of *Cdk2* and *Cdk4* or siRNA-mediated silencing of *Cdk2* and *Cdk4* leads to distinct centrosome cycle defects. (A) Proliferating E13.5 mouse embryonic fibroblasts of the indicated genotypes were fixed, processed, and coimmunostained with anti-pericentrin, anti- γ -tubulin, and the appropriate secondary antibodies. Averages \pm standard deviations of percentages of cells with one, two, and three centrosomes are shown. Exactly 8 wild-type (Wt), 3 *Cdk2*^{-/-}, 4 *Cdk4*^{-/-}, and 3 *Cdk2*^{-/-} *Cdk4*^{-/-} embryos were analyzed. The statistical significance of the averages ($P \leq 0.05$) was established by an unequal-variance *t* test. *t* test values for the percentages of cells in each population containing one centrosome relative to that of wild-type were 0.159885 for *Cdk2*^{-/-} MEFs, 0.000518 for *Cdk4*^{-/-} MEFs, and 0.000544 for *Cdk2*^{-/-} *Cdk4*^{-/-} MEFs. The *P* values for the percentages of cells in each population containing two centrosomes relative to that of wild-type MEFs were 0.122182 for *Cdk2*^{-/-} MEFs, 0.000172 for *Cdk4*^{-/-} MEFs, and 0.000528 for *Cdk2*^{-/-} *Cdk4*^{-/-} MEFs. The *P* values for the percentages of cells with ≥ 3 centrosomes relative to that of wild-type MEFs were 0.091487 for *Cdk2*^{-/-} MEFs, 0.06122 for *Cdk4*^{-/-} MEFs, and 0.000808 for *Cdk2*^{-/-} *Cdk4*^{-/-} MEFs. (B) MEFs of the indicated genotypes were grown in duplicate to confluence, followed by serum starvation for 60 h. Quiescent cells were stimulated with serum, and every 4 h for a period of 16 h, the numbers of cells with one and two centrosomes were scored. This experiment was repeated twice; results of a representative experiment are presented. The BrdU data are the same as those presented in Fig. 2A and B; they are shown here for purposes of clarity. (C) Western blots of proteins extracted from nontransfected wild-type cells, or from cells transfected with siRNAs against *Cdk2* or *Cdk4*, and probed with antibodies against *Cdk2* or *Cdk4*. The same membrane was probed with β -actin as a control. (D) Wild-type MEFs

as with the combinatorial knockouts, siRNA-mediated inhibition of Cdk2 or Cdk4 suppressed centrosome amplification in $p53^{-/-}$ MEFs (Fig. 4D). We conclude that Cdk2 and Cdk4 are individually required to mediate centrosome amplification.

In normal centrosome duplication, cells enter G_1 with a single centrosome composed of two centrioles: the mother centriole (older) and the daughter centriole (newer). To directly test whether Cdk2 and Cdk4 regulate centriole duplication, we performed a centriole reduplication assay. This assay involves challenging cells with hydroxyurea (HU) for 48 h, which inhibits DNA synthesis at late G_1 /early S phase. Since centrosomal checkpoints are functional in wild-type cells, centrosome reduplication is predicted to be absent in those cells (44). On the other hand, cells lacking certain checkpoint controls, such as those cells in which $p53$ is ablated, continue duplicating their centrioles within the centrosomes, resulting in multiple centrioles. For the centriole reduplication assay, proliferating MEFs of the indicated genotypes were either left untreated or treated with HU for 48 h. To determine the presence of centrioles, the cells were subjected to cold treatment and brief extraction prior to fixation. This treatment destabilizes microtubules nucleated at centrosomes; hence, centrioles can be microscopically visualized by immunostaining for α -tubulin, a major component of centrioles, at a high magnification. Coimmunostaining of cells subjected to cold treatment and brief extraction with anti- γ -tubulin (which detects the pericentriolar material [PCM]) and anti- α -tubulin revealed that wild-type cells stopped centrosome duplication after 48 h in culture in the presence of HU, while $p53^{-/-}$ cells continued centrosome duplication (Fig. 4E and F). On the other hand, ablation of either *Cdk2* or *Cdk4* in $p53^{-/-}$ cells completely halted centriole reduplication. The same experiment was repeated with 48-h cultures in the presence of mimosine or HU, with immunostaining done with γ -tubulin, and the results were the same: we detected the accumulation of centrosomes in $p53^{-/-}$ MEFs, and ablation of *Cdk2* or *Cdk4* prevented that accumulation (data not shown). These experiments demonstrated that ablation of *Cdk2* and *Cdk4* suppresses centrosome amplification by normalizing the centrosome reduplication defect triggered by ablation of $p53$. Importantly, this result identified one of the specific steps in the centrosome cycle affected by the absence of Cdk2 or Cdk4: centriole duplication.

Genetic ablation of *Cdk2* or *Cdk4* suppresses chromosome instability in $p53^{-/-}$ MEFs. Ablation of $p53$ generates chromosome instability through centrosome amplification (22, 23, 43) and by allowing the generation of reactive oxygen species, which are predicted to result in double-strand DNA breaks (60). To establish how the absence of Cdk2 and Cdk4 modu-

lates active chromosome instability in $p53^{-/-}$ MEFs, we used two assays: the micronucleus assay and γ -H2AX immunostaining. One of the initial cellular responses to the introduction of double-strand breaks is the phosphorylation of serine 139 of the carboxy-terminal tail of H2AX (56). The number of phosphorylated H2AX (γ -H2AX) molecules increases linearly with the severity of damage. Therefore, this assay represents a way to mark double-strand DNA breaks, a precursor to the chromosome breaks and recombinations leading to structural chromosomal abnormalities, the second major form of chromosome instability. Control experiments revealed that Adriamycin, a chemical that promotes DNA breaks, resulted in most cells in the population containing γ -H2AX foci (not shown). As shown in Fig. 5A and B, loss of $p53$ resulted in a significant elevation of the percentage of cells with γ -H2AX foci over that for wild-type controls. In contrast, ablation of *Cdk2* or *Cdk4* in $p53^{-/-}$ cells decreased the number of cells containing γ -H2AX relative to that observed for wild-type cells, consistent with published observations that loss of *Cdk2* reduces DNA repair (62).

Our second assay to detect active genomic instability was the micronucleus assay. A micronucleus is a chromosome or chromosome fragment missegregated during mitosis as a consequence of spindle damage or as a consequence of lost centromeric sequences (acentric chromosomes are unable to bind mitotic fibers and are excluded from the segregating chromatids) (31, 46, 69). Following cytokinesis, micronuclei appear in the cytoplasm as DNA-containing spheres surrounded by a nuclear membrane (Fig. 5C, arrows). The extent of micronucleus formation reflects the frequency of cells in a population actively losing whole chromosomes (a type of chromosome instability dependent on centrosome amplification), as well as the frequency of fragmented chromosomes, which arise as a result of DNA breaks (57, 58). Our results indicated that ablation of *Cdk2* or *Cdk4* reduced micronucleus formation in $p53^{-/-}$ MEFs to wild-type levels (Fig. 5D). We conclude that the absence of Cdk2 or Cdk4 prevents chromosome instability, an abnormal phenotype strongly associated with tumor biogenesis and progression (1, 11, 16, 17, 20).

Cdk2 and Cdk4 signal centrosome amplification and chromosome instability through a common phosphorylation site in nucleophosmin, Thr199. The nucleophosmin (NPM) protein, located at the centrosome, prevents premature centriole separation and duplication during early G_1 similarly to the way in which nuclear Rb prevents premature entry from early G_1 into S phase. Both proteins are phosphorylated in late G_1 by the Cdk2s to relieve negative regulation (37, 51). Unphosphorylated NPM binds to unduplicated centrosomes (centrosomes with

that either were left untransfected or were transfected with siRNAs against Cdk2 or Cdk4 were immunostained with anti- γ -tubulin antibodies, and frequencies were established by counting cells with one and two centrosomes in a population of at least 200 cells per group. Three independent MEF groups were used. The statistical significance of the averages ($P \leq 0.05$) was established by an unequal-variance t test. t test values for the percentage of cells in each population containing one centrosome relative to the percentage with two centrosomes were 0.215535 for wild-type MEFs, 0.003271 for MEFs transfected with a siRNA against Cdk2, and 0.008772 for those transfected with a siRNA against Cdk4. (E) Three independent *Cdk4*^{-/-} MEFs transfected with plasmids carrying either a control vector (pBABE-*hygro*) or pBABE-*hygro*-*Cdk2* and plated after selection with hygromycin were immunostained using anti- γ -tubulin antibodies and the appropriate secondary antibodies. Frequencies were established by counting cells with one and two centrosomes in a population of at least 200 cells per group. t test values of the percentage of cells in each population containing one centrosome relative to the percentage with two centrosomes were 0.345271 for pBABE-*hygro*-transfected MEFs and 0.136406 for pBABE-*hygro*-*Cdk2*-transfected MEFs.

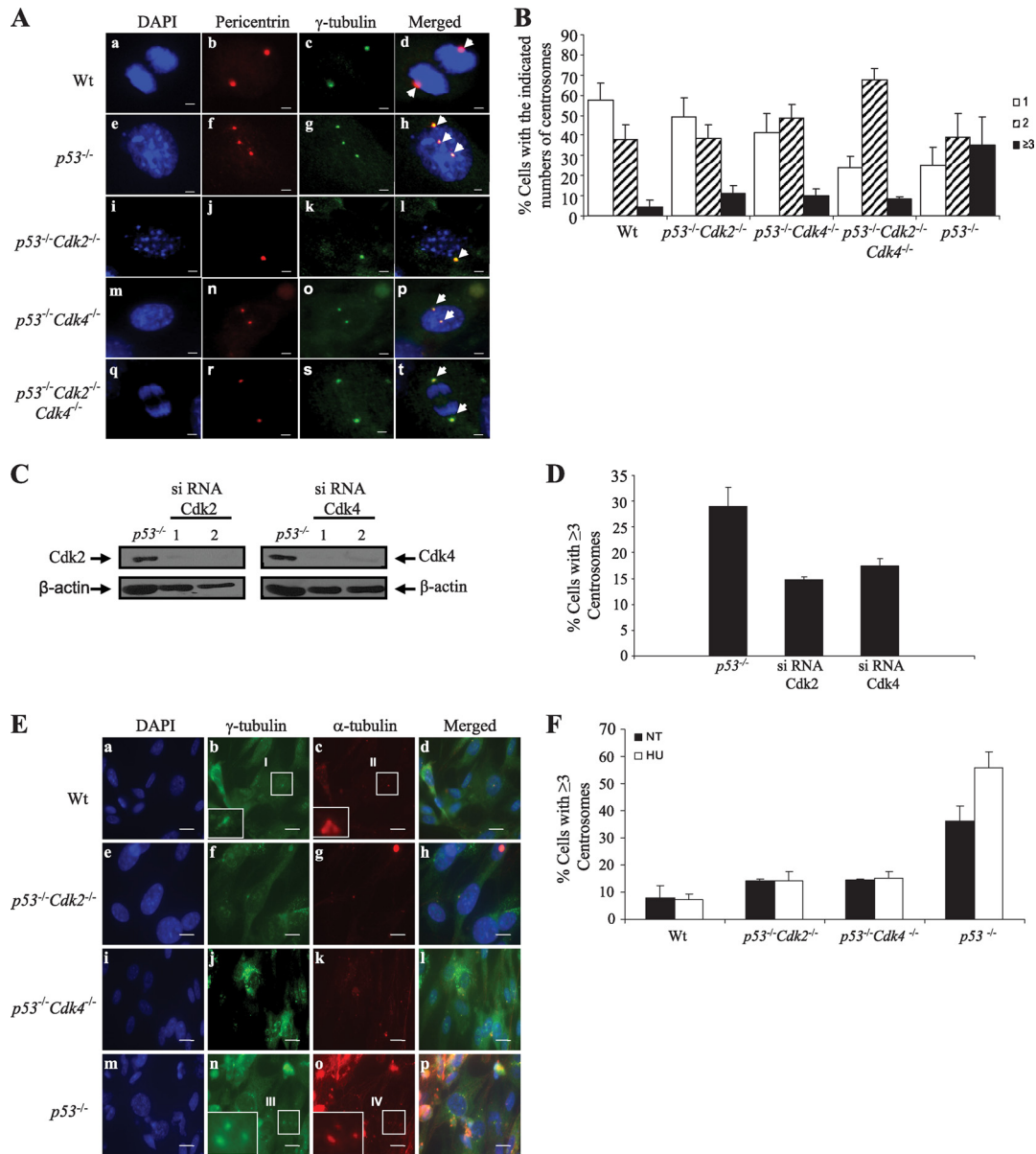


FIG. 4. Ablation or siRNA-mediated silencing of Cdk2 and Cdk4 prevents centriole reduplication and centrosome amplification in $p53^{-/-}$ MEFs. (A) MEFs of the indicated genotypes were coimmunostained with antibodies recognizing pericentrin (red) (b, f, j, n, and r) and γ -tubulin (green) (c, g, k, o, and s). Nuclei were stained with DAPI (blue) (a, e, i, m, and q). (d, h, l, p, and t) Overlay images of the pericentrin and γ -tubulin immunostaining. Wt, wild type. (B) Proliferating E13.5 mouse embryonic fibroblasts of the indicated genotypes were fixed, processed, and coimmunostained with anti-pericentrin, anti- γ -tubulin, and the appropriate secondary antibodies. The graph presents averages \pm standard deviations of the percentages of cells with one, two, and three or more centrosomes. Exactly 8 wild-type, 8 $p53^{-/-}$, 5 $p53^{-/-}$ Cdk2 $^{-/-}$, 5 $p53^{-/-}$ Cdk4 $^{-/-}$, and 4 $p53^{-/-}$ Cdk2 $^{-/-}$ Cdk4 $^{-/-}$ embryos were analyzed. *t* test values for the percentage of cells in each population containing one centrosome (relative to that for the wild type) were 0.017174 for $p53^{-/-}$ MEFs, 0.137854 for $p53^{-/-}$ Cdk2 $^{-/-}$ MEFs, 0.358121 for $p53^{-/-}$ Cdk4 $^{-/-}$ MEFs, and 3.95E-05 for $p53^{-/-}$ Cdk2 $^{-/-}$ Cdk4 $^{-/-}$ MEFs. *P* values for the percentage of cells in each population containing two centrosomes relative to that for wild-type MEFs were 0.860687 for $p53^{-/-}$ MEFs, 0.9713 for $p53^{-/-}$ Cdk2 $^{-/-}$ MEFs, 0.024679 for $p53^{-/-}$ Cdk4 $^{-/-}$ MEFs, and 2.69E-05 for $p53^{-/-}$ Cdk2 $^{-/-}$ Cdk4 $^{-/-}$ MEFs. *P* values for the percentage of cells with ≥ 3 centrosomes relative to that for wild-type MEFs were 0.006967 for $p53^{-/-}$ MEFs, 0.232114 for $p53^{-/-}$ Cdk2 $^{-/-}$ MEFs, 0.706722 for $p53^{-/-}$ Cdk4 $^{-/-}$ MEFs, and 0.051209 for $p53^{-/-}$ Cdk2 $^{-/-}$ Cdk4 $^{-/-}$ MEFs. (C) Western blots of extracts from untransfected $p53^{-/-}$ MEFs or $p53^{-/-}$ MEFs transfected with Cdk2- or Cdk4-specific siRNAs were probed with the indicated primary antibodies. (D) Frequencies of centrosome amplification in control $p53^{-/-}$ MEFs and in $p53^{-/-}$ MEFs in which Cdk2 or Cdk4 was knocked down. Three independent MEFs were used. Centrosomes were detected as for panels A and B. The statistical significance of the averages ($P \leq 0.05$) was established by an unequal-variance *t* test. *P* values for the percentage of cells with ≥ 3 centrosomes relative to that of control $p53^{-/-}$ MEFs were 0.002445 for MEFs transfected with a Cdk2-specific siRNA and 0.006696 for MEFs transfected with a Cdk4-specific siRNA. (E) Proliferating MEFs of the indicated genotypes (3 per group) were either left untreated (NT) or treated with 2 mM HU for 48 h. To determine the presence of centrioles, the cells were subjected to cold treatment and brief extraction prior to fixation. This treatment destabilizes microtubules nucleated at centrosomes; hence, centrioles can be microscopically visualized by immunostaining for α -tubulin (a major component of centrioles) at a high magnification. Cells were coimmunostained with anti- γ -tubulin polyclonal (green) (b, f, j, and n) and anti- α -tubulin monoclonal (red) (c, g, k, and o) antibodies and were counterstained with DAPI (blue) (a, e, i, and m). (d, h, l, and p) Overlaid images of γ -tubulin and α -tubulin immunostaining. (Insets) Magnified images of the areas indicated. (F) Frequencies of centriole reduplication were established by counting cells with ≥ 3 separated centrioles in a population of at least 200 cells per group. *P* values for HU-treated compared to NT cells were 0.791492 for wild-type MEFs, 1 for $p53^{-/-}$ Cdk2 $^{-/-}$ MEFs, 0.507158 for $p53^{-/-}$ Cdk4 $^{-/-}$ MEFs, and 0.012161 for $p53^{-/-}$ MEFs.

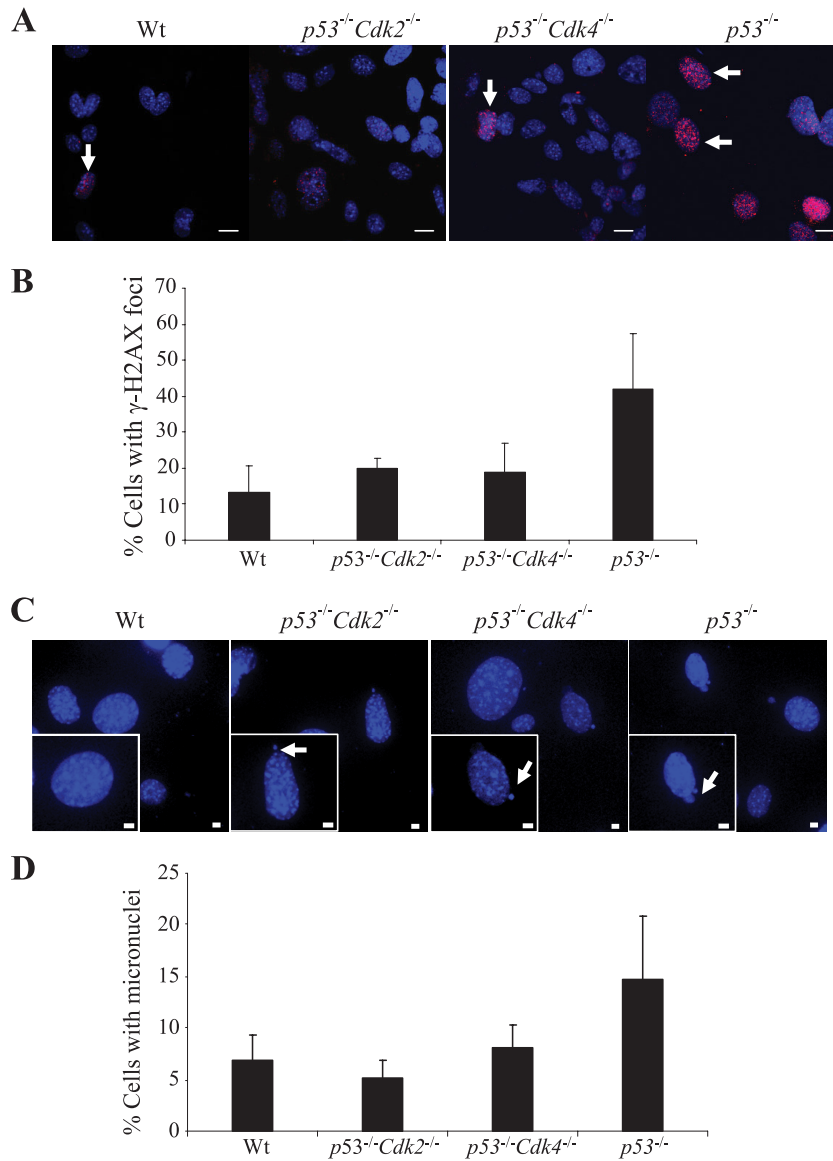


FIG. 5. Ablation of *Cdk2* and *Cdk4* inhibits chromosome instability in cells lacking p53. (A and B) The frequencies of γ -H2AX foci (arrows) in cells with the indicated genotypes were calculated. Bars, 10 μ m. The graph (B) shows the average percentage of γ -H2AX foci in each population of at least 200 cells. Error bars, standard deviations. Each group included 4 different MEFs. *P* values (relative to the wild-type control) were 0.015218 for $p53^{-/-}$ MEFs, 0.126173 for $p53^{-/-} Cdk2^{-/-}$ MEFs, and 0.346771 for $p53^{-/-} Cdk4^{-/-}$ MEFs. (C and D) Proliferating E13.5 mouse embryonic fibroblasts of the indicated genotypes were fixed, and nuclei were visualized with DAPI. Frequencies of micronucleus (insets and arrows) formation were calculated for at least 500 cells of each of the indicated genotypes. Each group included 4 different MEFs. *P* values (relative to the wild-type control) were 0.016122 for $p53^{-/-}$ MEFs, 0.137054 for $p53^{-/-} Cdk2^{-/-}$ MEFs, and 0.370282 for $p53^{-/-} Cdk4^{-/-}$ MEFs.

closely associated centrioles) to prevent premature centriole separation and duplication (51, 70). Upon phosphorylation by Cdk2 at the G₁/S transition on T199, NPM disassociates from the centrosome, allowing centriole separation and duplication (51, 70). Our laboratory previously correlated unregulated cyclin E/Cdk2 activity with phosphorylation of NPM at T199 (59). We demonstrated that cyclin E/Cdk2 phosphorylation of NPM^{T199} in G₀ rather than in late G₁ rendered NPM functionally inactive, since it no longer had the ability to bind centrosomes at G₀. This inability to bind centrosomes allowed a constitutive centrosome duplication cycle, where centrioles separated and duplicated uncontrollably, resulting in centro-

some amplification. Since the loss of *Cdk4* prevented centrosome amplification in $p53^{-/-}$ MEFs as efficiently as the ablation of *Cdk2*, we set out to establish whether they shared the same phosphorylation site in NPM to signal centrosome amplification and chromosome instability. Specifically, we tested whether suppression of centrosome amplification by ablation of *Cdk2* and *Cdk4* correlated with restoration of normal phosphorylation of NPM^{T199}. MEFs were serum starved to mimic G₀, and the phosphorylation status of NPM^{T199} was assessed by Western blotting (Fig. 6A). The following results provided important clues as to the mechanism by which ablation of *Cdk2* or *Cdk4* prevented centrosome amplification in $p53^{-/-}$ MEFs.

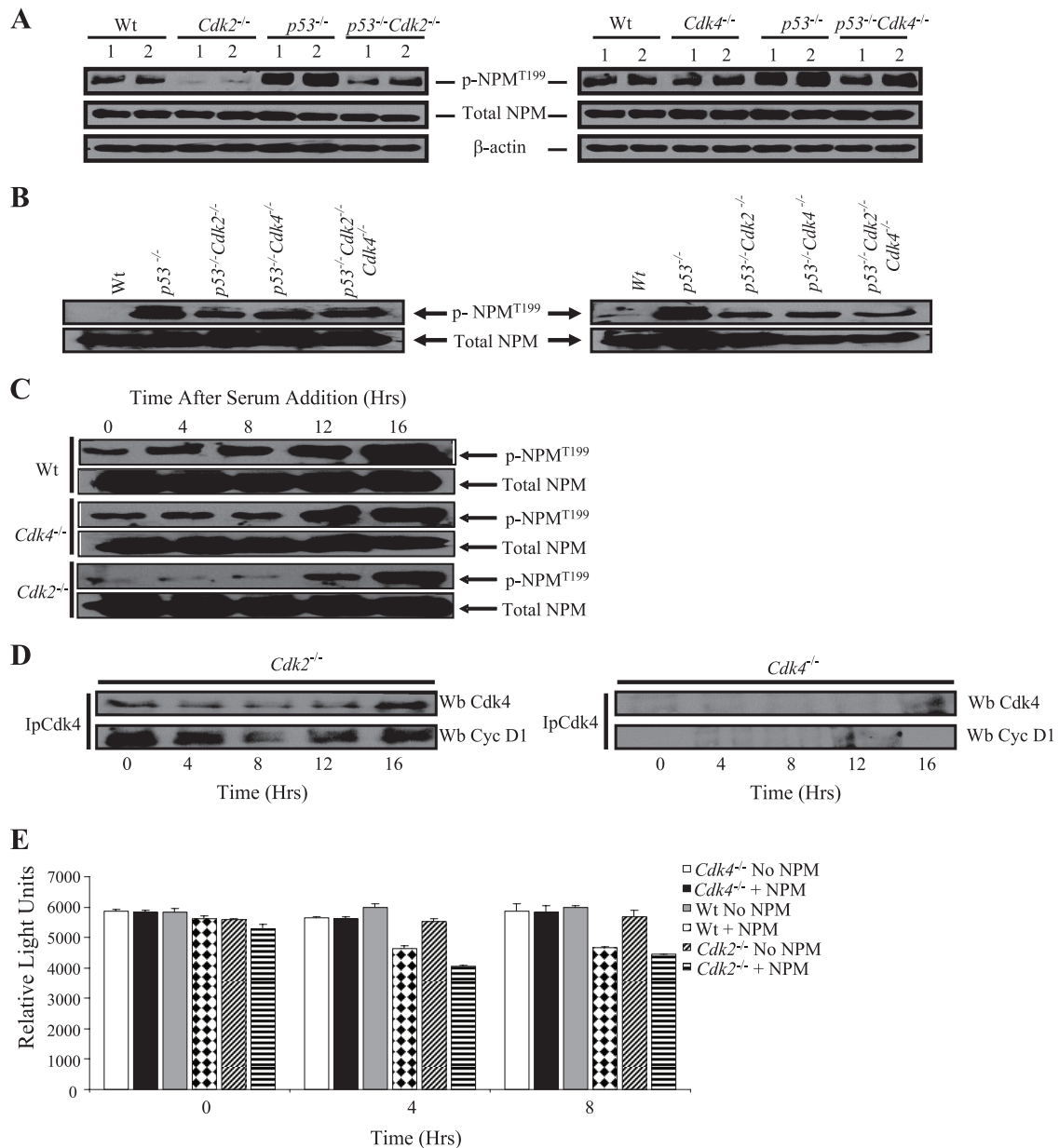


FIG. 6. Cdk2 and Cdk4 affect the centrosome cycle and centrosome amplification through NPM. (A) E13.5 MEFs of the indicated genotypes were serum starved for 60 h. Cells were preincubated with calyculin A, a serine/threonine phosphatase inhibitor. (Top) Western blot analysis of protein fractions of G_0 -arrested MEFs probed with antibodies against phospho-NPM^{T199} (p-NPM^{T199}). (Center and bottom) Blots were probed with antibodies against total nucleophosmin and β -actin to show equal loading. (B) MEFs of the indicated genotypes were treated with 2 mM HU for 48 h and were then preincubated with calyculin A, a serine/threonine phosphatase inhibitor, before protein extraction. Western blots of the protein extracts were probed with antibodies against NPM^{T199} or against total NPM (control). The MEFs for the left panel are independent of those for the right. (C) Western blot analyses of MEFs of the indicated genotypes that were serum arrested and released into the cell cycle for various times. Western blots of the protein extracts were probed with antibodies against NPM^{T199} or against total NPM (control). (D) Western blots of Cdk4 immunoprecipitated (Ip-Cdk4) from extracts of wild-type, $Cdk2^{-/-}$, or $Cdk4^{-/-}$ MEFs were probed with antibodies against Cdk4 and cyclin D1. (E) Cdk4 kinase assays of protein lysates from wild-type, $Cdk2^{-/-}$, and $Cdk4^{-/-}$ MEFs were carried out at various time points following serum addition. Results for the 0-, 4-, and 8-h time points are shown. The results are from three independent MEFs. The experiment was repeated at least twice, and results of a representative experiment are presented. The reaction mixtures either contained NPM peptide (+ NPM) or contained no NPM peptide (no NPM). Luminescence was recorded by the SpectraMax Gemini XS luminometer using the SoftMax program. P values of kinase assays comparing NPM to no NPM at each indicated time point were 0.107203829 for lysates from wild-type MEFs at 0 h, 0.037437678 for those from $Cdk2^{-/-}$ MEFs at 0 h, 0.000111335 for those from wild-type MEFs at 4 h, 0.002861355 for those from $Cdk2^{-/-}$ MEFs at 4 h, 0.000761355 for those from wild-type MEFs at 8 h, and 0.000449084 for those from $Cdk2^{-/-}$ MEFs at 8 h. The P values from the kinase assays performed with $Cdk4^{-/-}$ MEFs were greater than 0.05 at any given time point.

First, while wild-type cells displayed a baseline level of NPM phosphorylation, ablation of *Cdk2* resulted in a level of phosphorylation lower than that in wild-type cells. The relative level of phosphorylation of NPM in *Cdk4*^{-/-} MEFs was identical to that in wild-type MEFs. Second, ablation of *p53* resulted in constitutive phosphorylation of NPM^{T199}; importantly, the absence of Cdk2 or Cdk4 reduced the hyperphosphorylation of NPM^{T199} in *p53*^{-/-} MEFs to wild-type levels. These results suggested that ablation of *Cdk2* or *Cdk4* abrogated centrosome amplification in *p53*^{-/-} MEFs by restoring the normal phosphorylation of NPM^{T199} and reestablishing normal centrosome duplication licensing. To test whether the Thr-199 phosphorylation of NPM in *p53*^{-/-} MEFs was reduced by ablation of *Cdk2* or *Cdk4* under the conditions used for the centriole reduplication assay, MEFs were treated with HU for 48 h, followed by protein extraction. As shown in Fig. 6B, Western blotting indicated that in HU-arrested cells, ablation of *Cdk2* and/or *Cdk4* reduced the level of NPM^{T199} phosphorylation from that for *p53*^{-/-} MEFs.

NPM/B23 is a direct substrate of Cdk2 (51). To establish the status of NPM^{T199} phosphorylation in wild-type, *Cdk2*^{-/-}, and *Cdk4*^{-/-} MEFs throughout the cell cycle, we performed Western blotting at various time points following release from serum starvation (Fig. 6C). Phosphorylation of NPM^{T199} was moderately lower in *Cdk4*^{-/-} MEFs than in wild-type MEFs at 4 and 8 h. In contrast, phosphorylation of NPM^{T199} in *Cdk2*^{-/-} MEFs between 0 and 12 h was severely diminished. Because Western blotting suggested that NPM was indeed a target of Cdk4, we set out to demonstrate that Cdk4 can directly phosphorylate NPM. To that end, wild-type, *Cdk2*^{-/-}, and *Cdk4*^{-/-} MEFs were serum starved, stimulated by the addition of serum, and harvested at different time points, followed by protein extraction. The presence of Cdk4 (34 kDa) and its cofactor cyclin D1 (36 kDa) in Ip-Cdk4 was demonstrated by analysis of Western blots probed with anti-cyclin D1 and anti-Cdk4 antibodies (Fig. 6D). As expected, immunoprecipitations using antibodies against Cdk4 and cyclin D1 with *Cdk4*^{-/-} MEFs did not pull down any Cdk4 or cyclin D1, demonstrating the specificity of the antibodies. After showing that the antibodies specifically pulled down Cdk4, we performed luminescent kinase assays with the Kinase Glo kit (Promega), as previously described (67), immunoprecipitating cyclin D/Cdk4 complexes with antibodies against Cdk4 and incubating immunoprecipitates with purified NPM (Fig. 6E). In this assay, reduction of luminescence by the addition of kinase buffer and purified NPM peptide to the Cdk4 immunoprecipitates indicates kinase activity. To rule out the possibility that any contaminating Cdk2 activity would result in phosphorylation of NPM, we performed immunoprecipitation with synchronized *Cdk2*^{-/-} MEFs. As an additional control, we performed kinase assays with *Cdk4*^{-/-} MEFs. For extracts of wild-type or *Cdk2*^{-/-} MEFs from 4 and 8 h post-serum stimulation, considerable kinase activity was detected in samples containing NPM peptide compared to samples without the substrate (no NPM) (Fig. 6E). This phosphorylation was slightly reduced, but still statistically significant, in wild-type MEFs at 12 and 16 h after serum addition (data available on request). We then validated our experiment by including Ip-Cdk4 from extracts of *Cdk4*^{-/-} MEFs at the indicated time points; the extract from the *Cdk4*^{-/-} MEFs where no Cdk4 was precipitated showed no

kinase activity compared to samples without the substrate (no NPM). These results demonstrated that NPM is a Cdk4 target and that the maximal phosphorylation corresponded to the point of the cell cycle where centrosome cycle licensing occurs: mid/late G₁. However, unlike the phosphorylation of NPM by Cdk2, which occurs both in the nucleus and in the cytoplasm (59), due to a centrosome localization signal in cyclin E (41), phosphorylation of NPM by Cdk4 is predicted to be nuclear, because localization of Cdk4 and of cyclin D1 was strictly nuclear, and we did not find any Cdk4 or cyclin D1 within centrosomes (data available on request).

To gather more-direct evidence that the phosphorylation of NPM^{T199} by Cdk2 and Cdk4 is critical to centrosome amplification, plasmids encoding FLAG epitope-tagged wild-type NPM and the mutant NPM/B23 lacking the T199 phosphorylation site (NPM^{T199A}) were transfected into E13.5 MEFs derived from *p53* mutant embryos (Fig. 7). As control, the vector alone was transfected. Following neomycin selection, cells were examined for the level of expression of exogenous NPM/B23 by Western blot analysis using an anti-FLAG antibody. This analysis revealed that NPM- and NPM^{T199A}-transfected cells expressed similar protein levels (Fig. 7A). In addition, we set out to establish whether wild-type NPM or NPM^{T199A} modulated the frequencies of centrosome amplification in *p53*^{-/-} MEFs (Fig. 7B). This analysis revealed that while *p53*^{-/-} MEFs carrying the vector control and those expressing wild-type NPM had the same frequencies of centrosome amplification, the mutant lacking the G₁ Cdk phosphorylation site displayed greatly reduced frequencies of centrosome amplification. The extent of inhibition was almost identical to that in *p53*^{-/-} *Cdk2*^{-/-} or *p53*^{-/-} *Cdk4*^{-/-} MEFs (as presented in Fig. 4B). In summary, we have demonstrated that Cdk2 and Cdk4 hyperphosphorylate NPM and that this hyperphosphorylation is critical to centrosome amplification.

In addition, we investigated whether the suppression of centrosome amplification by NPM^{T199A} restored genomic stability. Cells expressing the vector and wild-type NPM had the same frequencies of micronucleus formation, while those expressing NPM^{T199A} had reduced frequencies (Fig. 7C). Likewise, NPM^{T199A} inhibited the formation of γ -H2AX double-stranded foci (Fig. 7D). We conclude from these experiments that ablation of either *Cdk2* or *Cdk4* prevents the generation of chromosome breaks and chromosome losses in *p53*^{-/-} MEFs. We propose a new paradigm for how centrosome amplification and chromosome instability arise in *p53*^{-/-} MEFs: the presence of both Cdk2 and Cdk4 is absolutely required in order to hyperphosphorylate NPM^{T199} to generate centrosome amplification and chromosome instability (Fig. 8).

DISCUSSION

In this study, we investigated the relative contributions of the G₁ Cdk—Cdk2 and Cdk4—to centrosome amplification in *p53*^{-/-} MEFs and to the regulation of the centrosome cycle. How do Cdk2 and Cdk4 affect the centrosome cycle? Ablation of *Cdk2* leads to moderate defects in centrosome duplication, suggesting redundancy in the control of this process (14); our results with asynchronously growing *Cdk2*^{-/-} MEFs confirmed those results. However, experiments measuring the centrosome cycle at various time points throughout the cell cycle in

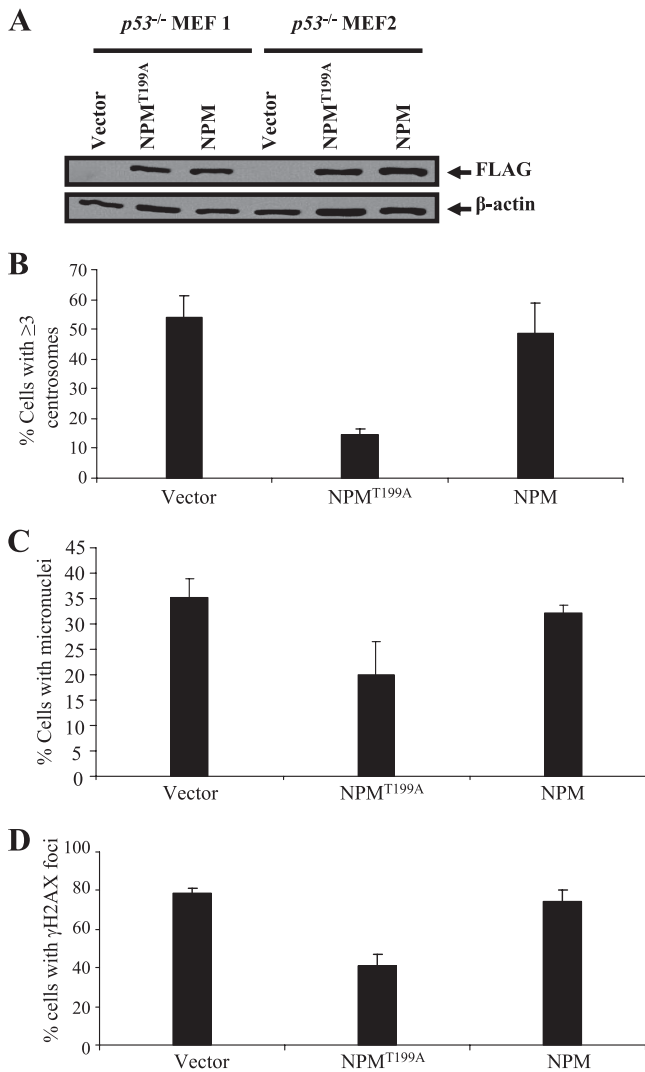


FIG. 7. NPM^{T199A} suppresses centrosome amplification and chromosome instability. (A) Passage 2 *p53*^{-/-} MEFs were transiently transfected with plasmids encoding FLAG epitope-tagged NPM and the NPM/B23 mutant (NPM^{T199A}). As a control, an empty vector was transfected. After neomycin selection, cell lysates were obtained and then probed with anti-FLAG antibodies. (B) The transfectants described in the legend to panel A were fixed, immunostained with anti- γ -tubulin polyclonal antibodies, and detected with Alexa Fluor 488-conjugated secondary antibodies. Cells were counterstained with DAPI. The number of cells with ≥ 3 centrosomes in a population of at least 200 cells was statistically analyzed by fluorescence microscopy. Each group included three transfected MEFs. *P* values (relative to the result for the transfected vector control) were 0.006963 for NPM^{T199A} and 0.560677 for wild-type NPM. (C) Proliferating E13.5 mouse embryonic fibroblasts of the indicated genotypes were fixed, and nuclei were visualized with DAPI. The frequencies of micronucleus formation in a population of 500 cells were calculated for the indicated genotypes. *P* values (relative to the result for the transfected vector control) were 0.011338 for NPM^{T199A} and 0.353737 for wild-type NPM. (D) Frequencies of γ -H2AX foci in cells with the indicated genotypes were calculated. *P* values (relative to the result for the transfected vector control) were 0.002591 for NPM^{T199A} and 0.476327 for wild-type NPM.

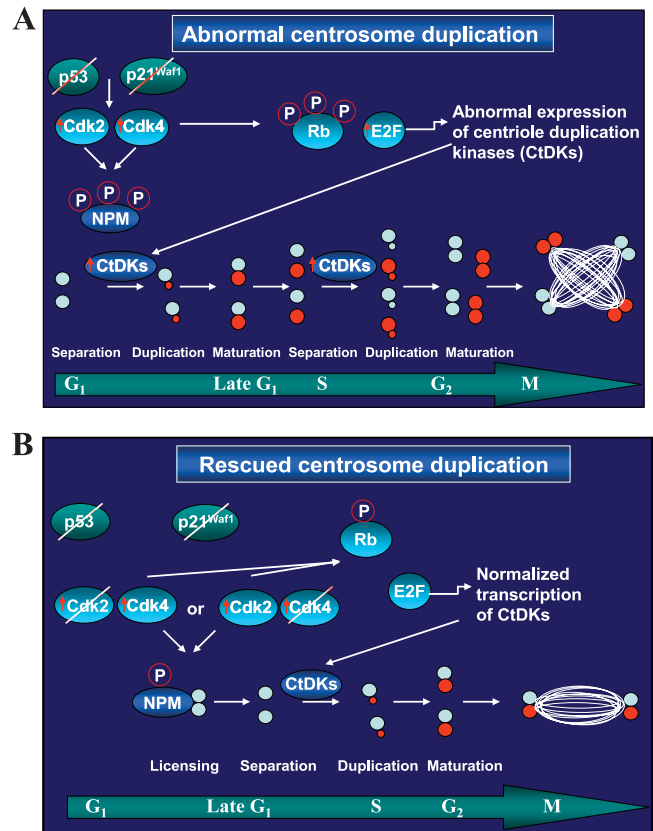


FIG. 8. Model explaining how the ablation of Cdk2 and Cdk4 prevents centrosome amplification. (A) Ablation of *p53* results in undetectable levels of *p21*^{Waf1}, leading to hyperactive Cdk2 and Cdk4. Hyperactive Cdk2 and Cdk4 cross talk to the centrosome in two modes. First, hyperactive Cdk2 and Cdk4 hyperphosphorylate Rb in the nucleus, leading to uncontrolled E2F-dependent transcription of molecules that influence various steps in the centrosome duplication cycle: those involved in centriole splitting, as well as centriole duplication kinases (CtdKs). Second, hyperactive Cdk2 and Cdk4 constitutively phosphorylate NPM^{T199}, resulting in excessive licensing of centrosome duplication. Uncontrolled expression of CtdKs and the inability of NPM to suppress normal centrosome duplication lead to faster centrosome duplication cycles within a single cell cycle, resulting in the formation of multiple centrosomes. (B) When Cdk2 or Cdk4 is deleted from *p53*^{-/-} MEFs, Rb is underphosphorylated, and the E2F-dependent transcription of CtdKs is restored. In addition, underphosphorylated NPM restores normal centrosome licensing and prevents excessive centriole duplication. This restricts the centrosome duplication cycle to one per cell cycle, thus resulting in normal centrosome numbers.

Cdk2^{-/-} and *Cdk4*^{-/-} MEFs, as well as transient downregulation of Cdk2 and Cdk4 using siRNAs, uncovered distinct centrosome cycle defects, suggesting that the functions of these Cdk2 and Cdk4 are nonredundant. For example, while *Cdk2* deficiency promoted the early separation and duplication of centrosomes, the absence of *Cdk4* promoted the accumulation of cells with one centrosome that failed to separate and duplicate. The accumulation of cells with one centrosome in *Cdk4*^{-/-} MEFs was not compensated for by passage, since the accumulation of cells with one centrosome was also observed in MEFs silenced with a siRNA directed against Cdk4. The accumulation of cells with one centrosome in *Cdk4*^{-/-} MEFs was not due to a block in G₁; these MEFs entered S phase with kinetics similar to

those of wild-type and *Cdk2*^{-/-} MEFs. On the other hand, inhibition of Cdk2 with siRNAs led to many cells in the population harboring two centrosomes, suggesting that that defect was compensated for in *Cdk2*^{-/-} MEFs by Cdk4, other Cdks, or centrosomal kinases. Our observations showed that concomitant ablation of *Cdk2* and *Cdk4* caused a severe accumulation of cells with two centrosomes; experiments following the cell and centrosome cycles in *Cdk2*^{-/-} *Cdk4*^{-/-} MEFs confirmed that centriole separation and duplication were premature. The fact that the centrosome cycle defect in *Cdk2*^{-/-} *Cdk4*^{-/-} MEFs was much more severe than that in other genetic groups suggests that Cdk2 and Cdk4 cooperate to regulate the centrosome cycle. The accumulation of cells with two centrosomes in asynchronously growing *Cdk2*^{-/-} *Cdk4*^{-/-} MEFs was not a complete block, since 40% of cells still contained one centrosome. Upon serum arrest, the ratio of the number of *Cdk2*^{-/-} *Cdk4*^{-/-} MEFs with one centrosome to that with two centrosomes was similar to that for wild-type MEFs, and upon serum addition, centrosomes separated and duplicated prematurely, indicating an active centrosome cycle in *Cdk2*^{-/-} *Cdk4*^{-/-} MEFs. In those MEFs, the cell and centrosome cycles were uncoupled: centriole separation and duplication preceded entry into S phase. Thus, as in the cell cycle, in the absence of Cdk2 and Cdk4, other Cdks or centrosome duplication kinases also play a role in driving the centrosome duplication cycle. That redundancy is also supported by the baseline phosphorylation of NPM^{T199} in *p53*^{-/-} *Cdk2*^{-/-} *Cdk4*^{-/-} MEFs cultured with HU, demonstrating that NPM^{T199} is phosphorylated by another kinase in the absence of Cdk2 and Cdk4. Those results indicated that the same redundancy operating within the kinases controlling S phase also exists in the regulation of the centrosome cycle.

Because NPM phosphorylation at Thr199 by Cdk2 is essential to signal entry into the centrosome cycle (51, 70), we assessed whether the centrosome defects observed in *Cdk2*^{-/-} and *Cdk4*^{-/-} MEFs were due in part to changes in NPM^{T199} phosphorylation. As reported previously, NPM^{T199} is a canonical target of Cdk2; our Western blot analyses revealed that at early time points (0 to 8 h) following the addition of serum to *Cdk2*^{-/-} MEFs, the phosphorylation of NPM^{T199} was greatly diminished from that for wild-type MEFs. The phosphorylation of NPM^{T199} increased in *Cdk2*^{-/-} MEFs at 12 h following serum addition. The fact that cyclin D1 levels peaked at 12 h of serum addition in *Cdk2*^{-/-} MEFs indicated that perhaps cyclin D1/Cdk4 was responsible for that phosphorylation. On the other hand, levels of NPM^{T199} were moderately reduced in *Cdk4*^{-/-} MEFs at early time points after serum addition (0 to 8 h). Our Cdk4 kinase assays using NPM as a substrate confirmed that NPM is indeed a Cdk4 target and that its phosphorylation is cell cycle regulated. In serum-arrested cells we did not detect phosphorylation of NPM; that phosphorylation commenced at 4 and 8 h following serum addition and was detected up to 16 h after serum addition. However, the fact that the reduction in NPM^{T199} phosphorylation was not nearly as great in *Cdk4*^{-/-} MEFs as in *Cdk2*^{-/-} MEFs suggests that the Cdk2 present in *Cdk4*^{-/-} MEFs actively phosphorylates NPM^{T199} and that the phosphorylation of NPM^{T199} is more efficiently carried out by Cdk2 than by Cdk4.

We speculate that cells have evolved compensatory mechanisms such that when they are devoid of Cdk2, Cdk4 can

indeed license the centrosome cycle. However, at this point we cannot rule out the possibility that Cdk2 phosphorylates NPM^{T199} to prime Cdk4 phosphorylation of NPM in other sites that would control centrosome licensing at late G₁. Indeed, there is evidence in the literature that phosphorylation of NPM at Thr234 and Thr237 by Cdk1 stabilizes NPM in the centrosome during M phase (9). A second possible reason why *Cdk4*^{-/-} MEFs display cells with one centrosome is that Cdk4 may directly phosphorylate and regulate the activity of a centriole separase, or it may regulate the Rb/E2F-dependent transcription of a centriole separase. Centriole separases are poorly understood; one centrosome separase normally active at M phase that displays centriole separase activity when ectopically expressed during interphase is Nek2A (18). A third possibility is that Cdk4 modulates other licenser factors besides NPM. The only other known centrosome-regulatory protein that may display licensing activity is CP110. Like NPM, CP110 is a phosphorylation target of Cdk2, and inhibition of its activity causes centrosome amplification (10). However, unlike NPM, which disassociates from centrosomes upon Cdk phosphorylation, CP110 is present in the centrosome to cap the synthesis of a new centriole (34). Thus, we think it unlikely that Cdk4 utilizes CP110 to license the centrosome cycle. Nevertheless, the activity inhibited by the ablation of Cdk4 that leads to the accumulation of cells with one centrosome is reversible, since ablation of Cdk2 (in *Cdk2*^{-/-} *Cdk4*^{-/-} MEFs), ectopic expression of Cdk2, or ablation of *p53* does not allow the accumulation of cells with one centrosome.

What causes the accumulation of two centrosomes in cells in which Cdk2 is knocked down, and in cells in which *Cdk2* and *Cdk4* are ablated? Our experiments following the cell and centrosome cycles in *Cdk2*^{-/-} MEFs and in *Cdk2*^{-/-} *Cdk4*^{-/-} MEFs revealed premature separation and duplication of centrosomes. The premature accumulation of cells with two centrosomes was present in early cell cycle stages of *Cdk2*^{-/-} MEFs, but the premature accumulation was restored at later passages cycle. Perhaps Cdk2 alone and Cdk2 and Cdk4 together impose negative regulation of centriole separases or of other centrosome kinases. For example, Cdk2 targets other than NPM may prevent the binding of centriole separases to the centriole pair. When *Cdk2* alone or both *Cdk2* and *Cdk4* are ablated, those centriole separases and/or centriole duplication kinases may associate with the centrosomes prematurely to signal early centriole separation and duplication. Other targets of Cdk2 that regulate various steps within the centrosome duplication cycle include CP110 and mMps-1 (10, 19). Plk4 has not been reported to be directly regulated by Cdk2 but requires Cdk2 activity for its maximum activity (26). Our future experiments will identify the centriole separases, centrosome cycle licensers, or centriole duplication kinases deregulated in *Cdk2*^{-/-}, *Cdk4*^{-/-}, and *Cdk2*^{-/-} *Cdk4*^{-/-} MEFs at the transcriptional or posttranslational level.

Our first major finding was that Cdk2 and Cdk4 regulate distinct steps in the centrosome cycle. The second major finding is that either Cdk2 or Cdk4 signals centrosome amplification in *p53*-null MEFs. Ablation of *p53* was proposed to lead to centrosome amplification, in part by preventing p21^{Waf1} from controlling Cdk2 (13, 39, 68). Using a genetic approach, we demonstrated that, indeed, Cdk2 is a major mediator of centrosome amplification in *p53*^{-/-} MEFs. A unique finding was

that ablation of *Cdk4* was equally effective at suppressing centrosome amplification. This was unexpected, since no one has reported elevated Cdk4 activity in *p53*^{-/-} MEFs. Western blot analysis assessing the relative levels of cyclins or CKIs that promote or inhibit Cdk2 or Cdk4 activities failed to identify any deregulated cell cycle-regulatory molecule that would promote Cdk4 activity. For example, levels of cyclin D1 were similar in all groups. On the other hand, p16 was upregulated in *p53*^{-/-} MEFs, which would be consistent with decreased Cdk4 activity.

How does Cdk4 mediate centrosome amplification in *p53*^{-/-} MEFs? At low concentrations, p21^{Waf1} is a specific inhibitor of Cdk2 and promotes the assembly of cyclin D/Cdk4 (35), while at higher concentrations it equally inhibits Cdk2, Cdk3, Cdk4, and Cdk6 (30, 35). In fact, there is precedent for the involvement of p21^{Waf1} in inhibiting Cdk2 and Cdk4 activities triggered by the Ras oncogene, since mice lacking p21^{Waf1} and overexpressing MMTV-H-Ras^{G12V} display higher Cdk2 and Cdk4 activities than those expressing H-Ras^{G12V} alone (4). Perhaps a similar scenario exists within the centrosome, as p21^{Waf1} may inhibit both Cdk2 and Cdk4 activities to regulate centrosome duplication; thus, ablation of *p53* and the concomitant absence of endogenous p21^{Waf1} may hyperactivate Cdk2 and Cdk4. Another possibility is that ablation of *Cdk2* or *Cdk4* changes the CKI/Cdk ratios within the cell, allowing more p21^{Waf1} to bind to and inhibit the Cdk that is still present in the cell; this compensatory mechanism has been observed in *Cdk4*^{-/-} MEFs, in which more p27^{Kip1} is available to bind and inhibit Cdk2 activity (72). However, observations that *p53*^{-/-} cells are devoid of detectable endogenous p21^{Waf1} render unlikely the possibility that more p21^{Waf1} is available to inhibit Cdk2 activity in *p53*^{-/-} *Cdk4*^{-/-} MEFs to prevent centrosome amplification. Other Cdk2s that may impose that kind of compensatory regulation are p16 and p57, which are overexpressed in *p53*^{-/-} MEFs. Nevertheless, since p16 and p57 did not accumulate upon transient knockdown of p53, and because the silencing of Cdk2 or Cdk4 was able to suppress centrosome amplification in the same cells, that level of indirect compensatory inhibition of Cdk2 or Cdk4 by the CKIs is unlikely. Still, because our experiments have not ruled out that type of compensatory mechanism, we will develop composite p53, Cdk, and CKI knockdowns and knockouts to directly address the individual roles of those CKIs in preventing the hyperactivity of the remaining Cdk.

The following evidence supports a direct role for Cdk2 and Cdk4 in mediating centrosome amplification in p53-null cells. First, concomitant loss of *Cdk2* and *Cdk4* in *p53*^{-/-} MEFs did not reduce the frequencies of centrosome amplification more than the ablation of each individual kinase, rendering unlikely the possibility that the compensatory mechanism imposed by CKIs on Cdk2 or Cdk4 kinase activities is responsible for the suppression of centrosome amplification when either kinase is ablated. Second, concomitant ablation of *Cdk2* and *Cdk4* in p53-deficient MEFs cultured in HU reduced NPM^{T199} hyperphosphorylation to the same extent as ablation of *Cdk2* or *Cdk4* alone. In addition, rendering the NPM^{T199} site nonphosphorylatable by mutating the Cdk2/Cdk4 phosphorylation site (NPM^{T199A}) suppressed centrosome amplification to the same extent as ablation of Cdk2 or Cdk4, demonstrating that ablation of Cdk2 or Cdk4 prevents maximum centrosome amplification.

Therefore, we conclude that both Cdk2 and Cdk4 are individually required to signal centrosome amplification in cells in which *p53* has been deleted.

How do Cdk2 and Cdk4 deregulate centrosome duplication, resulting in centrosome amplification? The model depicted in Fig. 8, based on published observations (44, 51, 59, 70) and the observations reported here, shows two ways in which the G₁ Cdk2s could affect centrosome amplification: first, by triggering unregulated phosphorylation of Rb and the release of E2Fs, and second, by the direct phosphorylation of centrosomal targets. Although we did not perform a wide screen for all the E2F targets that may result in centrosome amplification in *p53*^{-/-} MEFs, those E2F targets that we analyzed by Western blotting were unchanged (cyclins A, E, and D). Thus, in the present study, we tested the second scenario.

In this study, we resolved how Cdk2 and Cdk4 directly impinge on centrosome amplification through NPM. Our experiments indicated that ablation of *Cdk2* or *Cdk4* decreased NPM^{T199} hyperphosphorylation in *p53*^{-/-} MEFs arrested in G₀ (serum arrested) or in late G₁ (under an HU block). Those experiments suggested that restoration of the normal phosphorylation of NPM^{T199} normalized the licensing of the centrosome cycle, as well as the excessive centriole reduplication, in *p53*^{-/-} MEFs to prevent centrosome amplification. Our experiments showing that the introduction of a mutant NPM lacking Cdk2 and Cdk4 phosphorylation sites (NPM^{T199A}) abrogated centrosome amplification in *p53*^{-/-} MEFs to the same extent as the ablation of Cdk2 or Cdk4 demonstrated that, indeed, the Cdk2, Cdk4→NPM^{T199} pathway is central to the prevention of centrosome amplification. Based on our observations that Cdk2 and Cdk4 cross talk to the centrosome through the same phosphorylation site in NPM, Thr199, we propose a novel paradigm: that full-fledged phosphorylation of NPM by both Cdk2 and Cdk4 in *p53*^{-/-} MEFs is critical to the induction of centrosome amplification. How do Cdk2 and Cdk4 prevent centriole reduplication? Our experiments showing that ablation of *Cdk2* or *Cdk4* prevented centriole reduplication clearly identified an important step within the centrosome cycle that impinges on centrosome amplification. Western blot analyses showing that the hyperphosphorylation of NPM^{T199} is abrogated in *p53*^{-/-} *Cdk2*^{-/-}, *p53*^{-/-} *Cdk4*^{-/-}, and *p53*^{-/-} *Cdk2*^{-/-} *Cdk4*^{-/-} MEFs indicated that the restoration of normal centrosome cycle licensing is key to the prevention of centriole reduplication. Another putative mechanism is that the Cdk2s control the expression or activity of centriole duplication kinases, including Plk4, Plk2, and mMps-1. So far, Western blotting and real-time PCR have not detected increased levels of Plk4 in *p53*^{-/-} MEFs; we have yet to explore whether loss of p53 influences Plk4's kinase activity. Thus, future experiments will address how the activities of centriole duplication kinases are modulated by Cdk2 and Cdk4.

A third major finding was that ablation of *Cdk2* or *Cdk4* in *p53*^{-/-} MEFs inhibits two precursors to the two major types of chromosome instability: the formation of micronuclei (precursors to aneuploidy) and double-strand DNA breaks (precursors to chromosome rearrangements). We conclude that both G₁ Cdk2s are critical to centrosome amplification and chromosome instability in *p53*^{-/-} MEFs. Our data have important implications for cancer therapy, since the 50% of human cancers that harbor p53 mutations can be treated with small-

molecule inhibitors of Cdk2 or Cdk4, currently under development or undergoing clinical trials (63). By using inhibitors specific to either Cdk, we can remove the ability of *p53* ablation to generate chromosome instability, an abnormal phenotype strongly associated with a poor prognosis and resistance to chemotherapy (8, 15), thus stopping cancer progression in its tracks. One could accomplish this inhibition without the proliferative toxicity predicted to occur when Cdk2 and Cdk4 are concomitantly inhibited and without the mitotic and developmental defects triggered by Cdk1 inhibition (6, 61, 63).

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REFERENCES

- Albertson, D. G., C. Collins, F. McCormick, and J. W. Gray. 2003. Chromosome aberrations in solid tumors. *Nat. Genet.* **34**:369–376.
- Aleem, E., H. Kiyokawa, and P. Kaldis. 2005. Cdc2-cyclin E complexes regulate the G₁/S phase transition. *Nat. Cell Biol.* **7**:831–836.
- Ball, K. L., S. Lain, R. Fahraeus, C. Smythe, and D. P. Lane. 1997. Cell-cycle arrest and inhibition of Cdk4 activity by small peptides based on the carboxy-terminal domain of p21WAF1. *Curr. Biol.* **7**:71–80.
- Bears, D. J., R. J. Lee, D. A. Troyer, R. G. Pestell, and J. J. Windle. 2002. Differential effects of p21(WAF1/CIP1) deficiency on MMTV-ras and MMTV-myc mammary tumor properties. *Cancer Res.* **62**:2077–2084.
- Berthet, C., E. Aleem, V. Coppola, L. Tassarollo, and P. Kaldis. 2003. Cdk2 knockout mice are viable. *Curr. Biol.* **13**:1775–1785.
- Berthet, C., K. D. Klarman, M. B. Hilton, H. C. Suh, J. R. Keller, H. Kiyokawa, and P. Kaldis. 2006. Combined loss of Cdk2 and Cdk4 results in embryonic lethality and Rb hypophosphorylation. *Dev. Cell* **10**:563–573.
- Reference deleted.
- Castedo, M., A. Coquelle, I. Vitale, S. Vivet, S. Mouhamad, S. Viaud, L. Zitvogel, and G. Kroemer. 2006. Selective resistance of tetraploid cancer cells against DNA damage-induced apoptosis. *Ann. N. Y. Acad. Sci.* **1090**:35–49.
- Cha, H., C. Hancock, S. Dangi, D. Maignel, F. Carrier, and P. Shapiro. 2004. Phosphorylation regulates nucleophosmin targeting to the centrosome during mitosis as detected by cross-reactive phosphorylation-specific MKK1/MKK2 antibodies. *Biochem. J.* **378**:857–865.
- Chen, Z., V. B. Indjeian, M. McManus, L. Wang, and B. D. Dynlacht. 2002. CP110, a cell cycle-dependent CDK substrate, regulates centrosome duplication in human cells. *Dev. Cell* **3**:339–350.
- Coleman, W. B., and G. J. Tsongalis. 1999. The role of genomic instability in human carcinogenesis. *Anticancer Res.* **19**:4645–4664.
- Doxsey, S. 2002. Duplicating dangerously: linking centrosome duplication and aneuploidy. *Mol. Cell* **10**:439–440.
- Duensing, A., L. Ghanem, R. A. Steinman, Y. Liu, and S. Duensing. 2006. p21(Waf1/Cip1) deficiency stimulates centriole overduplication. *Cell Cycle* **5**:2899–2902.
- Duensing, A., Y. Liu, M. Tseng, M. Malumbres, M. Barbacid, and S. Duensing. 2006. Cyclin-dependent kinase 2 is dispensable for normal centrosome duplication but required for oncogene-induced centrosome overduplication. *Oncogene* **25**:2943–2949.
- Duesberg, P., R. Li, R. Sachs, A. Fabarius, M. B. Upender, and R. Hehlmann. 2007. Cancer drug resistance: the central role of the karyotype. *Drug Resist. Updat.* **10**:51–58.
- Duesberg, P., and D. Rasmick. 2000. Aneuploidy, the somatic mutation that makes cancer a species of its own. *Cell Motil. Cytoskeleton* **47**:81–107.
- Duesberg, P., D. Rasmick, R. Li, L. Winters, C. Rausch, and R. Hehlmann. 1999. How aneuploidy may cause cancer and genetic instability. *Anticancer Res.* **19**:4887–4906.
- Faragher, A. J., and A. M. Fry. 2003. Nek2A kinase stimulates centrosome disjunction and is required for formation of bipolar mitotic spindles. *Mol. Biol. Cell* **14**:2876–2889.
- Fisk, H. A., and M. Winey. 2001. The mouse Mps1p-like kinase regulates centrosome duplication. *Cell* **106**:95–104.
- Fukasawa, K. 2005. Centrosome amplification, chromosome instability and cancer development. *Cancer Lett.* **230**:6–19.
- Fukasawa, K. 2008. p53, cyclin-dependent kinase and abnormal amplification of centrosomes. *Biochim. Biophys. Acta* **1786**:15–23.
- Fukasawa, K., T. Choi, R. Kuriyama, S. Rulong, and G. F. Vande Woude. 1996. Abnormal centrosome amplification in the absence of p53. *Science* **271**:1744–1747.
- Fukasawa, K., F. Wiener, G. F. Vande Woude, and S. Mai. 1997. Genomic instability and apoptosis are frequent in p53 deficient young mice. *Oncogene* **15**:1295–1302.
- Geng, Y., W. Whoriskey, M. Y. Park, R. T. Bronson, R. H. Medema, T. Li, R. A. Weinberg, and P. Sicinski. 1999. Rescue of cyclin D1 deficiency by knockin cyclin E. *Cell* **97**:767–777.
- Geng, Y., Q. Yu, E. Sicinska, M. Das, J. E. Schneider, S. Bhattacharya, W. M. Rideout, R. T. Bronson, H. Gardner, and P. Sicinski. 2003. Cyclin E ablation in the mouse. *Cell* **114**:431–443.
- Habedanck, R., Y. D. Stierhof, C. J. Wilkinson, and E. A. Nigg. 2005. The Polo kinase Plk4 functions in centriole duplication. *Nat. Cell Biol.* **7**:1140–1146.
- Hanashiro, K., M. Kanai, Y. Geng, P. Sicinski, and K. Fukasawa. 2008. Roles of cyclins A and E in induction of centrosome amplification in p53-compromised cells. *Oncogene* **27**:5288–5302.
- Harbour, J. W., R. X. Luo, A. Dei Santi, A. A. Postigo, and D. C. Dean. 1999. Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G₁. *Cell* **98**:859–869.
- Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi, and S. J. Elledge. 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G₁ cyclin-dependent kinases. *Cell* **75**:805–816.
- Harper, J. W., S. J. Elledge, K. Keyomarsi, B. Dynlacht, L. H. Tsai, P. Zhang, S. Dobrowski, C. Bai, L. Connell-Crowley, E. Swindell, et al. 1995. Inhibition of cyclin-dependent kinases by p21. *Mol. Biol. Cell* **6**:387–400.
- Hennig, U. G., N. L. Rudd, and D. I. Hoar. 1988. Kinetochores immunofluorescence in micronuclei: a rapid method for the in situ detection of aneuploidy and chromosome breakage in human fibroblasts. *Mutat. Res.* **203**:405–414.
- Hinchcliffe, E. H., C. Li, E. A. Thompson, J. L. Maller, and G. Sluder. 1999. Requirement of Cdk2-cyclin E activity for repeated centrosome reproduction in *Xenopus* egg extracts. *Science* **283**:851–854.
- Hinchcliffe, E. H., and G. Sluder. 2002. Two for two: Cdk2 and its role in centrosome doubling. *Oncogene* **21**:6154–6160.
- Kleylein-Sohn, J., J. Westendorf, M. Le Clech, R. Habedanck, Y. D. Stierhof, and E. A. Nigg. 2007. Plk4-induced centriole biogenesis in human cells. *Dev. Cell* **13**:190–202.
- LaBaer, J., M. D. Garrett, L. F. Stevenson, J. M. Slingerland, C. Sandhu, H. S. Chou, A. Fattaey, and E. Harlow. 1997. New functional activities for the p21 family of CDK inhibitors. *Genes Dev.* **11**:847–862.
- Lacey, K. R., P. K. Jackson, and T. Stearns. 1999. Cyclin-dependent kinase control of centrosome duplication. *Proc. Natl. Acad. Sci. U. S. A.* **96**:2817–2822.
- Lundberg, A. S., and R. A. Weinberg. 1998. Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. *Mol. Cell Biol.* **18**:753–761.
- Malumbres, M., R. Sotillo, D. Santamaria, J. Galan, A. Cerezo, S. Ortega, P. Dubus, and M. Barbacid. 2004. Mammalian cells cycle without the D-type cyclin-dependent kinases Cdk4 and Cdk6. *Cell* **118**:493–504.
- Mantel, C., S. E. Braun, S. Reid, O. Henegariu, L. Liu, G. Hangoc, and H. E. Broxmeyer. 1999. p21(cip-1/waf-1) deficiency causes deformed nuclear architecture, centriole overduplication, polyploidy, and relaxed microtubule damage checkpoints in human hematopoietic cells. *Blood* **93**:1390–1398.
- Matsumoto, Y., K. Hayashi, and E. Nishida. 1999. Cyclin-dependent kinase 2 (Cdk2) is required for centrosome duplication in mammalian cells. *Curr. Biol.* **9**:429–432.
- Matsumoto, Y., and J. L. Maller. 2004. A centrosomal localization signal in cyclin E required for Cdk2-independent S phase entry. *Science* **306**:885–888.
- McDermott, K. M., J. Zhang, C. R. Holst, B. K. Kozakiewicz, V. Singla, and T. D. Tlsty. 2006. p16(INK4a) prevents centrosome dysfunction and genomic instability in primary cells. *PLoS Biol.* **4**:e51.
- Meraldi, P., R. Honda, and E. A. Nigg. 2002. Aurora-A overexpression reveals tetraploidization as a major route to centrosome amplification in p53^{-/-} cells. *EMBO J.* **21**:483–492.
- Meraldi, P., J. Lukas, A. M. Fry, J. Bartek, and E. A. Nigg. 1999. Centrosome duplication in mammalian somatic cells requires E2F and Cdk2-cyclin A. *Nat. Cell Biol.* **1**:88–93.
- Mikule, K., B. Delaval, P. Kaldis, A. Jurczyk, P. Hergert, and S. Doxsey. 2007. Loss of centrosome integrity induces p38–p53–p21-dependent G₁-S arrest. *Nat. Cell Biol.* **9**:160–170.
- Miller, B. M., H. F. Zitzelsberger, H. U. Weier, and I. D. Adler. 1991. Classification of micronuclei in murine erythrocytes: immunofluorescent

- staining using CREST antibodies compared to in situ hybridization with biotinylated gamma satellite DNA. *Mutagenesis* **6**:297–302.
47. **Montagna, C., E. R. Andrechek, H. Padilla-Nash, W. J. Muller, and T. Ried.** 2002. Centrosome abnormalities, recurring deletions of chromosome 4, and genomic amplification of HER2/neu define mouse mammary gland adenocarcinomas induced by mutant HER2/neu. *Oncogene* **21**:890–898.
 48. **Mussen, J. G., H. F. Horn, P. E. Carroll, M. Okuda, P. Tarapore, L. A. Donehower, and K. Fukasawa.** 2000. Synergistic induction of centrosome hyperamplification by loss of p53 and cyclin E overexpression. *Oncogene* **19**:1635–1646.
 49. **Nakayama, K., H. Nagahama, Y. A. Minamishima, M. Matsumoto, I. Nakamichi, K. Kitagawa, M. Shirane, R. Tsunematsu, T. Tsukiyama, N. Ishida, M. Kitagawa, K. Nakayama, and S. Hatakeyama.** 2000. Targeted disruption of Skp2 results in accumulation of cyclin E and p27(Kip1), polyploidy and centrosome overduplication. *EMBO J.* **19**:2069–2081.
 50. **Nelsen, C. J., R. Kuriyama, B. Hirsch, V. C. Negron, W. L. Lingle, M. M. Goggins, M. W. Stanley, and J. H. Albrecht.** 2005. Short term cyclin D1 overexpression induces centrosome amplification, mitotic spindle abnormalities, and aneuploidy. *J. Biol. Chem.* **280**:768–776.
 51. **Okuda, M., H. F. Horn, P. Tarapore, Y. Tokuyama, A. G. Smulian, P. K. Chan, E. S. Knudsen, I. A. Hofmann, J. D. Snyder, K. E. Bove, and K. Fukasawa.** 2000. Nucleophosmin/B23 is a target of CDK2/cyclin E in centrosome duplication. *Cell* **103**:127–140.
 52. **Ortega, S., I. Prieto, J. Odajima, A. Martin, P. Dubus, R. Sotillo, J. L. Barbero, M. Malumbres, and M. Barbacid.** 2003. Cyclin-dependent kinase 2 is essential for meiosis but not for mitotic cell division in mice. *Nat. Genet.* **35**:25–31.
 53. **Padmakumar, V. C., E. Aleem, C. Berthet, M. B. Hilton, and P. Kaldis.** 2009. Cdk2 and Cdk4 activities are dispensable for tumorigenesis caused by the loss of p53. *Mol. Cell. Biol.* **29**:2582–2593.
 54. **Ray, A., M. K. James, S. Laroche, R. P. Fisher, and S. W. Blain.** 2009. p27Kip1 inhibits cyclin D–cyclin-dependent kinase 4 by two independent modes. *Mol. Cell. Biol.* **29**:986–999.
 55. **Reddy, H. K., R. V. Mettus, S. G. Rane, X. Grana, J. Litvin, and E. P. Reddy.** 2005. Cyclin-dependent kinase 4 expression is essential for neu-induced breast tumorigenesis. *Cancer Res.* **65**:10174–10178.
 56. **Rogakou, E. P., D. R. Pilch, A. H. Orr, V. S. Ivanova, and W. M. Bonner.** 1998. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J. Biol. Chem.* **273**:5858–5868.
 57. **Saavedra, H. I., K. Fukasawa, C. W. Conn, and P. J. Stambrook.** 1999. MAPK mediates RAS-induced chromosome instability. *J. Biol. Chem.* **274**:38083–38090.
 58. **Saavedra, H. I., J. A. Knauf, J. M. Shirokawa, J. Wang, B. Ouyang, R. Elisei, P. J. Stambrook, and J. A. Fagin.** 2000. The RAS oncogene induces genomic instability in thyroid PCCL3 cells via the MAPK pathway. *Oncogene* **19**:3948–3954.
 59. **Saavedra, H. I., B. Maiti, C. Timmers, R. Altura, Y. Tokuyama, K. Fukasawa, and G. Leone.** 2003. Inactivation of E2F3 results in centrosome amplification. *Cancer Cell* **3**:333–346.
 60. **Sablina, A. A., A. V. Budanov, G. V. Ilyinskaya, L. S. Agapova, J. E. Kravchenko, and P. M. Chumakov.** 2005. The antioxidant function of the p53 tumor suppressor. *Nat. Med.* **11**:1306–1313.
 61. **Santamaria, D., C. Barriere, A. Cerqueira, S. Hunt, C. Tardy, K. Newton, J. F. Caceres, P. Dubus, M. Malumbres, and M. Barbacid.** 2007. Cdk1 is sufficient to drive the mammalian cell cycle. *Nature* **448**:811–815.
 62. **Satyanarayana, A., M. B. Hilton, and P. Kaldis.** 2008. p21 inhibits Cdk1 in the absence of Cdk2 to maintain the G₁/S phase DNA damage checkpoint. *Mol. Biol. Cell* **19**:65–77.
 63. **Shapiro, G. I.** 2006. Cyclin-dependent kinase pathways as targets for cancer treatment. *J. Clin. Oncol.* **24**:1770–1783.
 64. **Sharma, N., C. Timmers, P. Trikha, H. I. Saavedra, A. Obery, and G. Leone.** 2006. Control of the p53-p21/CIP1 axis by E2f1, E2f2 and E2f3 is essential for G₁/S progression and cellular transformation. *J. Biol. Chem.* **281**:36124–36131.
 65. **Sherr, C. J., and J. M. Roberts.** 2004. Living with or without cyclins and cyclin-dependent kinases. *Genes Dev.* **18**:2699–2711.
 66. **Sugihara, E., M. Kanai, S. Saito, T. Nitta, H. Toyoshima, K. Nakayama, K. I. Nakayama, K. Fukasawa, M. Schwab, H. Saya, and M. Miwa.** 2006. Suppression of centrosome amplification after DNA damage depends on p27 accumulation. *Cancer Res.* **66**:4020–4029.
 67. **Tagliati, F., A. Bottoni, A. Bosetti, M. C. Zatelli, and E. C. degli Uberti.** 2005. Utilization of luminescent technology to develop a kinase assay: Cdk4 as a model system. *J. Pharm. Biomed. Anal.* **39**:811–814.
 68. **Tarapore, P., H. F. Horn, Y. Tokuyama, and K. Fukasawa.** 2001. Direct regulation of the centrosome duplication cycle by the p53-p21Waf1/Cip1 pathway. *Oncogene* **20**:3173–3184.
 69. **Thomson, E. J., and P. E. Perry.** 1988. The identification of micronucleated chromosomes: a possible assay for aneuploidy. *Mutagenesis* **3**:415–418.
 70. **Tokuyama, Y., H. F. Horn, K. Kawamura, P. Tarapore, and K. Fukasawa.** 2001. Specific phosphorylation of nucleophosmin on Thr(199) by cyclin-dependent kinase 2–cyclin E and its role in centrosome duplication. *J. Biol. Chem.* **276**:21529–21537.
 71. **Toledo, F., and G. M. Wahl.** 2006. Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. *Nat. Rev. Cancer* **6**:909–923.
 72. **Tsutsui, T., B. Hesabi, D. S. Moons, P. P. Pandolfi, K. S. Hansel, A. Koff, and H. Kiyokawa.** 1999. Targeted disruption of CDK4 delays cell cycle entry with enhanced p27(Kip1) activity. *Mol. Cell. Biol.* **19**:7011–7019.
 73. **Ussar, S., and T. Voss.** 2004. MEK1 and MEK2, different regulators of the G₁/S transition. *J. Biol. Chem.* **279**:43861–43869.
 74. **Vogelstein, B., D. Lane, and A. J. Levine.** 2000. Surfing the p53 network. *Nature* **408**:307–310.
 75. **Wu, L., C. Timmers, B. Maiti, H. I. Saavedra, L. Sang, G. T. Chong, F. Nuckolls, P. Giangrande, F. A. Wright, S. J. Field, M. E. Greenberg, S. Orkin, J. R. Nevins, M. L. Robinson, and G. Leone.** 2001. The E2F1, E2F2, and E2F3 transcription factors are essential for cellular proliferation. *Nature* **414**:457–462.
 76. **Xiong, Y., G. J. Hannon, H. Zhang, D. Casso, R. Kobayashi, and D. Beach.** 1993. p21 is a universal inhibitor of cyclin kinases. *Nature* **366**:701–704.
 77. **Yu, Q., Y. Geng, and P. Sicinski.** 2001. Specific protection against breast cancers by cyclin D1 ablation. *Nature* **411**:1017–1021.