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Alternative Cyclin D1 Splice Forms Differentially Regulate the DNA Damage Response

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Abstract

The DNA damage response (DDR) activates downstream pathways including cell cycle checkpoints. The *cyclin D1* gene is overexpressed or amplified in many human cancers and is required for gastrointestinal, breast, and skin tumors in murine models. A common polymorphism in the human *cyclin D1* gene is alternatively spliced, resulting in cyclin D1a and D1b proteins that differ in their carboxyl terminus. Cyclin D1 overexpression enhances DNA damage–induced apoptosis. The role of cyclin D1 and the alternative splice form in regulating the DDR is not well understood. Herein cyclin D1a overexpression enhanced the DDR as characterized by induction of γH2AX phosphorylation, the assembly of DNA repair foci, specific recruitment of DNA repair factors to chromatin, and G_2-M arrest. Cyclin D1 deletion in fibroblasts or small interfering RNA–mediated reduction of endogenous cyclin D1 in colon cancer cells reduced the 5-fluorouracil–mediated DDR. Mechanistic studies showed that cyclin D1a, like DNA repair factors, elicited the DDR when stably associated with chromatin. *Cancer Res; 70(21); 8802–11. ©2010 AACR.*

Introduction

The ability to sense DNA damage is determined through activation of the serine/threonine kinase, ataxia-telangiectasia mutated (ATM), DNA-dependent protein kinase (DNA-PK), and ataxia-telangiectasia and Rad3 related (ATR; ref. 1). Replication stress induces ATR activation, whereas both ATM and ATR are induced in response to double-stranded DNA break repair. The rapid targeting of DNA repair factors near the site of damage gives rise to nuclear foci (2). The early DNA damage sensor complex involves MRE11/Rad50/NBS1 (MRN), the transducer proteins MDC1 and 53BP1, and the phosphotidylinositol 3-kinase ATM/DNA-PK/ATR which phosphorylate H2AX and the Chk1/Chk2 cell cycle kinases (3). Phosphorylation of histone H2AX on serine residues of the carboxyl terminus (Ser139), producing γH2AX, recruits proteins that sense or signal the presence of DNA damage, inducing the response that leads to DNA repair (4).

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The role of the DNA damage response (DDR) in tumorigenesis is complex. Defects of the MRN complex occur at the preinvasive carcinoma *in situ*, and γH2AX activation is commonly observed among familial breast tumors (5). Activated oncogenes are capable of inducing both double-stranded DNA break (DSB) and a DDR in NIH3T3 cells. These oncogenes include *myc, ras, mos, cdc25A, E2F1*, and *cyclin E* (6–8). DNA damage is also a feature of premalignant tissue (9, 10), suggesting that oncogenic stress in nonmalignant cells may contribute to tumorigenesis. Preferential activation of the DDR occurs in early, preinvasive lesions including ductal carcinoma *in situ* of the breast (9, 10).

Activation of cell cycle control proteins is also an early feature of tumorigenesis. The cyclins encode essential components of the cell-cycle machinery, binding and activating their specific cyclin-dependent kinase partners (11). The abundance of cyclin D1 has been shown to play a role in cell type–dependent radiation-induced sensitivity. *Cyclin D1*$^{-/-}$ mouse embryonic fibroblasts (MEF) have enhanced apoptosis evoked by γ irradiation (12), and cyclin D1 expression also inhibited UV-induced apoptosis in the presence of p300 (13). In contrast, breast cancer cell lines overexpressing cyclin D1 showed enhanced apoptosis in response to γ irradiation (14, 15), suggesting cell type–specific differences governing cyclin D1–mediated apoptosis. The abundance of cyclin D1 mediates a G_1 cell-cycle arrest, as interference with cyclin D1 degradation prevents G_1 arrest in cell exposed to γ irradiation–induced DNA damage (12) and G_2-M arrest (16). Collectively these studies suggest that overexpression of cyclin D1 enhances γ irradiation–induced apoptosis, but the role of endogenous cyclin D1 and the molecular mechanisms by which cyclin D1 may mediate the DDR are not well understood.

The *cyclin D1* gene encodes the regulatory subunit of a holoenzyme that phosphorylates the pRb protein. In addition, cyclin D1 promotes cell migration, regulates cellular metabolism, conveys transcriptional functions, and is recruited to DNA in the context of local chromatin (17). The human *cyclin D1* gene is polymorphic (reviewed in ref. 18). The polymorphism (A870G), located at the splice donor region at the exon 4–intron 4 boundary, modulates the efficiency of alternate splicing between exons 4 and 5. As a result of the altered splicing that occurs, the coding region downstream is altered such that the amino acid sequence of the COOH terminus of cyclin D1 is altered (18). Thus, two isoforms of cyclin D1 are produced: the canonical isoform termed cyclin D1a and the alternately spliced isoform termed cyclin D1b. These proteins are distinct in their COOH termini. Clinical studies have associated this polymorphism with an increased risk of colon and rectal cancer, early-onset squamous cell carcinoma, head and neck cancer, and transitional cell carcinoma of the bladder (18, 19). Both isoforms encode regulatory subunits that can stimulate CDK4/6 activity; however, cyclin D1b has a reduced capacity to phosphorylate pRb and advance cell cycle progression (20). The role of cyclin D1a versus the cyclin D1b isoform in the DNA damage response has not previously been examined. It may be important in understanding the differences in transforming capacity of the two isoforms (20) and/or targeting therapy for cancer patients.

The current studies were conducted to determine whether cyclin D1 isoforms may affect the DDR signal pathway. Small interfering RNA (siRNA) to endogenous cyclin D1 and isoform-specific reconstitution overexpression experiments in *Cyclin D1*$^{-/-}$ MEFs/3T3s allowed the identification of an isoform-specific DDR. Recent studies have indicated that the physical tethering of these DNA repair factors to chromatin is sufficient to induce the DDR signaling cascade (21). The current studies showed that cyclin D1a, but not cyclin D1b, recruitment to chromatin was sufficient to elicit the DDR.

Materials and Methods

Plasmids

Cyclin D1a and cyclin D1b were amplified by PCR from MSCV-cyclin D1a-IRES-GFP and MSCV-cyclin D1b-IRES-GFP (22) and cloned at the COOH-terminus of the Cherry-lacR-NLS vector (21) in *Kpn*I/*Xma*I sites. The primers used were the following: cyclin D1 forward: cggggtaccgaacaccagctcctgtgct; cyclin D1a reverse: tccccccgggtcagatgtccacgtcccgca; cyclin D1b reverse: tccccccgggtcaccctttgggggccttg. All plasmid DNA constructs were verified by sequencing.

Cell culture, treatment, and transfection

Cyclin D1$^{+/+}$ and *Cyclin D1*$^{-/-}$ primary mouse MEFs and 3T3 cell cultures, and retroviral infected *Cyclin D1*$^{-/-}$ MEFs and 3T3 cells were prepared as described previously (22–24). *p21*$^{Cip1+/+}$ and *p21*$^{Cip1-/-}$ MEFs were obtained from Dr. Philip Leder (Harvard Medical School, Boston, MA) and Dr. Dale S. Haines (Temple University School of Medicine, Philadelphia, PA). All MEFs, 3T3s, human kidney 293T cells, MCF-7 human breast cancer cells, and HCT116 human colon cancer cells were maintained in DMEM containing penicillin and streptomycin (100 mg of each/liter) and supplemented with 10% fetal bovine serum. The NIH2/4 stable cell line that contains 256 repeats of the lac operator sequence (lacO) stably integrated on chromosome 3 (25) was maintained in DMEM with 10% fetal bovine serum and 400 μg/mL hygromycin (Invitrogen). The NIH2/4 stable cell line was transfected using the Nucleofector kit for immortalized cell lines (Amaxa, Nucleofector R) as described previously (21). For the analysis of cyclin D1–mediated responses after DNA damage, MEFs or 3T3 cells were treated with ionizing radiation at the indicated dosage and times after treatment or doxorubicin (Sigma) at the indicated dosage and period of time of treatment.

Immunofluorescence

Immunofluorescence was conducted as described previously (22).

Immunoprecipitation and Western Blotting

Immunoprecipitation and Western blotting were conducted as described previously (22).

Neutral pH comet assay

Neutral pH comet assays were conducted using the Comet-Assay Kit (Trevigen). After treatment with doxorubicin or control, cells were harvested and mixed with low-melting temperature agarose. After lysis, electrophoresis was conducted at 1 V/cm for 20 minutes. Slides were stained with SYBG green dye for 10 minutes and visualized on a Zeiss LSM 510 META confocal microscope with a ×20 objective. The relative length and intensity of SYBG green–stained DNA tails to heads was proportional to the amount of DNA damage present in the individual nuclei and was measured by Olive tail moment using TriTek Comet Score software (TriTek; ref. 26).

Additional materials and methods are discussed in Supplementary Information.

Results

Endogenous cyclin D1 mediates the DDR

A surrogate assay for the measurement of DSBs in the cell is the comet assay. When cells are electrophoresed in neutral pH, the image looks like a comet with a distinct head composed of intact DNA and a tail consisting of damaged DNA (27). The comet assay conducted at neutral pH detects mainly DSBs. Comet assays were conducted to examine the role of endogenous cyclin D1 in the DDR. *Cyclin D1*$^{+/+}$ and *Cyclin D1*$^{-/-}$ 3T3 cells were treated with 2 μmol/L doxorubicin for 16 hours. The percentage of cells with a comet tail was calculated (Fig. 1A). After doxorubicin treatment about 14.5% cells had a comet tail in *Cyclin D1*$^{+/+}$ cells, but only 1.4% cells had a comet tail in *Cyclin D1*$^{-/-}$ cells. These results suggest that endogenous cyclin D1 increases the DDR. Histone H2AX phosphorylation on a serine residue at the carboxyl terminus (Ser139), producing γH2AX, is a sensitive marker for DSBs. Western blot analysis was conducted to

detect γH2AX in *Cyclin D1*$^{+/+}$ and *Cyclin D1*$^{-/-}$ MEFs treated with doxorubicin for 16 hours (Fig. 1B). After doxorubicin treatment γH2AX increased more in *Cyclin D1*$^{+/+}$ cells than in *Cyclin D1*$^{-/-}$ cells. To further determine if endogenous cyclin D1 plays a role in DDR we used specific cyclin D1 siRNA to knock down endogenous cyclin D1 in the HCT116 colon cancer cell line. Cyclin D1 siRNA reduced cyclin D1 levels, and reduced 5-fluorouracil (FU)-induced γH2AX phosphorylation. Rad51 levels were also reduced upon the reduction in cyclin D1 protein levels (Fig. 1C). Collectively, these studies suggest that endogenous cyclin D1 contributes to the DDR.

Cyclin D1a induces the cellular DDR and DSBs

To determine the specific requirement for cyclin D1a versus cyclin D1b in the DDR we conducted *Cyclin D1*$^{-/-}$ cell reconstitution overexpression analysis. *Cyclin D1*$^{-/-}$ cells were transduced with a retroviral expression vector encoding either cyclin D1a or cyclin D1b to overexpress each isoform. Two types of analysis were conducted to assess the relative abundance of the D-type cyclin in the reconstitution by Western blot analysis. As cellular transduction was high, the relative abundance of cyclin D1a and cyclin D1b was approximately 3- to 4-fold greater than endogenous cyclin D1 level (Supplementary Fig. S1A and B). At a single cell level the relative abundance of cyclin D1a and cyclin D1b was similar to the abundance of endogenous cyclin D1b (Supplementary Fig. S1C and D). (The difference in relative abundance per cell versus total abundance by Western blot is because endogenous cyclin D1a expression fluctuates during the cell cycle and is at low levels during early G_1 and late S and G_2-M.) These cell populations were treated with doxorubicin (2 μmol/L) to induce DNA damage. Cyclin D1a enhanced comet formation 4-fold compared with the control vector ($P < 0.01$). In contrast, although cyclin D1b was expressed at similar levels to cyclin D1a (Supplementary Fig. S1), comet activity was induced only about 80% (Fig. 2A). Cyclin D1a increased the DDR assessed by comet assay significantly more than did cyclin D1b ($P < 0.01$; Fig. 2A).

The DDR response is capable of inducing a cell cycle arrest at the G_1-S or G_2-M boundary. To determine whether the cyclin D1a–mediated DDR was associated with cell-cycle arrest, propidium iodide (PI) staining and fluorescence-activated cell sorting (FACS) analysis was conducted. Doxorubicin increased the proportion of cells in the G_2-M phase in *Cyclin D1*$^{-/-}$ MEFs (Fig. 2B). Reintroduction of cyclin D1a into *Cyclin D1*$^{-/-}$ MEFs increased the proportion of G_2-M cells from 27.7% to 49.2% and enhanced doxorubicin-mediated induction of G_2-M from 36.7% to 64.3% (Fig. 2B). In contrast, cyclin D1b failed to change the basal or doxorubicin-induced G_2-M phase (27.7% to 28.8%, 36.7% to 36.6%; Fig. 2B).

Phosphorylation of H2AX (known as γH2AX) is the most studied chromatin modification induced by double-strand breaks. DSB induction of γH2AX results in the formation of large foci that are important for the accumulation and retention of DSB repair factors. Assemblage of nuclear repair foci containing γH2AX was assessed in *Cyclin D1*$^{-/-}$ MEFs

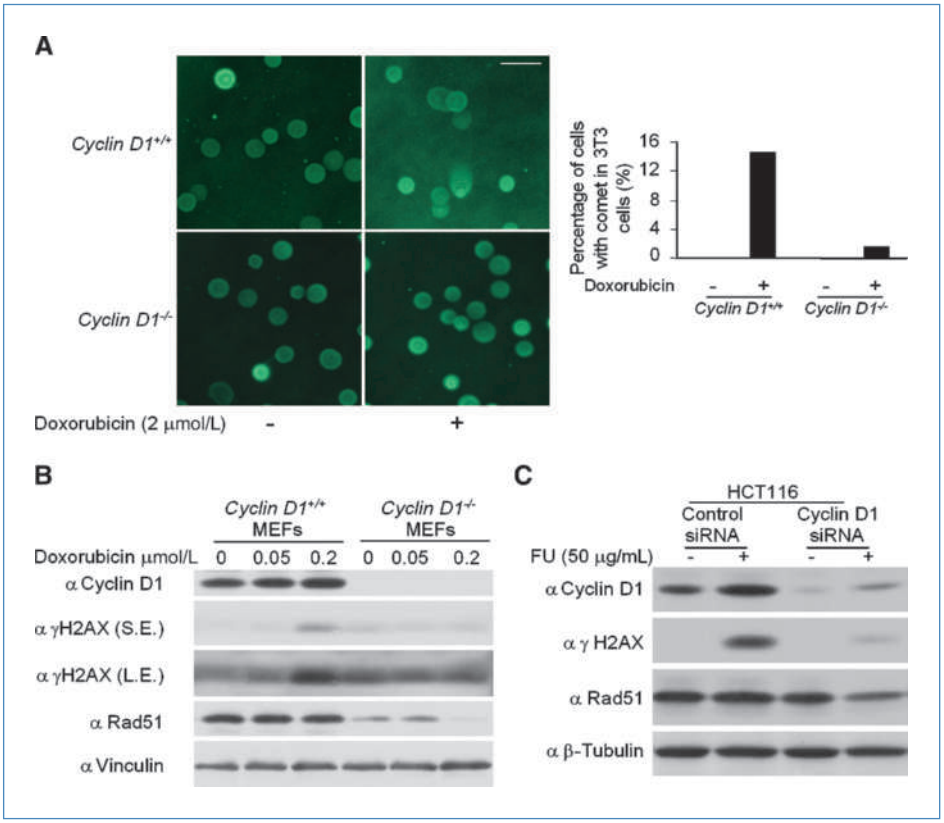

Figure 1. Endogenous cyclin D1 increases the DDR. A, the comet assay was conducted as a single-cell DNA damage assay at neutral pH. A neutral pH comet assay detects mainly DSBs. *Cyclin D1*$^{+/+}$ and *Cyclin D1*$^{-/-}$ 3T3 cells were treated with 2 μmol/L doxorubicin for 16 hours. Scale bar, 100 μm. Right, percentage of cells with comet tail. B, Western blot analysis of *cyclin D*$^{+/+}$ and *Cyclin D1*$^{-/-}$ MEFs treated with doxorubicin for 16 hours. Antibodies are directed to γH2AX (Ser139), Rad51. Vinculin expression was used as a protein loading control. S.E., shorter exposure; L.E., longer exposure. C, knocking down endogenous cyclin D1 decreases FU-induced γH2AX in HCT116 colon cancer cell line. Western blot analysis of the HCT116 colon cancer cell line transfected with control or cyclin D1 siRNA prior to treatment with FU for 24 hours.

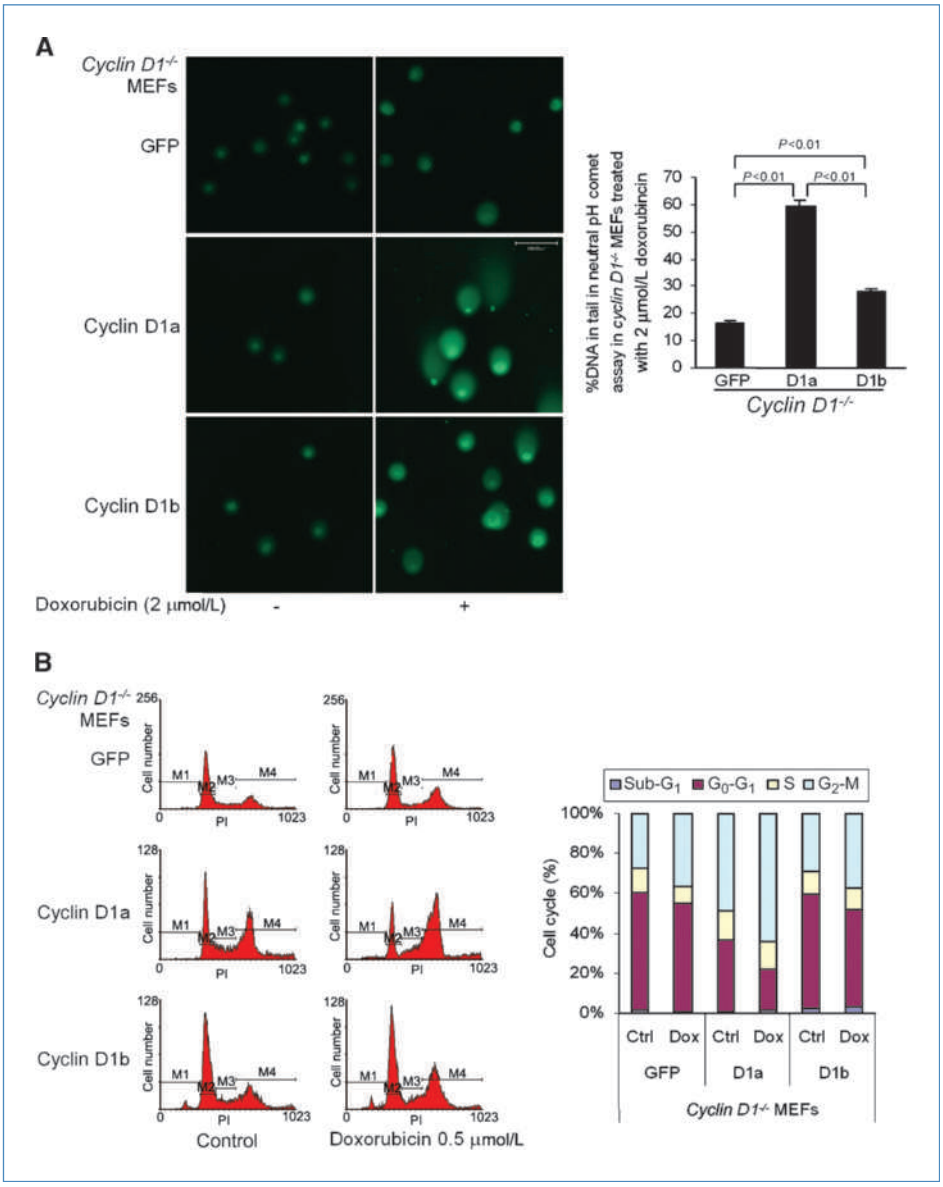

Figure 2. Alternate cyclin D1 splice forms differentially regulate the DDR and a G_2 delay. A, neutral pH comet assay was conducted as a single-cell DNA damage assay. The tail of the DNA comet reflects the amount of damaged DNA and was quantified using TriTek Comet Score software. Data are mean ± SE of 100 cells per condition. *Cyclin D1*$^{-/-}$ MEFs were transduced with retroviral expression vectors encoding either the cyclin D1a or cyclin D1b splice forms, and treated with 2 μmol/L doxorubicin for 16 hours. GFP, green fluorescent protein. Scale bar, 100 μm. B, FACS analysis of *Cyclin D1*$^{-/-}$ cells rescued with cyclin D1a or cyclin D1b viral expression vectors. Cells were treated with 0.5 μmol/L doxorubicin for 24 hours prior to being stained with PI and cell cycle analysis by FACS. DOX, doxorubicin.

transduced with each cyclin D1 isoform. Cyclin D1a enhanced the basal- (Fig. 3A) and radiation-induced γH2AX (Fig. 3B). Western blot analysis showed that cyclin D1a enhanced more doxorubicin-mediated γH2AX (Fig. 3C). Cyclin D1b also induced γH2AX, but quantitatively the effect was significantly less ((Fig. 3C, lane 4 versus lane 6), γH2AX L.E., S.E.). Western blot analysis of *Cyclin D1*$^{-/-}$-transduced cells with an antibody directed to the FLAG epitope confirmed the expression of cyclin D1a and cyclin D1b in the *Cyclin D1*$^{-/-}$ MEFs (Fig. 3B and C).

γH2AX has been reported to occur after DDR in all phases of the cell cycle (28) or at the site of replication fork breakage in the S phase of SV40-transformed Hela cells (29, 30). Cyclin D1 has been shown to inhibit S-phase entry in some studies (31) or promote S-phase entry in other studies (32). The induction of DNA synthesis by cyclin D1 could potentially contribute to the DDR induction of replication forks. In the current studies S phase was increased from 12.2% to 14.3% (Fig. 2B). The kinetics of DDR induction was rapid (15 minutes) and preceded the effect on DNA synthesis (>6 hours). Cyclin D1 induction of DNA synthesis requires the serum-dependent association with Cdk (33). To examine whether the effect of cyclin D1a on the DDR could be further uncoupled from the effect on DNA synthesis we examined the effect of cyclin D1 on the DDR in the absence of serum. The *Cyclin D1*$^{+/+}$ and *Cyclin D1*$^{-/-}$ cells had reduced DNA synthesis in the absence of serum (Supplementary Fig. S2A). Cyclin D1 significantly enhanced the DDR as assessed by γH2AX induction (Supplementary Fig. S2B) in the absence of serum. *Cyclin D1*$^{-/-}$ cells showed no increase in DDR in the absence of serum.

To further examine whether the effect of cyclin D1a to induce DNA synthesis can be uncoupled from its ability to induce the DDR we conducted FACS analysis (bromodeoxyuridine/PI) to assess S phase of doxorubicin-treated *Cyclin D1*$^{-/-}$ 3T3 cells transduced with cyclin D1a or cyclin D1b (Supplementary Fig. S3). Cyclin D1a enhanced the DDR induced by doxorubicin, but did not enhance S-phase entry. Three lines of evidence therefore suggest that induction of the DDR by cyclin D1a can be uncoupled from its induction of DNA synthesis. As cyclin D1 induced the DDR within 15 minutes (Fig. 3B), preceding DNA synthesis, cyclin D1 induces the DDR in the absence of serum (Supplementary Fig. S2), and cyclin D1 induces the DDR of doxorubicin without increasing S phase (Supplementary Fig. S3). These studies suggest that the ability of cyclin D1 to induce the DDR can be dissociated from its ability to induce DNA synthesis.

The cyclin D1a splice form binds to p21^{CIP1} and regulates the induction of p21^{CIP1} abundance

p21^{CIP1} is an essential target of p53 which governs components of the genotoxic response (34). We had observed that the abundance of p21^{CIP1} was increased by transduction of *Cyclin D1*$^{-/-}$ cells with cyclin D1a (Fig. 3B and C). p21^{CIP1} abundance was induced 1 hour after radiation and this induction was enhanced upon expression of cyclin D1a versus cyclin D1b (Fig. 3B). Correspondingly, the abundance of p21^{CIP1} was reduced in MEFs, bone marrow macrophages, and 3T3 cells lacking cyclin D1 (Fig. 4A). Cyclin D1b induced the basal level of p21^{CIP1} but did not enhance the DDR induction of p21^{CIP1} compared with vector control (Fig. 3B, p21^{CIP1} L.E., lane 4 versus 12).

The induction of p21^{CIP1} by cyclin D1 increased the possibility that p21^{CIP1} may contribute to activation of DSB repair. To determine the mechanism by which cyclin D1a enhanced the DDR, *p21*$^{Cip1-/-}$ MEFs were transduced with retroviral expression vectors encoding cyclin D1a or cyclin D1b and treated with doxorubicin. The expression of cyclin D1a and cyclin D1b was confirmed by Western blotting (Fig. 4B). Cyclin D1 antibody (clone DCS-6) was used to detect both the endogenous cyclin D1 and the transduced cyclin D1 isoforms. Cyclin D1a enhanced basal and doxorubicin-induced phosphorylation of γH2AX (Fig. 4B) and the expression of Rad51. The relative induction of γH2AX and the expression of Rad51 were greater with cyclin D1a compared with cyclin D1b. The enhancement of doxorubicin-mediated γH2AX phosphorylation and Rad51 expression was abrogated in *p21*$^{Cip1-/-}$ cells (Fig. 4B).

The increased abundance of p21^{CIP1} in *Cyclin D1*$^{+/+}$ versus *Cyclin D1*$^{-/-}$ cells increased the possibility that cyclin D1 may physically associate with p21^{CIP1} to regulate the abundance

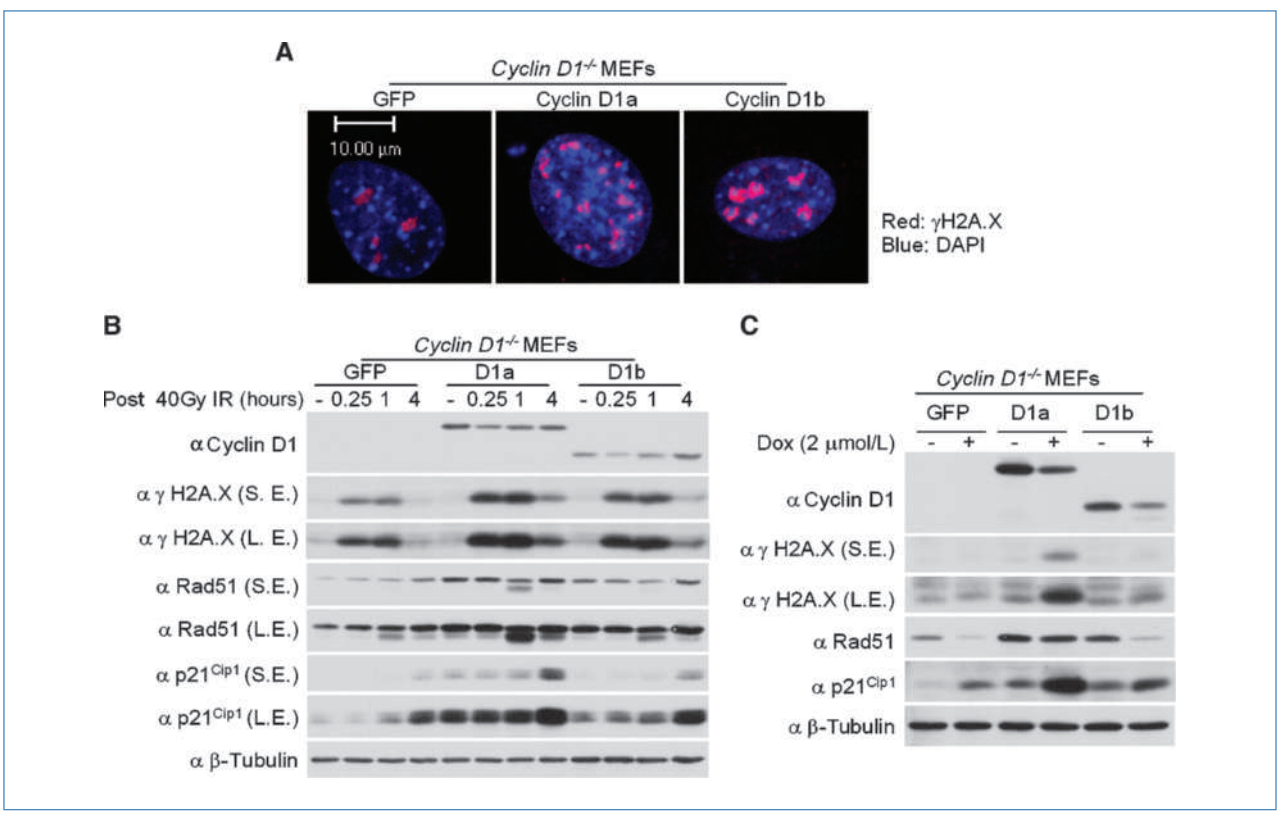

Figure 3. Induction of γH2AX foci by cyclin D1a isoform. A, confocal microscopy for γH2AX with nuclear staining using 4′, 6-diamidino-2-phenylindole (DAPI). *Cyclin D1*$^{-/-}$ MEFs were transduced with cyclin D1 expression vectors as indicated. Scale bar, 10 μm. B and C, Western blot analysis of *Cyclin D1*$^{-/-}$ cells transduced with cyclin D1 isoform–specific retrovirus as indicated. Antibodies are directed to γH2AX (Ser139). Cells were treated with 40 Gy irradiation (B) or doxorubicin (C). IR, ionizing radiation.

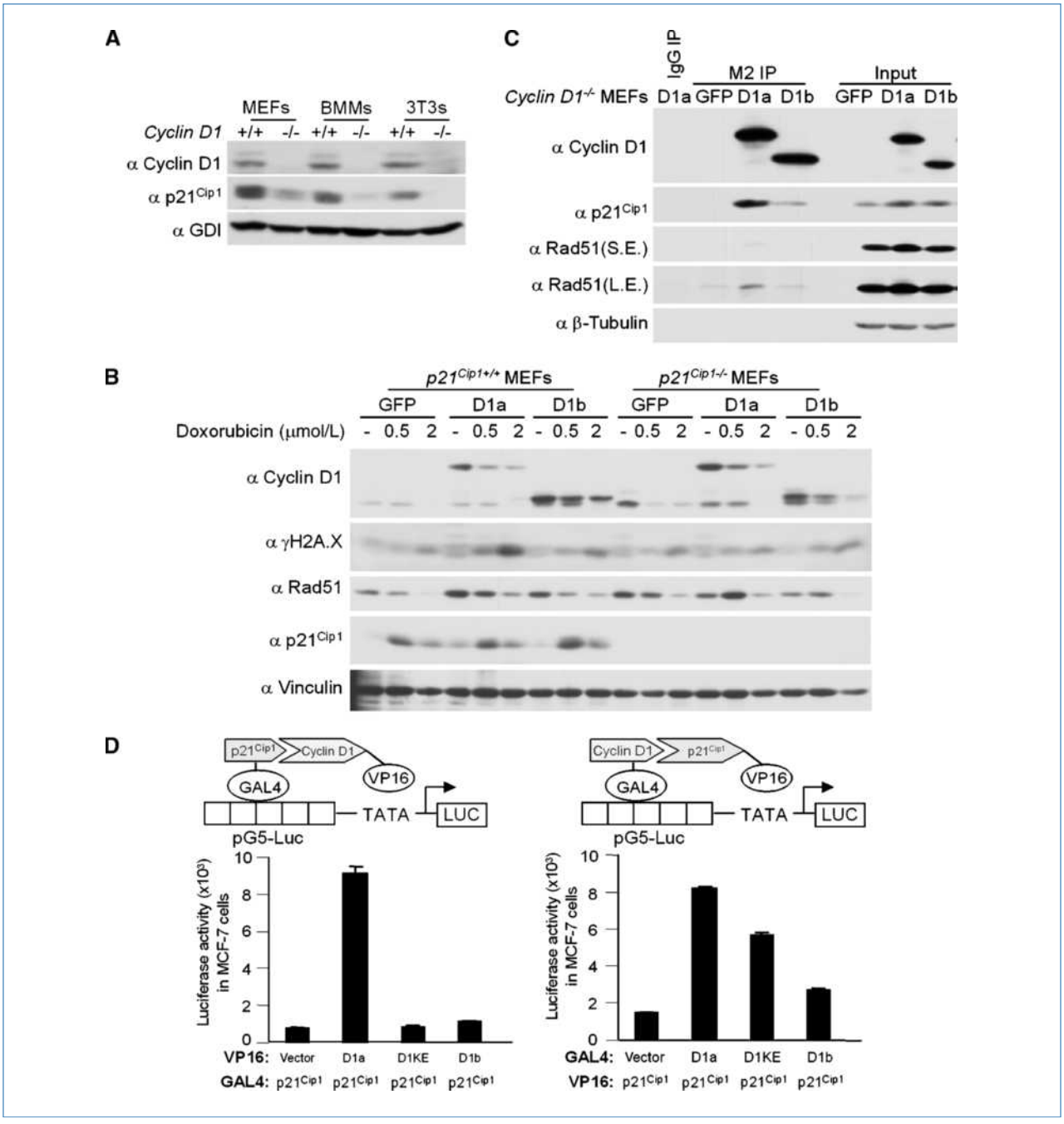

Figure 4. Cyclin D1a–mediated cellular DDR involves p21^{CIP1}. A, Western blot analysis of *Cyclin D1*$^{+/+}$ and *Cyclin D1*$^{-/-}$ cells indicates reduction in p21^{CIP1} abundance in *Cyclin D1*$^{-/-}$ cells. BMM, bone marrow macrophages. B, *p21*$^{Cip1-/-}$ cells transduced with isoform-specific cyclin D1 retrovirus vectors and treated with the DDR-inducing agent doxorubicin. Cyclin D1 antibody (clone DCS-6) was used to detect both the endogenous and the transduced cyclin D1 isoforms. The induction of γH2AX (Ser139) by cyclin D1a is attenuated in *p21*$^{Cip1-/-}$ cells. C, immunoprecipitation (IP)-Western blot analysis of cyclin D1 isoforms indicates coassociation of cyclin D1 with endogenous p21^{CIP1} and Rad51. D, mammalian two-hybrid shows preferential coassociation of p21^{CIP1} with cyclin D1a. Data are mean ± SE luciferase activity.

of p21^{CIP1}. To examine the interaction between cyclin D1 and p21^{CIP1}, immunoprecipitation, Western blotting, and mammalian two-hybrid analysis were conducted (Fig. 4C and D). The amino terminal FLAG epitope was used to immunoprecipitate equal amounts of cyclin D1a or cyclin D1b, and sequential Western blotting was conducted with an antibody to the p21^{CIP1} protein (Fig. 4C). Cyclin D1a bound to p21^{CIP1}. The relative abundance of p21^{CIP1} associated with cyclin D1b was reduced approximately 90% compared with cyclin D1a (Fig. 4C).

The $p21^{CIP1}$ cDNA was linked to the Gal4 DNA binding domain, and the interaction with cyclin D1 was assessed in the context of a cyclin D1-VP16 fusion expression plasmid. Cyclin D1a enhanced $p21^{CIP1}$-Gal4 activity ~9-fold (Fig. 4D, left). Point mutation of the CDK binding site of cyclin D1 reduced or abrogated interaction with the $p21^{CIP1}$-Gal4 hybrid (Fig. 4D, left). The alternate splice form of cyclin D1 (cyclin D1b) failed to interact significantly with the $p21^{CIP1}$-Gal4 hybrid (Fig. 4D, left). Reciprocal analysis was conducted in which the cyclin D1 cDNA was linked to GAL4 and the $p21^{CIP1}$ cDNA was linked to VP16 to assess interaction using a multimeric Gal4 DNA binding site linked to a luciferase reporter (Fig. 4D, right). Cyclin D1a and $p21^{CIP1}$ coexpression enhanced reporter activity 5-fold. This activity was reduced 80% using the cyclin D1b cDNA as bait in the two-hybrid assay (Fig. 4D, right).

Cyclin D1 induces formation of repair factors to chromatin

To examine further the mechanism by which cyclin D1a induced the DDR we considered recent studies showing that the stable association of DDR factors with chromatin can trigger and amplify the DDR signal via an ATM- and DNA-PK–dependent manner (21). We had previously shown that cyclin D1a was in the context of local chromatin in chromatin immunoprecipitation assays at DNA transcription factor binding sites (35, 36). We examined the possibility that cyclin D1a tethered to chromatin may function in a similar manner as DDR factors to activate the DDR. DNA repair factors fused to the *Escherichia coli* lac-repressor (lacR) and tagged with Cherry-red fluorescent protein were examined in an NIH3T3 cell line that contains 256 repeats of the lacO stably integrated into chromosome 3, known as NIH2/4 (25). Fusion proteins accumulated at the lacO array as distinct nuclear foci: immobilization of ATM, NBS1, or MDC1 was sufficient to activate the DDR as evidenced by phosphorylation of H2AX at the lacO site (Fig. 5A–C). Phosphorylation of H2AX at the lacO site was enhanced by NBS1, MDC1, or ATM alone as previously shown (21). Immobilization of cyclin D1b did not affect H2AX phosphorylation. Immobilization of cyclin D1a in chromatin, however, enhanced H2AX phosphorylation substantially (Fig. 5B and C). Thus, immobilization of cyclin D1a in the context of local chromatin is sufficient to activate the DDR.

In the absence of DNA damage neither cyclin D1a nor cyclin D1b can recruit Rad51 to local chromatin (Supplementary Fig. S4). Rad51 is involved in recombination repair of DSBs. Additional experiments were conducted to address if both cyclin D1 isoforms could recruit Rad51 to local chromatin in response to DNA damage. NIH2/4 cells were transfected with Cherry-lacR-NLS-cyclin D1a, Cherry-lacR-NLS-cyclin D1b, or vector control Cherry-lacR-NLS. Twenty-four hours later cells were treated with 0.4 μmol/L doxorubicin for 3 hours. Then immunofluorescence staining was conducted using specific antibody to Rad51. We found that only cyclin D1a recruits Rad51 to local chromatin in response to DNA damage (Fig. 5D).

Cyclin D1a enhancement of doxorubicin-induced γH2AX involves DNA-PK, c-*jun*-NH-kinase, and casein kinase 2

The current studies showed that cyclin D1a increases H2AX phosphorylation induced by γ irradiation or chemotherapy agents (doxorubicin or FU) in fibroblasts or in the colon cancer line HCT116 (Figs. 1 and 3). To examine further the mechanism by which cyclin D1 induced γH2AX, we treated 3T3 cells with doxorubicin combined with inhibitors of the DDR signaling pathway. Western blot analysis was conducted to detect γH2AX. Compared with vehicle control, the DNA-PK inhibitor (NU7026), casein kinase 2 (CK2) inhibitor (TBB), and c-*jun*-NH-kinase (JNK) inhibitor (SP600125) reduced doxorubicin-induced γH2AX in cyclin D1a–transduced cells (Supplementary Fig. S4B; $P < 0.05$), but not in control vector–transduced cells (Supplementary Fig. S4C). In contrast, the ATM inhibitor (KU55933) did not show a significant change. These findings suggest cyclin D1a induction of doxorubicin-induced γH2AX involves DNA-PK, JNK, and CK2 (Supplementary Fig. S4C).

Discussion

Cyclin D1a enhances the DDR

The current studies provide evidence for an important new function of cyclin D1a overexpression in amplifying the cellular DDR. Induction of the DDR by cyclin D1a was evidenced by enhanced formation of damaged double-stranded DNA assessed by comet assay activity, induction of Rad51 foci, and formation and induction of γH2AX phosphorylation. Cyclin D1–mediated DDR was observed with the cyclin D1a versus the cyclin D1b isoform. The physiologic relevance of the amplification of DDR by cyclin D1a was evidenced by the findings that siRNA to endogenous cyclin D1 reduced the DDR phenotype. The importance of cyclin D1a in the DDR was shown using distinct activators of the DDR, including γ irradiation, double-stranded DNA damage–inducing agents (doxorubicin, FU), and the targeting of single DNA repair factors to chromatin.

Previous studies on cyclin D1 in regulating the DDR suggest cell type–dependent functions. Forced expression of cyclin E, but not cyclin D1, in rat embryo fibroblasts induced aneuploidy but did not affect gene amplification (37). Forced expression of a degradation-defective mutant of cyclin E induced more aneuploidy (37). In subsequent studies, genome-wide microarray analysis showed that cyclin D1 induced expression of genes involved in DNA replication and DNA damage checkpoints, suggesting a role for cyclin D1 in the DDR. Cyclin D1a induced the mRNA expression of minichromosome maintenance 3 (MCM3), MCM4, replication factor C (activator 1) 4 (Rfc4), cell division cycle 6 homology (Cdc6), cell division cell associated 7 (Cdc7), and H2Afx (H2A Histone family member X) in MEFs (35). Mammary gland–targeted cyclin D1–inducible antisense transgenics showed that endogenous cyclin D1 maintained expression of MCM2, Rfc2, Cdc20, Rad51, and histone 1 in the mammary epithelium (38). In subsequent studies forced expression of cyclin D1 induced expression of genes regulating the DDR,

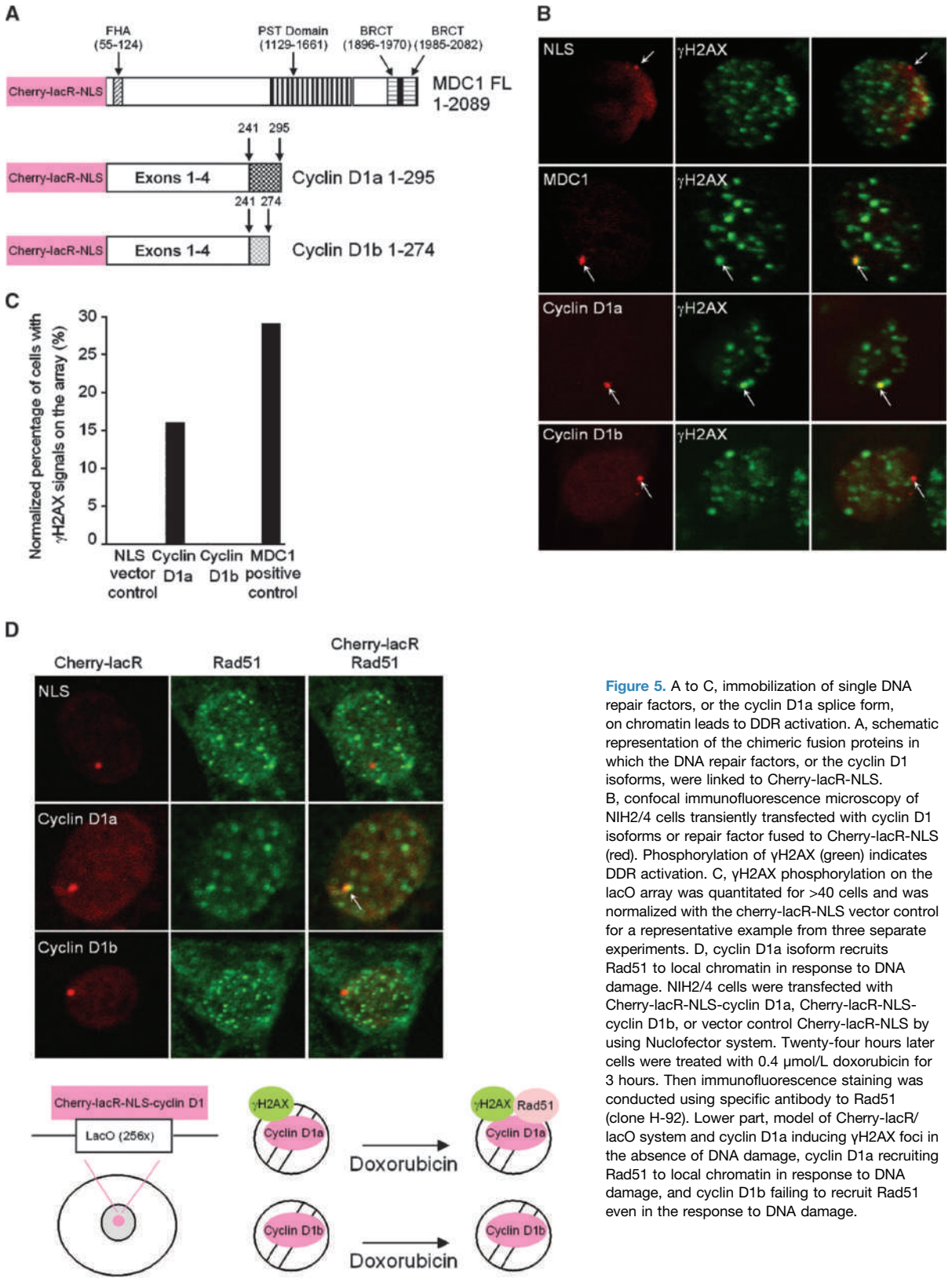

Figure 5. A to C, immobilization of single DNA repair factors, or the cyclin D1a splice form, on chromatin leads to DDR activation. A, schematic representation of the chimeric fusion proteins in which the DNA repair factors, or the cyclin D1 isoforms, were linked to Cherry-lacR-NLS. B, confocal immunofluorescence microscopy of NIH2/4 cells transiently transfected with cyclin D1 isoforms or repair factor fused to Cherry-lacR-NLS (red). Phosphorylation of γH2AX (green) indicates DDR activation. C, γH2AX phosphorylation on the lacO array was quantitated for >40 cells and was normalized with the cherry-lacR-NLS vector control for a representative example from three separate experiments. D, cyclin D1a isoform recruits Rad51 to local chromatin in response to DNA damage. NIH2/4 cells were transfected with Cherry-lacR-NLS-cyclin D1a, Cherry-lacR-NLS-cyclin D1b, or vector control Cherry-lacR-NLS by using Nuclofector system. Twenty-four hours later cells were treated with 0.4 μmol/L doxorubicin for 3 hours. Then immunofluorescence staining was conducted using specific antibody to Rad51 (clone H-92). Lower part, model of Cherry-lacR/lacO system and cyclin D1a inducing γH2AX foci in the absence of DNA damage, cyclin D1a recruiting Rad51 to local chromatin in response to DNA damage, and cyclin D1b failing to recruit Rad51 even in the response to DNA damage.

as MCM3 and Cdc7 (39) expression was increased in mammary tumors derived from mammary epithelial cell–targeted cyclin D1a transgenic mice. Forced expression of a degradation-defective cyclin D1a mutant induced the DDR in murine lymphoid tissue (40). Thus, prior indirect evidence implicated cyclin D1 in enhancing gene expression governing the DDR in a number of different cell types.

Cyclin D1 induction of the DDR requires $p21^{CIP1}$

Herein cyclin D1 bound to, and augmented the abundance of, $p21^{CIP1}$. In $p21^{Cip1-/-}$ MEFs, cyclin D1 expression failed to increase doxorubicin-induced γH2AX (Fig. 5B). This suggests that cyclin D1 may increase the DDR through $p21^{CIP1}$. Previous studies showed that downregulation of $p21^{CIP1}$ inhibited Rad51 foci formation (41). Expression of Rad51, which is involved in the repair of DSBs induced by cisplatin and other platinum agents (42, 43), was induced by cyclin D1a. Elevated Rad51 levels are found in tumor cell lines and primary tumors (34), and correlated with resistance to drug and radiation therapy, tumor recurrence (44), and poor prognosis (45–47). The functions of a number of tumor suppressor genes have been linked to elevated Rad51 levels, suggesting that increased Rad51 activity may promote tumorigenesis (34, 44).

Cyclin D1a recruitment to local chromatin elicits the DDR

The current studies showed that the recruitment of cyclin D1a, but not cyclin D1b, is sufficient to activate the DDR. The recruitment of DNA repair factors to chromatin is also sufficient to elicit the DDR characterized by γH2AX phosphorylation. In previous studies, cyclin D1a reintroduction into *Cyclin D1*$^{-/-}$ cells resulted in recruitment of cyclin D1 to chromatin at a peroxisome proliferator-activated receptor response element, associated with local deacetylation of core histones, in particular of H3Lys9 (35). Cyclin D1a recruits SUV39 and HP1α (35) to local chromatin and HP1β mobilization is thought to promote chromatin changes that initiate the DDR (48). The recruitment of DNA repair factors to chromatin induces a G_2-M delay (25). In previous studies DNA-damaging agents, and as shown here, cyclin D1a, induced a G_2-M arrest. As cyclin D1a enhanced recruitment of DNA repair factors to chromatin this effect may contribute to the G_2-M arrest.

Cyclin D1 is expressed early in a variety of human cancers and premalignant diseases, including colonic polyps and breast ductal carcinoma *in situ*. Activation of the DDR occurs in human cancer (49). The current studies suggest that cyclin D1a may contribute to the induction of DDR in tumors. The induction of DDR occurred more with cyclin D1a than with cyclin D1b. Clinical studies will be important to determine the role of cyclin D1 isoforms in therapeutic stratification of patients receiving therapy inducing the DDR.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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