Loss of Cyclin-Dependent Kinase 2 (CDK2) Inhibitory Phosphorylation in a CDK2AF Knock-In Mouse Causes Misregulation of DNA Replication and Centrosome Duplication

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Cyclin-dependent kinase 1 (CDK1) inhibitory phosphorylation controls the onset of mitosis and is essential for the checkpoint pathways that prevent the G₂- to M-phase transition in cells with unreplicated or damaged DNA. To address whether CDK2 inhibitory phosphorylation plays a similar role in cell cycle regulation and checkpoint responses at the start of the S phase, we constructed a mouse strain in which the two CDK2 inhibitory phosphorylation sites, threonine 14 and tyrosine 15, were changed to alanine and phenylalanine, respectively (CDK2AF). This approach showed that inhibitory phosphorylation of CDK2 had a major role in controlling cyclin E-associated kinase activity and thus both determined the timing of DNA replication in a normal cell cycle and regulated centrosome duplication. Further, DNA damage in G₁ CDK2AF cells did not downregulate cyclin E-CDK2 activity when the CDK inhibitor p21 was also knocked down. We were surprised to find that this was insufficient to cause cells to bypass the checkpoint and enter the S phase. This led to the discovery of two previously unrecognized pathways that control the activity of cyclin A at the G₁ DNA damage checkpoint and may thereby prevent S-phase entry even when cyclin E-CDK2 activity is deregulated.

Inhibitory phosphorylation on cyclin-dependent kinase 1 (CDK1) is an evolutionarily conserved regulatory pathway that controls the onset of mitosis (54, 63, 64). Myt-1- and Wee1-related kinases phosphorylate Cdk1 at two adjacent residues within its catalytic pocket, thereby preventing ATP binding and catalytic activity. The Cdc25-related proteins dephosphorylate these same residues, causing Cdk1 activation. These pathways are interconnected by positive- and negative-feedback loops: CDK1 phosphorylates and activates Cdc25 and phosphorylates and inactivates Wee1. Together, these create a bistable switch that results in the sudden, all-or-none activation of CDK1 at the onset of mitosis (21). Disruption of this pathway, for example, by expressing a mutant of CDK1 that lacks the inhibitory phosphorylation sites, dampens the normal operation of this cell cycle switch, and downstream events happen prematurely (30, 41) but less robustly (56, 62). This mutation also causes cells to bypass the checkpoint pathway that prevents mitosis when chromosomes are damaged or not fully replicated (18, 37, 60).

The components of the pathway that regulate Cdk1 by inhibitory phosphorylation at the G₂- to M-phase transition are conserved in a Cdk2 regulatory pathway that operates at the transition from G₁ into S phase. The Cdk1 inhibitory phosphorylation sites are conserved in the Cdk2 protein. The same Myt-1 and Wee-1 kinases that phosphorylate CDK1 also phosphorylate CDK2. In higher eukaryotes, the CDC25 phosphatases are a small gene family. In mammals, CDC25A, CDC25AB, and CDC25AC have all been implicated in the regulation of mitosis, and both CDC25A and CDC25B have been shown to dephosphorylate and activate CDK2 (40).

Dephosphorylation of CDK1 triggers the onset of mitosis and is a regulatory switch at the G₂ DNA damage checkpoint. In contrast, the role(s) of CDK2 inhibitory phosphorylation in controlling cell cycle progression is less well understood. CDK2 is phosphorylated on inhibitory threonine 14 and tyrosine 15 residues in proliferating cells, and dephosphorylation of those sites *in vitro*

substantially increases CDK2 activity (25, 31, 68). CDC25A activity is upregulated at the G₁- to S-phase transition by three mechanistically distinct CDK2-initiated positive-feedback loops (16, 35, 76). This is analogous to the regulation of CDC2 and CDC25 at mitosis, which suggests that the CDK2/CDC25A circuit may control a similar switch for the G₁- to S-phase transition.

In support of the idea that CDC25A regulates the transition into S phase, microinjection of antibodies against CDC25A blocks the cell cycle in the G₁ phase (38, 76). However, this was not confirmed by more-specific siRNA silencing experiments (46), perhaps due to functional redundancy among CDC25 family members (20, 45). On the other hand, ectopic overexpression of CDC25A causes premature cell cycle activation of CDK2 and advanced entry into S phase (7, 69). However, the effect of CDC25A overexpression on tyrosine phosphorylation of CDK2 is uncertain. In one report (69), tyrosine phosphorylation of CDK2 paradoxically was shown to increase in CDC25A-overexpressing cells, raising the issue of whether the direct *in vivo* physiological target of CDC25A was CDK2. Moreover, constitutive expression in *Drosophila* of a CDK2 mutant lacking the conserved inhibitory phosphorylation sites (CDK2AF) causes no phenotype abnormalities, which raises further questions about the physiological importance of CDK2 inhibitory phosphorylation as a critical regulatory path-

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way (44). However, the relevance of this to mammalian cell biology has been unclear, because *Drosophila* lacks CDC25A, which has been specifically implicated in CDK2 regulation in mammalian cells.

Tyrosine phosphorylation of CDK2 is also thought to be important for establishing DNA damage-induced cell cycle checkpoints, both at the G₁- to S-phase transition and within S-phase cells. DNA damage in G₁- and S-phase cells induces ATM- and ATR-dependent pathways that, via the Chk1 and Chk2 kinases, phosphorylate and ultimately inactivate CDC25A (3, 8, 19, 40, 48, 53). This correlates with decreased CDK2 activity, which is attributed both to an increase in CDK2 tyrosine phosphorylation (19, 48) and to p53-dependent induction of the CDK2 inhibitor p21 (57, 70). Enforced overexpression of CDK2AF or CDC25A overrides the checkpoint response to DNA damage in G₁- and S-phase cells, resulting in radioresistant DNA synthesis (13, 19). However, expression of a stabilized form of CDC25A that is not downregulated after DNA damage is insufficient to override the checkpoint response in S-phase cells (29, 71).

Activation of cyclin E-Cdk2 coordinately initiates both centrosome and chromosome duplication (32, 33, 42, 49). The mechanisms that couple centrosome and chromosome duplication and the mechanisms that limit centrosome duplication to just one occurrence per cell cycle are not well understood. Cyclin E-CDK2 is localized to centrosomes (50), as is CDC25B (17). Moreover, expression of a stabilized mutant of CDC25A causes centrosome amplification (71).

The importance of CDK2 tyrosine phosphorylation as a mechanism to control centrosome duplication has not been directly investigated. Moreover, whether CDK2 inhibitory phosphorylation regulates the onset of DNA replication, both in an unperturbed cell cycle and in response to DNA damage, remains uncertain. Experiments that have implicated this pathway in cell cycle and checkpoint control in mammalian cells have utilized ectopic overexpression of CDC25A, CDK2, or other cell cycle regulators and therefore may not be representative of normal cell physiology. We report here our analysis of a mouse strain in which the endogenous CDK2 gene has been replaced by homologous recombination with an allele that lacks both inhibitory phosphorylation sites (CDK2AF). We have addressed whether the absence of inhibitory CDK2 phosphorylation affects murine development and the regulation of DNA replication and centrosome duplication during a normal cell cycle and at the G₁ DNA-damage checkpoint.

MATERIALS AND METHODS

Constructs, antibodies, and reagents. The 4317G9 targeting vector and Mox2Cre mice were as described previously (6). pLXSN-MTcyclin A (Δ 80) was provided by Taku Chibazakura (Tokyo University of Agriculture, Tokyo, Japan) and pBP-p21-shRNA by Bruce E. Clurman (Fred Hutchinson Cancer Research Center, Seattle, WA). Anti-cyclin A (H432) and anti-Myc (sc-789) were purchased from Santa Cruz Biotechnology; anti-p21 (catalog no. 556431) and antibromodeoxyuridine (anti-BrdU; catalog no. 556028) from BD Pharmingen; anti-Grb2 (catalog no. 610112) from BD Transduction Laboratories; antipericentrin (catalog no. PRB-432C) from Covance; anti- γ -tubulin (T6557) and anti- α -tubulin (T9026) from Sigma-Aldrich; Alexa 488-conjugated secondary antibody from Invitrogen; and cyanin-3-conjugated secondary antibody from Jackson ImmunoResearch Laboratories.

Generation of CDK2 AF knock-in mice. To generate the CDK2^{T14A Y15F} allele, a 2,070-bp NheI genomic fragment containing the first coding exon of the CDK2 gene was inserted into a pcDNA3.1 vector. Thr14 and Tyr15

were mutated to Ala and Phe by the use of a QuikChange site-directed mutagenesis kit (Stratagene). DNA sequencing was performed to verify the mutagenesis. The mutant genomic fragment was then inserted into the 4317G9 targeting vector on one side of the simian virus 40-neo (SV40-neo) cassette (used as a positive-selection marker) flanked by LoxP sites. A 4,221-bp NheI-BamHI genomic fragment (with an engineered BamHI site upstream of the NheI site) containing exon II to exon V of the CDK2 gene was inserted on the other side of the SV40-neo cassette (Fig. 1A). Herpes simplex virus thymidine kinase (HSV-TK) and diphtheria toxin (DTA) were both used as negative-selection markers. Targeting vectors were electroporated into AK7.1 ES cells, and after positive and negative selections were applied, resistant clones were screened by PCR and Southern blot hybridization using a StuI-SacI probe upstream of exon I (Fig. 1A). The correctly targeted clone was used for injection into blastocysts and generation of chimeric mice. Germ line transmission was obtained, and the SV40-neo cassette was removed by crossing CDK2^{+/AF} mice with Mox2Cre mice (73). Genotyping was performed by PCR using tail DNA and the following primers: 5'-GCCTCACTAGCGCTCCATGG-3' and 5'-TTCTGGGTAGTTAGGGTGTGG-3'.

Tissue culture and retroviral infections. Primary mouse embryonic fibroblasts (MEFs) were prepared as described previously (5). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine growth serum (HyClone) at 37°C in 5% CO₂ and 3% O₂. Retroviral infection was performed as described previously (65). Briefly, viruses were produced from Phoenix ecotropic cells by transient transfection. MEFs were subjected to two rounds of infection and selected for 72 h in puromycin (4 μ g/ml) or 5 days in G418 (200 μ g/ml).

G₁- to S-phase progression and UV-induced G₁- to S-phase checkpoint. Confluent cells were synchronized by incubation in DMEM supplemented with 0.1% bovine growth serum for 72 h. Cells were then subjected to trypsinization and split 1:2 into medium containing 10% bovine growth serum. Nocodazole (40 ng/ml) was added to prevent cells from entering a second cell cycle. At 6 h after release, the medium was removed and cells were exposed to 40 J of UV irradiation (Stratagene Stratalinker 2400). The same medium was readded to the cells with BrdU (100 μ M) to label replicated DNA. Cells were harvested and fixed followed by BrdU antibody and propidium iodide (PI) staining, as previously described (12). The data were analyzed using CELLQUEST analysis software. The percentages of BrdU-positive cells were plotted. For detection of cell cycle distribution in asynchronous cells, continuously proliferating cells (60 to 70% confluent) were pulse-labeled with BrdU for 1 h.

Immunoprecipitation, histone H1 kinase assay, and Western blotting. MEFs were suspended in cell lysis buffer consisting of 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween 20, 10% glycerol, 1% Nonidet P-40, 10 mM β -glycerophosphate, 10 mM NaF, 1 mM Na₃VO₄, and protease inhibitor cocktail (Calbiochem catalog no. 535140) (1:200 dilution). The cell suspension was sonicated, and insoluble material was removed. Protein concentrations were determined using Quick Start Bradford dye reagent (Bio-Rad catalog no. 500-0205). Immunoprecipitation was carried out by incubating 2 μ g of antibody and 20 μ l of protein A-agarose beads (IPA-300; Repligen) with 150 μ g of extracts in a 0.5-ml volume diluted with lysis buffer and rotated at 4°C for 2 h. The beads were then washed once with lysis buffer and once with kinase buffer containing 50 mM HEPES (pH 7.5), 10 mM MgCl₂, 10 mM NaF, and 10 mM β -glycerophosphate. Beads were incubated in 20 μ l of kinase buffer supplemented with 2 μ g of histone H1 and 5 μ Ci of [γ -³²P]ATP at 30°C for 30 min. Beads were then eluted by adding Laemmli loading buffer containing sodium dodecyl sulfate (SDS). Phosphorylated histone H1 was detected by exposure to X-ray film after being resolved in 12% SDS-polyacrylamide gels. Cell lysate or immunoprecipitates were subjected to Western blotting.

Quantitative real-time reverse transcription-PCR (RT-PCR). RNA was prepared from cells suspended in TRIzol reagent (Invitrogen). RNA (1 μ g) was then reverse transcribed using poly(T) oligonucleotides and Superscript II reverse transcriptase according to the manufacturer's in-

FIG 1 Construction of CDK2AF mouse. (A) Diagrams of the murine CDK2 gene (WT [wild type]), targeting vector 4317G9 containing CDK2 long arm and short arm, which bears T14-A and Y15-F CDK2 mutations, and the predicted knock-in allele. The DNA probe used to identify homologous recombinants between 4317G9 and the CDK2 gene is shown. PCR primers used to distinguish the WT CDK2 from CDK2AF allele are also shown. (B) PCR analysis of genomic DNA from CDK2^{+/+}, CDK2^{+/AF}, and CDK2^{AF/AF} mice. (C) Mean body weight of both male and female CDK2^{+/+}, CDK2^{+/AF}, and CDK2^{AF/AF} mice from 4 to 12 weeks of age ($\pm$ standard errors of the means [SEM]). (D) Cyclin A-associated H1 kinase assay of thymus of CDK2^{+/+}, CDK2^{+/AF}, and CDK2^{AF/AF} mice and cyclin E- and cyclin A-associated kinase activities from MEFs. Confluent early-passage MEFs were synchronized in quiescence by contact inhibition and serum depletion. Cells were then replated in medium supplemented with 10% serum. BrdU was added 6 h after plating, and cells were collected at the indicated time points. IP, immunoprecipitation. (E) The corresponding cells were collected for BrdU staining followed by flow cytometry analysis. BrdU-positive cells were gated to measure the percentage of cells in S phase.

structions (Invitrogen). cDNA was diluted (1:4), and 2.5 μ l of cDNA was amplified in a 25- μ l reaction volume by real-time PCR. The amount of cDNA was compared with a mouse cDNA standard and then normalized to the amount of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA. The TaqMan gene expression assay probes (Applied Biosystems) were as follows: for cyclin A, Mm00438064_m1; for cyclin E, Mm01266311_m1; and for GAPDH, Mm03302249_g1.

Immunofluorescence. To view centrosomes after DNA damage in the G₁- to S-phase progression, cells were seeded on glass coverslips and treated as described below, followed by fixation in cold methanol for 15 min. Coverslips were stored in phosphate-buffered saline (PBS) at 4°C until staining. Cells were permeabilized for 5 min in 0.2% Triton X-100, rinsed in PBS, and blocked for 30 min in block buffer containing PBS and 1% fetal bovine serum. Coverslips were incubated for 1.5 h with anti-pericentrin antibody. The coverslips were washed in PBS and incubated with Alexa Fluor 488-conjugated secondary antibody for 30 min. After being washed in PBS, coverslips were incubated with antibody to γ -tubulin for 1 h. Coverslips were rinsed in PBS and incubated with cyanin-3-conjugated secondary antibody for 30 min. The coverslips were washed in PBS and incubated with Hoechst dye (1 μ g/ml)-PBS for 10 min. After being washed in PBS, coverslips were mounted on glass slides. All the incubations were done at room temperature, and antibodies were diluted 1/500 in block buffer. Centrosomes were visualized on a Nikon Eclipse 800 microscope, and images were obtained with a 40 $\times$ objective using a Spot charge-coupled-device camera (Diagnostic Instrument). For each sample, centrosomes in at least 100 cells were counted.

RESULTS

Construction and phenotypes of CDK2AF mice. Threonine 14 and tyrosine 15 are the two sites of inhibitory phosphorylation in CDK2. To understand the biological roles of the pathways that inactivate CDK2 via phosphorylation of these amino acids, we engineered mutations of thr14 to Ala and tyr15 to Phe (T14A and Y15F) into the mouse CDK2 gene (Fig. 1A; see also Materials and Methods). We confirmed accurate homologous recombination by Southern blotting (data not shown), genomic PCR (Fig. 1B), and genomic sequencing (not shown). Note that the PCR product from the mutated allele was approximately 100 bp larger than the wild-type allele because excision of the Neo cassette from intron 1 by the Cre recombinase left behind a single loxP site (Fig. 1A).

We interbred mice heterozygous for the CDK2AF allele in a mixed-strain background (129S1/SvImJ $\times$ C57BL/6). From a total of 40 progeny, we obtained 5 homozygous CDK2AF (12%), 14 heterozygous (35%), and 21 wild-type (53%) siblings. These results suggested that the CDK2A/F allele might cause prenatal lethality. However, an aging cohort of 18 CDK2AF homozygous mice had the same life span as wild-type littermates and exhibited no gross pathology other than slightly reduced body weight (Fig. 1C and data not shown).

The CDK2AF allele had a dominant effect on male fertility. In the mixed-strain background, CDK2AF heterozygous males were able to produce a limited number of offspring, including CDK2AF homozygous mice, when mated to CDK2AF heterozygous females. However, upon backcrossing into the 129S1/SvImJ and C57BL/6 backgrounds, we discovered that CDK2AF heterozygous males were completely infertile. This was associated with testicular atrophy and complete depletion of germ cells (unpublished data). We observed no effect of the CDK2AF mutation on female fertility, and histological examination of other tissues in CDK2AF homozygous male and female mice revealed no additional pathology.

The CDK2AF mutation caused increased CDK2 activity and accelerated entry into S phase. We determined the effect of the CDK2AF mutation on CDK2 activity in murine embryo fibroblasts (MEFs) prepared from homozygous and heterozygous CDK2AF and wild-type littermate embryos (12 to 13 days postcoitum [dpc]). Early-passage (precrisis) MEFs were synchronized in quiescence by growth to high density (contact inhibition) and serum depletion for 72 h. Cells were then released to enter the cell cycle by replating at lower density in medium supplemented with serum. BrdU was added 6 h after release to continuously label replicated DNA. Cyclin E- and cyclin A-associated CDK activities were measured at regular intervals, and the entry of the cells into S phase was determined by incorporation of BrdU into nuclear DNA.

We observed that both cyclin E- and cyclin A-associated CDK activities were prematurely activated and markedly elevated in both homozygous and heterozygous CDK2AF MEFs (Fig. 1D) and that this correlated with earlier entry into the S phase (Fig. 1E). We also noted that the CDK2AF mutation was dominant with respect to these effects and did not alter the relative timing of cyclin E- and cyclin A-associated kinase activities (cyclin E-associated kinase activity peaked before cyclin A-associated kinase activity). Consistent with these observations in MEFs, we observed that cyclin A-associated kinase activity was increased in highly proliferative tissues *in vivo* (the thymus and spleens) of homozygous CDK2 A/F mice (Fig. 1D and data not shown). We concluded that inhibitory phosphorylation of CDK2 normally constrains CDK2 activation during exit from quiescence and thus determines the timing of entry into S phase. Interestingly, the CDK2AF mutation had no effect on the cell cycle distribution of continuously proliferating cells (Fig. 2C), indicating that the role of inhibitory CDK2 phosphorylation in determining the timing of S-phase entry may be less important in the transition from mitosis to the S phase than it is during exit from quiescence.

Redundant mechanisms control the onset of DNA replication by the DNA damage checkpoint. Our observation that inhibitory phosphorylation of CDK2 restricts entry into S phase was consistent with the idea that this same pathway underlies, at least in part, the checkpoint pathways that prevent the G₁/S transition after DNA damage (19, 48). To directly test this hypothesis, we asked whether the UV-induced DNA damage checkpoint was defective in CDK2AF MEFs. MEFs were synchronized in quiescence by contact inhibition and serum depletion, released into G₁ by replating at low density in medium supplemented with serum and nocodazole, and 6 h later, during the mid-G₁ phase, exposed to 40 J/m² of UV irradiation. BrdU was added at this point. Compared to untreated cells, both wild-type and CDK2AF cells exposed to UV irradiation showed a delay in the transition into S phase (Fig. 2A and B). Both the magnitude of the checkpoint response (a 70% reduction in S-phase cell numbers at 18 h after release from quiescence; see Fig. 2A) and its duration (a 5- to 6-h delay in the start of the S phase) were approximately equivalent in the two genotypes (Fig. 2B).

In addition to inhibitory phosphorylation of CDK2, a parallel pathway decreases CDK2 activity in response to DNA damage by increasing the level of the CDK inhibitory protein p21^{Cip1} through stabilization of p53 (10, 15, 34). We therefore asked whether these two pathways redundantly controlled cyclin E-CDK2 activity and S-phase entry in response to DNA damage. We knocked down p21 protein amounts in wild-type and CDK2AF MEFs by stable infec-

FIG 2 Analysis of G_1 DNA damage response in CDK2AF MEFs. (A) Cells were treated as described for Fig. 1D and E but were exposed to UV irradiation in early G_1 phase (see Materials and Methods). The percentage of BrdU-positive cells was determined by flow cytometry, and representative profiles from cells 18 h after release are shown. (B) Cells were treated as described for panel A; the percentage of cells of the indicated genotypes in S phase is shown. (C) Percentage of S-phase cells in continuously proliferating CDK2^{+/+} and CDK2AF cells pulse-labeled with BrdU.

tion of the cells with a vector expressing a p21 short hairpin RNA (shRNA) (Fig. 3A). Cells exiting quiescence were exposed to UV irradiation in mid- G_1 phase, as before, and at regular intervals thereafter we measured cyclin E-associated CDK activity, the amount of p21 bound to cyclin E, and the onset of DNA synthesis (Fig. 3B).

In control wild-type MEFs, the amount of cyclin E protein was unaffected by UV irradiation at 18 h, but the amount of p21 bound to cyclin E increased, and this correlated with 6-h delays in both the appearance of cyclin E-associated kinase activity and the start of the S phase. In CDK2AF MEFs, cyclin E protein behaved similarly, although the amount of cyclin E-associated kinase activity in cells recovering from the checkpoint arrest was much greater than in wild-type MEFs. Knockdown of p21 had no effect on the G_1 DNA damage checkpoint in wild-type MEFs. Although only basal amounts of p21 were detected in complexes with cyclin E after UV irradiation in p21-knockdown MEFs, both cyclin E-associated kinase activation and entry into S phase were delayed 6 h just as in control MEFs.

Surprisingly, the cell cycle delay after UV-induced DNA damage was also not affected by knockdown of p21 in CDK2AF MEFs. Only very small amounts of p21 were bound to cyclin E after knockdown of p21 in CDK2AF MEFs, and the amounts did not increase after DNA damage. This correlated with a failure to delay the onset of cyclin E-associated kinase activity in UV-irradiated cells; at 18 h after release from quiescence, the amount of cyclin E-associated kinase activity in these UV-irradiated cells was nearly the same as in unirradiated cells. Moreover, this was equivalent to the amount of cyclin E-associated activity in wild-type cells at the onset of the S phase. Although cyclin E was active, the delay in S-phase entry caused by UV damage was unperturbed. We concluded that the CDK2 phosphorylation and p21 pathways were together sufficient to account for delay of cyclin E-associated kinase activity induced by DNA damage but insufficient to explain the delayed onset of the S phase. These observations implied that DNA damage in G_1 cells induced not only CDK2 inhibitory phosphorylation and CDK2 inhibitory proteins but also additional checkpoint pathways that regulated cell cycle progression.

Identification of a third DNA damage checkpoint pathway which controls expression of cyclin A protein. We therefore investigated the regulation of cyclin A by DNA damage, because cyclin A-Cdk2 can initiate S phase even in the absence of cyclin E-Cdk2 (28). In contrast to what we had observed for cyclin E, we found that UV-induced DNA damage delayed the accumulation of both cyclin A protein and its associated kinase activity independently of the CDK2 genotype and expression of p21 (Fig. 4A). The delay in expression and activation of cyclin A paralleled the delay in the onset of the S phase (similar to the data shown in Fig. 3B). Thus, cyclin A was also a target of the DNA damage checkpoint.

Cyclin A gene transcription, and hence cyclin A protein levels, are regulated by the E2F family of transcription factors. It has been proposed that DNA damage in G_1 MEFs results in hypophosphorylation of the Retinoblastoma (Rb) protein, due to decreased expression of cyclin D1, which in turn inhibits E2F-mediated transcription of the cyclin A gene (43). To test whether this might explain the delayed cyclin A accumulation in our experiments, we compared cyclin A transcript levels in control and UV-irradiated wild-type and CDK2AF-synchronized MEFs. We observed that UV irradiation did not decrease the accumulation of cyclin A mRNA (Fig. 4B), ruling out the possibility that modulation of

FIG 3 Effect of p21 knockdown in CDK2AF cells. (A) Extracts of the indicated genotypes, with or without knockdown of p21 with shRNA, were immunoblotted to detect p21 and Grb2 (loading control). (B) Cells released from serum starvation, with or without UV irradiation in G₁ phase, were collected at the indicated times. A portion of the cells was subjected to BrdU staining and flow cytometry to measure the percentage of cells in S phase. The remaining cells were lysed and either immunoprecipitated with a cyclin E antibody to measure cyclin E-associated kinase activity, followed by Western blotting with p21 antibody, or immunoblotted for cyclin E and tubulin (loading control).

cyclin A gene transcription by the Rb-E2F pathway was the cause of the decreased expression of cyclin A protein. Consistent with this, we also found no effect of UV irradiation on the abundance of cyclin D1 protein at the time of the transition from G₁ to S phase in synchronized MEFs (not shown). Interestingly, the amounts of cyclin A mRNA were higher in CDK2AF MEFs compared to wild-type cells, consistent with their elevated CDK2 activity and accelerated G₁- to S-phase progression. These observations suggested that there was a previously unrecognized DNA damage-dependent checkpoint pathway that prevented accumulation of the cyclin A protein.

Identification of a fourth DNA damage checkpoint pathway.

We then asked if this triad of checkpoint pathways—inhibitory CDK2 phosphorylation, induction of the CDK2 inhibitory protein p21, and inhibition of cyclin A protein accumulation—was sufficient to explain the block to S-phase entry induced by UV irradiation of G₁-phase MEFs. The cyclin A protein is unstable in G₁-phase cells due to ubiquitination by the Cdh1 form of the anaphase-promoting complex (APC) (24). In late G₁ phase, the APC is inactivated by interaction with Emi1, which results in the stabilization of cyclin A and S-phase entry (36). We therefore speculated that the instability of cyclin A in UV-irradiated cells might be due to persistent activation of the APC and predicted that we could bypass this checkpoint pathway by constitutively

expressing a form of cyclin A that was resistant to APC-mediated ubiquitination.

The APC recognizes cyclin A via its N-terminal destruction box (D box) (27). Therefore, we ectopically expressed a mutant form of cyclin A, MT-cyclin AΔ80, in which the N-terminal 80 amino acids, including the D box, are deleted and replaced with a myc epitope tag (MT). For these experiments, we intercrossed CDK2AF knock-in mice with p21 null mice in order to create MEFs that both lacked p21 and expressed the CDK2AF protein. In this way, only a single manipulation, ectopic expression of MT-cyclin AΔ80, was necessary to engineer cells that constitutively bypassed all three checkpoint pathways.

We first compared the UV-induced DNA damage checkpoint in p21^{-/-}; CDK2^{+/+} and p21^{-/-}; CDK2^{+/AF} MEFs (Fig. 5). As we had seen previously using p21 shRNAs, UV irradiation delayed the induction of cyclin A-associated kinase and S-phase entry. We then constitutively expressed cyclin A protein in these MEFs by stable transduction with a vector encoding MT-cyclin AΔ80. MT-cyclin AΔ80 assembled into a catalytically active complex and accelerated S-phase entry in unirradiated MEFs, which demonstrated that this form of the cyclin A protein was both biochemically and biologically active. However, the kinase activity associated with constitutively expressed MT-cyclin AΔ80 was still downregulated by UV irradiation in p21^{-/-}; CDK2^{+/AF} MEFs,

FIG 4 Analysis of cyclin A expression and associated kinase activity in CDK2AF cells. (A) Serum-starved and released cells, with or without UV irradiation in G_1 phase, were collected at the indicated time points. Cells were lysed and extracts either immunoprecipitated with a cyclin A antibody, followed by histone H1 kinase assay, or immunoblotted for cyclin A and Grb2 (loading control). (B) Real-time RT-PCR was performed to determine cyclin A mRNA abundance in serum-starved and released and UV-irradiated CDK2^{+/+} and CDK2AF MEFs.

and entry into S phase was delayed. We concluded that UV irradiation induced a previously uncharacterized checkpoint pathway that prevented activation of cyclinA-Cdk2 and was independent of p21, inhibitory CDK2 phosphorylation, and cyclin A protein expression. The mechanism of this pathway is unknown and under investigation, but there was not increased association of p27 with cyclin A (not shown).

CDK2 inhibitory phosphorylation regulates centrosome duplication. Entry into S phase coordinately initiates both DNA replication and centrosome duplication. Conversely, when cell cycle checkpoints prevent the transition from G_1 to S phase, these processes are coordinately delayed. Since both are initiated by CDK2, we asked whether CDK2 inhibitory phosphorylation regulates centrosome duplication.

Wild-type and CDK2AF homozygous MEFs were made quiescent by serum depletion and then released to synchronously reenter the cell cycle. In mid- G_1 phase, the cells were irradiated with UV, which delayed entry into S phase by 4 to 6 h. Centrosomes were identified and counted by coimmunostaining with antibodies that recognize pericentrin and γ -tubulin (Fig. 6B). We found that CDK2AF MEFs had an elevated number of centrosomes and that this abnormality was exacerbated by the UV-induced checkpoint delay in G_1 phase (Fig. 6A). Thus, in mock-treated cells, between 10% and 15% of CDK2AF MEFs had more than 4 centrosomes, compared to less than 5% of wild-type MEFs. This increased to between 25% and 30% of CDK2AF MEFs with more than 4 centrosomes after UV irradiation, compared to 10% of control MEFs. Centrosome numbers were essentially unaffected

by knockdown of p21. Immunostaining for α -tubulin demonstrated that the extra centrosomes in these cells were functional, as they organized multipolar spindles at mitosis (Fig. 6C).

We concluded that CDK2 inhibitory phosphorylation was essential to control centrosome duplication, both in a normal cell cycle and especially after checkpoint-induced cell cycle arrest. This contrasted with the control of DNA replication, where additional pathways that targeted cyclin A compensated for the absence of CDK2 inhibitory phosphorylation and p21 induction and maintained the integrity of the DNA replication checkpoint.

DISCUSSION

The initiation of cell division is marked by the coordinate onset of both centrosome and chromosome duplication, both of which require Cdk2 (33, 52). It is therefore thought that the initiation of cell division is regulated by pathways that control Cdk2 activity. Here, we have focused on the regulation of cell division by the inhibitory phosphorylation of Cdk2. Our approach was to analyze cell cycle progression in mice with an engineered knock-in mutation of Cdk2 in which both inhibitory phosphorylation sites were changed to nonphosphorylatable residues (CDK2AF). We first determined to what extent the CDK2AF mutation altered the magnitude and timing of Cdk2 activation, both in an unperturbed cell cycle and in response to the G_1 DNA damage checkpoint. We then asked if the changes in Cdk2 activation caused by this mutation affected the regulation of either centrosome duplication or chromosome replication. Our primary conclusions are that inhibitory phosphorylation of CDK2 has a major role in controlling the

FIG 5 Effect of expression of stabilized cyclin A in CDK2^{+/AF}; p21^{-/-} MEFs. Cells were serum starved and released, with or without UV irradiation in G₁ phase, as described above. The percentage of cells in S phase was measured. Kinase activities associated with cyclin A and stabilized, exogenous cyclin A were measured at the indicated time points. Expression of the stabilized cyclin A (MT-cyclin A Δ 80) was determined by immunoblotting with anti-Myc epitope tag antibody.

activities of cyclins E and A and thus both determines the timing of S-phase entry in a normal cell cycle and regulates centrosome duplication. However, inhibitory CDK2 phosphorylation is fully redundant with at least three other pathways that control the activity of cyclin A at the G₁ DNA damage checkpoint. Therefore, the regulation of DNA replication in response to DNA damage is unperturbed when CDK2 inhibitory phosphorylation is prevented.

Our initial observation was that the CDK2AF mutation caused a dose-dependent increase in the amounts of cyclin E- and cyclin A-associated kinase activities during the late G₁ and S phases of a synchronized cell cycle. These late G₁- and S-phase cyclin-dependent kinase activities are predominantly if not exclusively due to their association with Cdk2. The increased amount and earlier onset of Cdk2 activity correlated with an earlier onset of DNA replication. We concluded that, when cells were stimulated by mitogens to exit quiescence and enter the cell cycle, the timing of CDK2 activation determined the timing of S-phase entry. We suggest that a rate-limiting step for progression from quiescence to S phase is overcoming an inhibitory threshold established by constitutive phosphorylation of Cdk2 on T14 and Y15. This rate-limiting step may be cyclin synthesis.

It might seem paradoxical to conclude that CDK2 inhibitory phosphorylation regulates the initiation of cell duplication, when germ line deletion of the CDK2 gene has no apparent effect on mouse development or cell cycle progression (4, 55). However, it is sometimes misleading to use a mutant phenotype to infer the normal function of a gene. For example, in this case, the absence of CDK2 protein allows "compensatory" cyclin-CDK complexes to assemble (e.g., cyclin A-CDK1) that functionally substitute for the missing CDK2 (66). In fact, chemical genetic approaches show that specific pharmacological inhibition of CDK2 blocks the pro-

liferation of the same cells in which depletion of CDK2 is without effect (52). Thus, the consequences of dysregulating the endogenous CDK2 pathway and the effect of chemically inhibiting endogenous CDK2 both support the conclusion that a normal function of CDK2 is to control the transition from G₁ to S phase.

Unexpectedly, the CDK2AF mutation did not affect the cell cycle distribution of continuously proliferating cells. Moreover, cyclin E-associated kinase activity was not elevated in continuously proliferating CDK2AF cells (not shown). Differences between the transitions from mitosis to S phase and from quiescence to S phase have been previously observed. For example, cyclin E null cells are substantially delayed in exiting from quiescence but then proliferate normally through successive mitogen-driven cell cycles (28). The E2F transcriptional program is differently regulated under the two sets of conditions, as is the repertoire of CDK inhibitory proteins and the level of expression of G₁ cyclins (12). These data suggest that the rate-limiting steps for S-phase entry differ when the S phase is approached from mitosis versus quiescence. The pathway from quiescence to S phase may be uniquely dependent on cyclin E accumulation and activation.

A DNA damage-triggered checkpoint pathway negatively controls the initiation of cell division at the transition from G₁ to S phase. ATM and ATR kinases respond to DNA damage and coordinately activate proteins that both pause progression through the cell cycle and repair the damaged DNA (3, 59). One cell cycle inhibitory pathway triggered by ATM/ATR operates by stabilization of p53, which in turn promotes transcription of the p21Cip1 gene and repression of the CDC25A gene (10, 14, 15, 34). In parallel, activation of the Chk2 kinase causes ubiquitin-dependent degradation of the Cdc25A phosphatase, thereby increasing inhibitory phosphorylation of Cdk2 (13, 48). It is currently thought

FIG 6 Centrosome duplication in CDK2AF cells. (A) Percentage of cells with more than 2 (left panel) or 4 (right panel) centrosomes in CDK2^{+/+} and CDK2AF MEFs infected with control vector or p21 shRNA. Cells were synchronized and released as described, with or without UV irradiation in G₁ phase. Cells were fixed 72 h after serum stimulation. Centrosomes were visualized by double immunostaining with antipericentrin and anti- γ -tubulin. At least 100 cells were counted for each sample. Dapi, 4',6'-diamidino-2-phenylindole. (B) Representative images of multiple centrosomes in CDK2AF MEFs. (C) Proliferating CDK2AF MEFs were fixed and immunostained with anti-pericentrin and anti- α -tubulin. Representative images are shown.

that these two pathways are, together, sufficient to explain the regulation of CDK activity by the G₁ DNA damage checkpoint.

We observed that the CDK2AF allele did not prevent the downregulation of cyclin E- and cyclin A-associated Cdk2 activity that occurs in response to UV-induced DNA damage and did not affect the number of cells pausing at the G₁- to S-phase transition or the duration of the ensuing cell cycle delay. This was consistent with the idea that p21 and Cdk2 phosphorylation pathways may redundantly enforce the G₁- to S-phase delay caused by DNA damage. We directly tested this idea by using stable expression of a p21shRNA to decrease p21 protein expression. Knockdown of p21 decreased UV-triggered assembly of inhibitory p21-Cdk2

complexes but on its own had no effect on the downregulation of Cdk2 activity or the cell cycle checkpoint response.

Most interestingly, knockdown of p21 in CDK2AF cells completely prevented downregulation of cyclin E-associated kinase activity by DNA damage. Therefore, these two pathways acting in concert were sufficient to explain the checkpoint regulation of cyclin E-associated kinase activity. Remarkably, however, downregulation of cyclin A-associated kinase activity remained fully responsive to DNA damage, and the cell cycle delay caused by DNA damage at the G₁- to S-phase transition was unperturbed. Therefore, other DNA damage checkpoint pathways must be operative and sufficient, on their own, to

downregulate cyclin A-associated kinase activity and bring about a cell cycle delay.

By studying CDK2AF; p21shRNA (or p21 null) cells, we uncovered the existence of two new pathways that regulate cyclin A-associated kinase activity in response to DNA damage. The first was downregulation of cyclin A protein. This was not secondary to a decrease in cyclin A transcription, because cyclin A mRNA abundance was not diminished. Decreased accumulation of cyclin A protein was possibly due to protein turnover by the Cdh1-APC E3 ubiquitin ligase. We ectopically expressed a form of cyclin A that was resistant to APC-mediated degradation, which allowed stable expression of cyclin A protein at the G₁ DNA damage checkpoint. Nevertheless, this too was insufficient to allow accumulation of cyclin A-associated kinase activity after DNA damage and insufficient to cause bypass of the DNA damage checkpoint (even in the CDK2AF; p21^{-/-} background). We point out that the mechanism for turnover of cyclin A protein at the G₁ DNA damage checkpoint remains to be characterized. UV irradiation did not prevent the accumulation of Emi1 protein that normally occurs in late G₁ phase, and UV irradiation did not affect expression of Cdh1 protein (not shown).

These results showed that G₁ DNA damage checkpoint pathways cause inactivation of cyclin A-Cdk2 in a manner distinct from (or in addition to) those that regulate cyclin E-Cdk2. We also did not detect increased amounts of a related CDK inhibitory protein, p27, bound to cyclin A (data not shown). A cyclin A-specific inhibitory protein, Roughex, has been characterized in *Drosophila*, but no homolog is known to exist in mammals (1, 75). It is possible that mammalian cells have a functional homolog of Roughex that is yet to be identified. But, inhibition may, in principle, involve decreased activating phosphorylation on threonine 160 or decreased binding of cyclin A to Cdk2. These possibilities are under investigation.

Genetic experiments show that cyclin A is not required for the cell cycle; either cyclin E-Cdk2 or cyclin A-Cdk2 is alone sufficient for progression from G₁ through S phase (39). It was therefore surprising that activation of cyclin E-CDK2 in UV-irradiated cells (in CDK2AF cells, with p21 knocked down) was not sufficient to cause cells to bypass the checkpoint and enter S phase. As discussed above, inhibition of a protein may uncover an essential function that had been missed in a genetic null due to the operation of compensatory pathways. Hence, whether maintenance of the checkpoint is due to inhibition of cyclin A-CDK2 or to other mechanisms remains to be determined.

In contrast to the multiple pathways that control the initiation of DNA replication by Cdk2, we found that inhibitory Cdk2 phosphorylation had a primary, nonredundant role in controlling centrosome duplication. In CDK2AF cells stimulated to reenter the cell cycle from quiescence, we found that the percentage of cells with abnormally elevated numbers of centrosomes was substantially greater. After UV irradiation, more than 60% of CDK2AF cells had more than 2 centrosomes and more than 30% had more than 4 centrosomes (Fig. 6A). This represented overduplication of functional centrosomes, as evidenced by their assembly of multipolar spindles (Fig. 6B).

Chromosome duplication occurs exactly once per cell cycle, and this is extremely robust with respect to perturbations in the cell division cycle. Chromosomes do not reduplicate when the cell cycle is delayed or blocked. For example, we show here that the G₁ DNA damage checkpoint activates at least four distinct pathways,

all of which constrain CDK2 activity and, thus, the initiation of chromosome replication. Equally stringent pathways operate in S phase and in G₂ cells to ensure that chromosome duplication and cell duplication remain coordinated.

Centrosome duplication, like chromosome duplication, is initiated by Cdk2 (32, 42, 49, 51). Also, similar to chromosomal DNA, centrosomes possess an intrinsic mechanism that ordinarily prevents their reduplication within a single cell cycle (77). However, the coupling between centrosome duplication and cell cycle progression is much less robust with respect to conditions that impair cell cycle progression. Centrosome amplification has been observed in cells arrested in S or G₂ phase by DNA damage (2) and after inhibition protein synthesis (2, 26, 72).

Our results suggest a mechanism underlying the difference between the control of chromosome duplication and the control of centrosome duplication. Whereas chromosome duplication usually requires both cyclin A-CDK2 and cyclin E-CDK2 activities, cyclin E-CDK2 is thought to be sufficient to initiate centrosome duplication (23, 33). We have shown that the regulation of cyclin E-CDK2 is less tightly controlled than regulation of cyclin A-CDK2. Loss of inhibitory CDK2 phosphorylation and decreased p21 expression are, together, sufficient to allow cyclin E-CDK2 activation at the G₁ DNA damage checkpoint. On the other hand, at least two additional regulatory pathways prevent cyclin A-CDK2 activation (and hence the onset of DNA replication) under those same conditions. The subpopulation of cyclin E-CDK2 that participates in centrosome duplication may be physically separated from p21, which is a nuclear protein, and therefore may be primarily regulated by inhibitory phosphorylation alone. Therefore, in cells expressing CDK2AF, centrosome-localized cyclin E may be sufficiently active to allow premature centrosome duplication. Uncoupling of chromosome and centrosome duplication in CDK2AF cells is more severe at the DNA damage checkpoint, likely because cyclin A-CDK2 activation would be further delayed relative to cyclin E-CDK2.

Centrosome amplification is characteristic of aggressive tumor cells and is mechanistically linked to activation of specific oncogenes or loss of tumor suppressor genes (11, 47, 78). Loss of p53, for instance, has been shown to cause centrosome amplification and genetic instability (22, 74). It is interesting, in this regard, that p53 is not only a transcriptional activator of p21 but also a transcriptional repressor of CDC25A (14, 61). Thus, loss of p53 would not only decrease p21 expression but also increase expression of CDC25A. The latter may be largely responsible for the increase in centrosome number, but this may be augmented to some extent by concomitant p21-deficiency.

CDK2AF mice live a normal life span and display no pathology other than slightly reduced body weight and infertility in male mice. We can therefore remove the caveat that the similar results in *Drosophila* might be species specific due to absence of CDC25A (44). It may be surprising that mice with the CDK2AF mutation, which alters cell cycle kinetics and promotes centrosome amplification, were not tumor prone (67). Indeed, increased expression of CDC25A, which specifically targets the inhibitory phosphorylation sites in CDK2, is found in many human tumors (9). Nevertheless, our results are consistent with previous studies which showed that overexpression of CDC25A was not sufficient to cause a tumor phenotype in transgenic mice but accelerated tumorigenesis when combined with other oncogenes (58) or after radiation-induced DNA damage (71).

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