Overall Cdk activity modulates the DNA damage response in mammalian cells

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In response to DNA damage, cells activate a phosphorylation-based signaling cascade known as the DNA damage response (DDR). One of the main outcomes of DDR activation is inhibition of cyclin-dependent kinase (Cdk) activity to restrain cell cycle progression until lesions are healed. Recent studies have revealed a reverse connection by which Cdk activity modulates processing of DNA break ends and DDR activation. However, the specific contribution of individual Cdks to this process remains poorly understood. To address this issue, we have examined the DDR in murine cells carrying a defined set of Cdks. Our results reveal that genome maintenance programs of postreplicative cells, including DDR, are regulated by the overall level of Cdk activity and not by specific Cdks.

Introduction

Eukaryotic cells possess a repertoire of DNA repair systems that are used depending on the nature of the lesion. Also, the type of repair process is also reliant on cell proliferation, and similar lesions may be dealt with differently depending on whether they occur in a quiescent or a dividing cell or even on the cell cycle phase when they are detected (Branzei and Foiani, 2008). Regardless of the repair mechanism, a mandatory step of the DNA damage response (DDR) in proliferating cells is to arrest the cell cycle. This is mediated via a checkpoint cascade that ultimately leads to inhibition of the Cdks, the enzymes responsible for driving cell division. DNA lesions are recognized by a network of sensor and mediator factors that result in the rapid recruitment of ataxia telangiectasia mutated (ATM) and ATM-Rad3 related (ATR) to the site of DNA damage (Harper and Elledge, 2007). These kinases activate Chk1 and Chk2 (Falck et al., 2005), which ultimately activate numerous cellular pathways including cell cycle arrest (Matsuoka et al., 2007).

In dividing cells, Cdk activity is modulated by overlapping mechanisms including availability of cyclins, regulatory phosphorylation by upstream kinases (CAK, Wee1, and Myt1) and phosphatases (Cdc25), as well as binding of protein inhibitors (Malumbres and Barbacid, 2005). These pathways are directly or indirectly modulated by the DDR. Early within this response, Chk1/Chk2 inactivates the Cdc25 phosphatases that cancel the inhibitory phosphorylations on the Cdks (Mailand et al., 2000). In addition, p53 and Mdm2 are targeted by several DDR kinases including ATM, ATR, DNAPK, Chk2, and possibly Chk1, resulting in the activation of p53 transcriptional program and ultimately in the accumulation of the Cdk inhibitor $p21^{Cip1}$ (Lukas et al., 2004). Also, decreased Cdk activity results in diminished transcriptional activity of the E2F family members responsible for the synthesis of cyclins, thus leading to sustained inhibition of Cdk activity as long as the repair activity is in progress.

Recent data have also placed Cdk activity upstream of the DDR. In *Saccharomyces cerevisiae*, Cdk activity is required for the processing of double strand breaks (DSBs) and for efficient checkpoint response (Aylon et al., 2004; Ira et al., 2004). Likewise, addition of broad-range Cdk inhibitors such as roscovitine to human cells abolished ATR/Chk1 damage–dependent phosphorylation and blocked DSB repair by homologous recombination (HR; Jazayeri et al., 2006). Moreover, ATR/Chk1 activation and HR-mediated repair are restricted to postreplicative cells (Cuadrado et al., 2006; Jazayeri et al., 2006), suggesting that S- and G2-specific Cdk phosphorylation events could be necessary to "license" this pathway. Because many of the components of

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Abbreviations used in this paper: ATM, ataxia telangiectasia mutated; ATR, ATM-Rad3 related; AurB, aurora B; DDR, DNA damage response; DSB, double strand break; HR, homologous recombination; IR, γ-irradiation; MEF, murine embryonic fibroblast; MMS, methyl methanesulfonate; NCS, neocarzinostatin; RPA, replication protein A; shRNA, short hairpin RNA; TKO, triple knockout.

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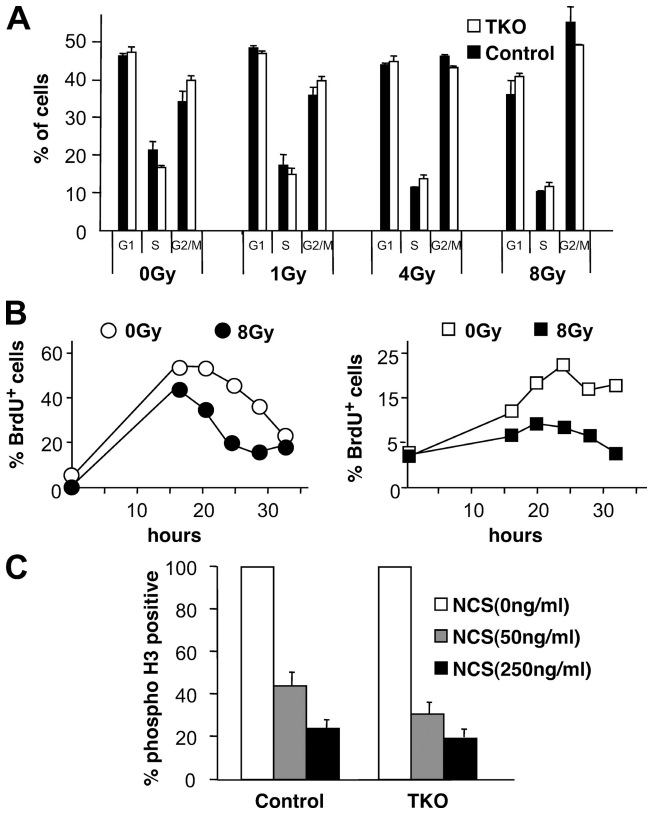

Figure 1. **Functional G1/S and G2/M checkpoints in the absence of interphase Cdks.** (A) Cell cycle distribution of *Cdk4*$^{+/+}$;*Cdk2*$^{+/+}$;*Cdk6*$^{-/-}$ control and TKO MEFs 10 h after IR with the indicated doses. (B) Percentage of quiescent *Cdk4*$^{+/+}$;*Cdk2*$^{+/+}$;*Cdk6*$^{-/-}$ control (left) and TKO (right) MEFs in S phase after serum stimulation and 2-h pulses of BrdU. Cells were either nonirradiated or exposed to 8 Gy of IR before serum stimulation. (C) Fraction of phospho-H3–positive *Cdk4*$^{+/+}$;*Cdk2*$^{+/+}$;*Cdk6*$^{-/-}$ control and TKO MEFs either untreated or 45 min after NCS. Error bars indicate mean ± SD (n = 3).

the DDR harbor putative Cdk phosphorylation sites, identification of those Cdk substrates that participate in the DDR has attracted significant attention. One potential candidate is CtIP (Sae2 in yeast). To allow HR, the DNA ends of DSBs need to be converted to single-strand DNA, an essential step abrogated upon Cdk inhibition. In mammals, the resection step is dependent on CtIP (Sartori et al., 2007). Furthermore, a S267E phosphomimetic mutant of a Cdk phosphorylation site on Sae2 alleviates the need for Cdk activity in DSB resection (Huertas et al., 2008). A similar outcome resulted from the T847E substitution in human CtIP (Huertas and Jackson, 2009). Collectively, these data implicate Cdk activity in at least the crucial resection step during DSB repair. Yet, it is likely that Cdk-mediated control of the DDR relies on several targets. Indeed, additional Cdk targets involved in the DDR, such as BRCA1 and 2, Rad9, Crb2, and ATRIP, as well as topoisomerases and helicases have been described previously (Ruffner et al., 1999; Liberi et al., 2000; Caspari et al., 2002; St Onge et al., 2003; Myers et al., 2007; Venere et al., 2007).

Unfortunately, available data on mammalian cells largely rely on the use of broad-range Cdk inhibitors. Thus, it remains unclear whether there are unique requirements for individual Cdks in the regulation of the DDR. We have now used murine embryonic fibroblasts (MEFs) lacking interphase Cdks to determine whether checkpoint responses to DNA damage and repair of DSBs are functional in their absence. We have also interrogated the role of Cdk1 and Cdk2 in the activation of the DDR. Our results suggest a high degree of functional redundancy in Cdk-mediated control of the DDR and argue against specific roles of individual Cdks.

Results and discussion

Genome stability of MEFs lacking interphase Cdks

Cdk inhibition in cultured cells results in activation of the DDR (Maude and Enders, 2005). Thus, we decided to examine whether MEFs lacking all interphase Cdks (Santamaría et al., 2007) as a result of genetic ablation (Cdk2, Cdk4, and Cdk6) and natural mutation (Cdk3) presented endogenous accumulation of DNA damage. These cells, designated as triple knockout (TKO) MEFs, did not show increased ATM or ATR kinase activities as measured by phosphospecific antibodies against the Ser^{1981} and Ser^{345} residues of ATM and Chk1, respectively (Fig. S1 A). One of the early markers for the induction of the DDR is the formation of phosphorylated γ-H2AX foci. These foci appear within minutes after γ-irradiation (IR) and mark the DSBs (Rogakou et al., 1999). To rule out the possibility that the DDR could be activated in a small number of cells, and thus preclude detection by Western blot analysis, we measured the levels of γ-H2AX by high throughput microscopy (Murga et al., 2007). We also failed to detect activation of the DDR in the TKO MEFs (Fig. S1 B). Thus, loss of all interphase Cdks does not result in activation of the DDR.

Functional G1/S and G2/M checkpoints in the absence of interphase Cdks

Checkpoint activation ultimately converges on the inhibition of Cdk activities to restrain cell cycle progression. Thus, it is possible that the absence of interphase Cdks affects how cells arrest their cycle. We next addressed whether the G1/S and G2/M checkpoints were functional in TKO MEFs. Proliferating cells were submitted to different doses of IR and the S phase population determined by FACS. 10 h after irradiation, TKO and control MEFs displayed similar dose–response reductions in DNA synthesis (Fig. 1 A). These observations suggested a functional G1/S checkpoint. However, this response could be underestimated because of the lower proliferation rate of TKO MEFs (Santamaría et al., 2007). Thus, we tested the functionality of this checkpoint in serum-deprived cells. Primary MEFs were serum starved for 72 h before IR. Entry into S phase was monitored by BrdU incorporation upon serum stimulation. Both TKO and control cells displayed a significant reduction in the number of BrdU-positive cells when compared with the nonirradiated controls, indicating a functional G1/S checkpoint (Fig. 1 B).

Next, we examined whether TKO cells had a functional G2/M checkpoint. To this end, MEFs were exposed to the radiomimetic drug neocarzinostatin (NCS) and determined the number of cells positive for the mitotic marker phosphorylated histone H3 (Xu et al., 2001). No significant differences between TKO and control MEFs were observed (Fig. 1 C). Thus, cells

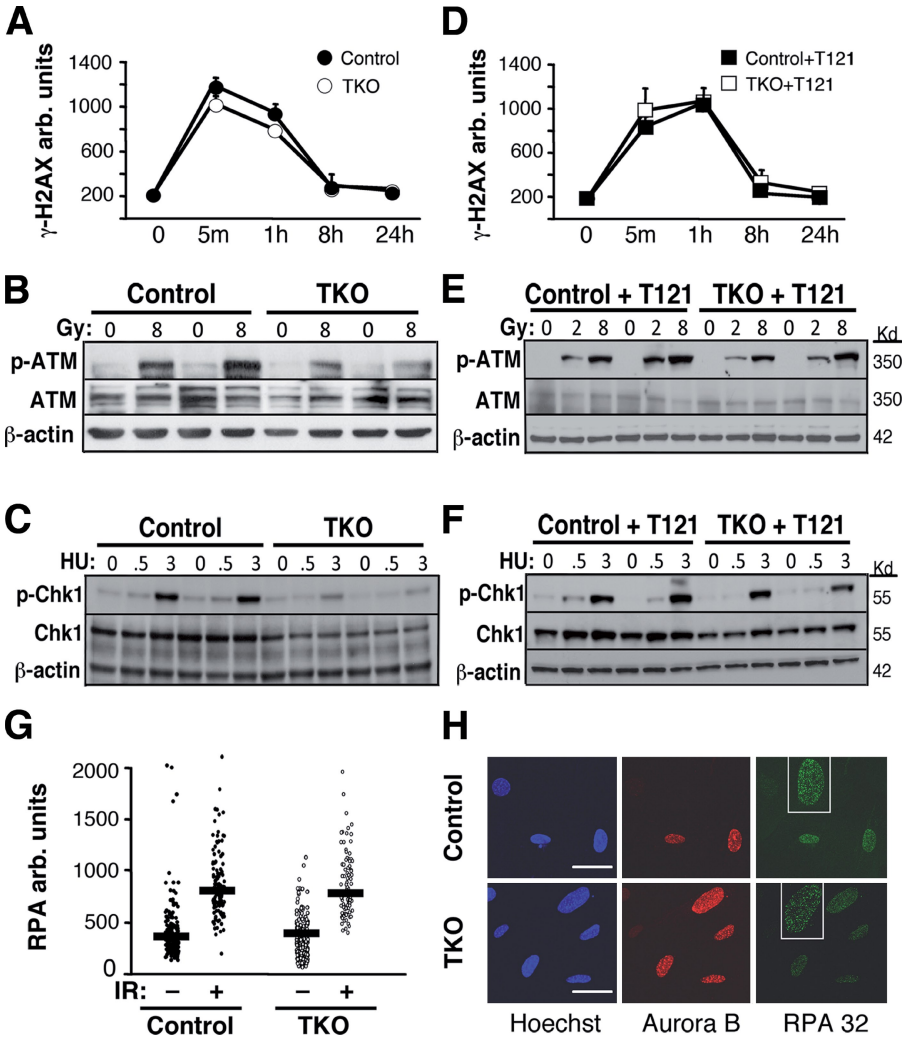

Figure 2. **DNA repair and checkpoint activation in TKO MEFs.** (A) Cells were treated for 1 h with 50 ng/ml NCS, washed, and analyzed by high throughput microscopy over time. The intensity of the γ-H2AX signal per nucleus was measured for *Cdk4*$^{+/+}$;*Cdk2*$^{+/+}$;*Cdk6*$^{-/-}$ control and TKO MEFs at the indicated times. (B) Whole cell extracts were prepared from *Cdk4*$^{+/+}$;*Cdk2*$^{+/+}$;*Cdk6*$^{-/-}$ control and TKO MEFs 45 min after 8 Gy of IR. Extracts were blotted with antibodies against ATM-Ser^{1981} or ATM as indicated in Materials and methods. Samples from two independent experiments were loaded. (C) *Cdk4*$^{+/+}$;*Cdk2*$^{+/+}$;*Cdk6*$^{-/-}$ control and TKO MEFs were treated for 2 h with hydroxyurea (HU) at the indicated concentrations (millimolars). Extracts were blotted with antibodies against Chk1-Ser^{345} or Chk1 as indicated in Materials and methods. Samples from two independent experiments were loaded. (D) *Cdk4*$^{+/+}$;*Cdk2*$^{+/+}$;*Cdk6*$^{-/-}$ control and TKO MEFs were infected with retroviral particles expressing the T121 fragment (remaining information is as described in A). (E) Whole cell extracts were prepared from *Cdk4*$^{+/+}$;*Cdk2*$^{+/+}$;*Cdk6*$^{-/-}$ control and TKO MEFs expressing the T121 fragment 45 min after IR with 2 and 8 Gy. Extracts were blotted with antibodies against ATM-Ser^{1981} or ATM as indicated in Materials and methods. Samples from two independent experiments were loaded. (F) *Cdk4*$^{+/+}$;*Cdk2*$^{+/+}$;*Cdk6*$^{-/-}$ control and TKO MEFs expressing the T121 fragment were treated for 2 h with hydroxyurea at the indicated concentrations (millimolars). Extracts were blotted with antibodies against Chk1-Ser^{345} or Chk1 as indicated in Materials and methods. Samples from two independent experiments were loaded. (G) *Cdk4*$^{+/+}$;*Cdk2*$^{+/+}$;*Cdk6*$^{-/-}$ control and TKO MEFs were preextracted 3 h after IR, and chromatin-bound RPA were analyzed by high throughput microscopy. The results are the mean of three independent experiments. Horizontal bars mark median values. (H) *Cdk4*$^{+/+}$;*Cdk2*$^{+/+}$;*Cdk6*$^{-/-}$ control and TKO MEFs were submitted to IR, maintained in culture for 3 h, and preextracted before incubation with the indicated antibodies and confocal analysis. Insets on RPA32 fields show magnified views of a positive cell. Error bars indicate mean ± SD (n = 3). Bars, 15 μM.

devoid of interphase Cdks maintain the functionality of their G1/S and G2/M checkpoints.

Proficient DDR and repair capabilities in TKO MEFs

Appearance and clearance of γ-H2AX foci have been used as surrogate readouts for initiation and completion of DNA repair (Riballo et al., 2004). Thus, we quantified γ-H2AX foci in cells exposed to NCS for 1 h using high throughput microscopy. TKO and control MEFs reached a maximum intensity with parallel kinetics after washing out NCS (Fig. 2 A). Moreover, they displayed similar decay until they reached basal levels. However, the γ-H2AX signal in TKO MEFs was slightly lower than in control cells and was accompanied by decreased activation of ATM- and ATR-dependent phosphorylation (Fig. 2, B and C). The expression of Chk1 and other DNA repair factors is under the control of the E2F program and is restricted to actively proliferating cells (Kaneko et al., 1999; Ren et al., 2002). Indeed, total levels of Chk1 were reduced in TKO cells (Fig. 2 C). Thus, it is possible that the lower proliferation rate of TKO MEFs could account for the suboptimal activation of the DDR. To examine this possibility, we restored the proliferation rate of TKO MEFs to wild-type levels by inactivating the Rb protein family (Santamaría et al., 2007). This was achieved by retroviral delivery of T121, a fragment of the SV40 large T antigen that antagonizes the three Rb family members but not p53 (Sáenz-Robles et al., 1994). When the DNA repair was assayed on T121-infected TKO MEFs, the kinetics and peak levels of the γ-H2AX signal were indistinguishable from controls (Fig. 2 D). Also, phosphorylation levels of ATM and Chk1 in response to IR and replication stress were recovered (Fig. 2, E and F). Thus, ablation of interphase Cdks leads to a minor reduction on DDR activation that does not impact on DNA repair. Moreover, this is only a consequence of the reduced proliferation rate of these cells rather than a direct effect of Cdk activity on the DDR and on DNA repair.

Cdk activity has also been proposed to promote the DDR by stimulating end resection of DSBs (Jazayeri et al., 2006). Thus, we exposed primary TKO and control MEFs to IR and monitored foci formation of the single-stranded DNA-binding protein replication protein A (RPA) as a surrogate marker of DNA end resection.

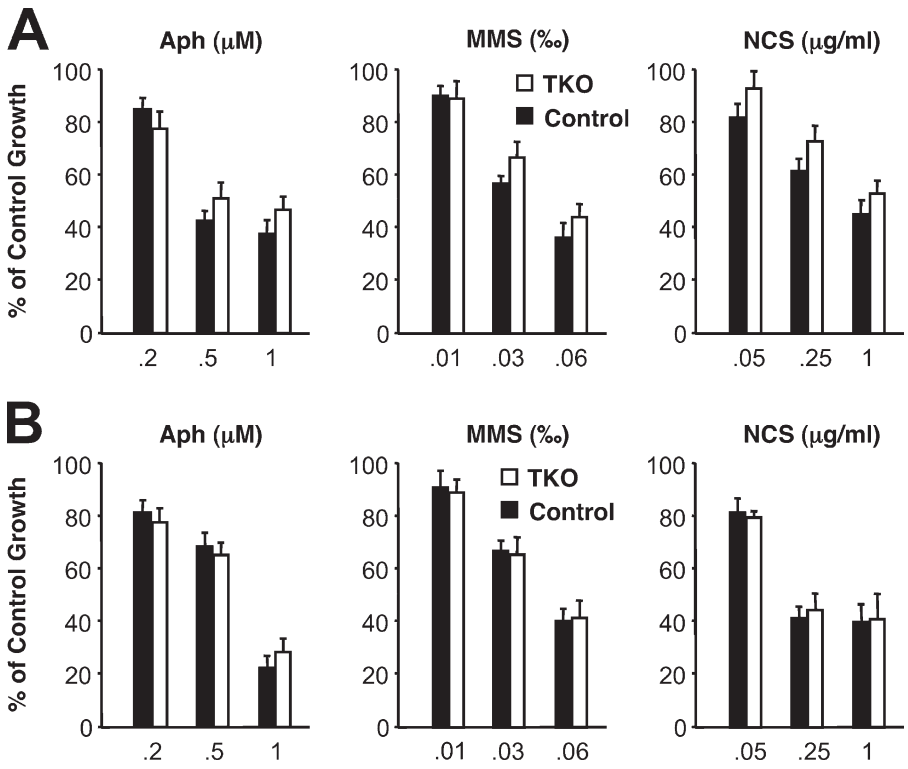

Figure 3. **TKO MEFs are not hypersensitive to DNA-damaging agents.** (A) *Cdk4*$^{+/+}$;*Cdk2*$^{+/+}$;*Cdk6*$^{-/-}$ control and TKO MEFs were grown for 5 d in the presence of aphidicolin (Aph), MMS, or NCS at the indicated concentrations. Graphs represent the variation in cell number at the end of the experiment normalized to the untreated controls. The results are the mean of two (control MEFs) and four (TKO MEFs) independent experiments. (B) *Cdk4*$^{+/+}$;*Cdk2*$^{+/+}$;*Cdk6*$^{-/-}$ control and TKO MEFs expressing the T121 fragment (remaining information is as described in A). Error bars indicate ± SD.

Analysis of chromatin-bound RPA by high throughput microscopy revealed a comparable response (Fig. 2 G). Furthermore, when individual cells were observed by confocal microscopy, the nuclear distribution of RPA foci in TKO cells was indistinguishable from controls. In both cases, all cells permissive for HR-mediated repair, those in late S or G2 as determined by aurora B (AurB)–positive staining, showed chromatin-bound RPA foci (Fig. 2 H).

Finally, we measured the DNA repair capabilities of TKO MEFs by examining their response to DNA-damaging agents. Cells were exposed to genotoxic chemicals including methyl methanesulfonate (MMS), NCS, and aphidicolin. We monitored total cell numbers because the most common cellular responses to DNA damage are perturbations in cell cycle progression and/or cell death. TKO MEFs did not display exacerbated sensitivity to any of the treatments (Fig. 3 A). Moreover, TKO MEFs showed increased tolerance to the genotoxic treatment. This observation was a result of their lower proliferation rate. Indeed, the response of T121-expressing TKO cells was comparable with that observed in the controls even at suboptimal doses of the drugs (Fig. 3 B). These observations indicate that TKO MEFs are not hypersensitive to DNA-damaging agents, further illustrating that cells can repair DNA efficiently in the absence of interphase Cdks. These observations are at variance with a previous study indicating that Cdk2 is required for proper repair of damaged DNA (Satyanarayana et al., 2008).

Increased Cdk activity does not stimulate the DDR

The aforementioned results suggest that a minimum level of Cdk activity is sufficient to promote DNA repair and checkpoint activation. Yet these observations do not establish whether the DDR is sensitive to augmented Cdk activity. Thus, we generated MEFs with increased Cdk activity as a result of the absence of two Cdk inhibitors, p21^{Cip1} and p27^{Kip1} (Cheng et al., 1999). *p21*$^{-/-}$;*p27*$^{-/-}$ MEFs were exposed to IR, and the levels of Chk1 phosphorylation on Ser^{345} followed over time. We observed that *p21*$^{-/-}$;*p27*$^{-/-}$ primary MEFs displayed more robust Chk1-Ser^{345} phosphorylation than wild-type controls (Fig. S2 A). In addition, these MEFs responded more efficiently to increasing doses of IR (Fig. S2 B). This is likely because of the fact that a higher percentage of *p21*$^{-/-}$;*p27*$^{-/-}$ MEFs were at the S and G2 phases of the cell cycle as determined by their increased levels of Chk1 and AurB (Fig. S2 B). This was confirmed by FACS analysis (Fig. S2 C). Thus, the enhanced Chk1-Ser^{345} phosphorylation could reflect a higher proportion of cells in the responsive phases of the cycle instead of an intrinsic enhancement of the DDR. To confirm this assumption, we reasoned that the putative hypersensitive DDR observed in *p21*$^{-/-}$;*p27*$^{-/-}$ MEFs should be paralleled by more efficient activation of the G2/M checkpoint. However, both genotypes showed equal dose–response behavior of the G2/M checkpoint (Fig. S2 D). Thus, increased Cdk activity does not promote a faster resection of DSBs or a hypersensitive ATR/Chk1-dependent checkpoint activation. Instead, these results support the concept that DDR proficiency is not affected by increased Cdk activity.

Net Cdk activity controls HR-mediated repair

Cdk specificity has been claimed to be part of the regulatory circuit that restricts HR-mediated repair to the S and G2 phases (Branzei and Foiani, 2008). Because interphase Cdks seemed dispensable for efficient DNA repair, we investigated whether

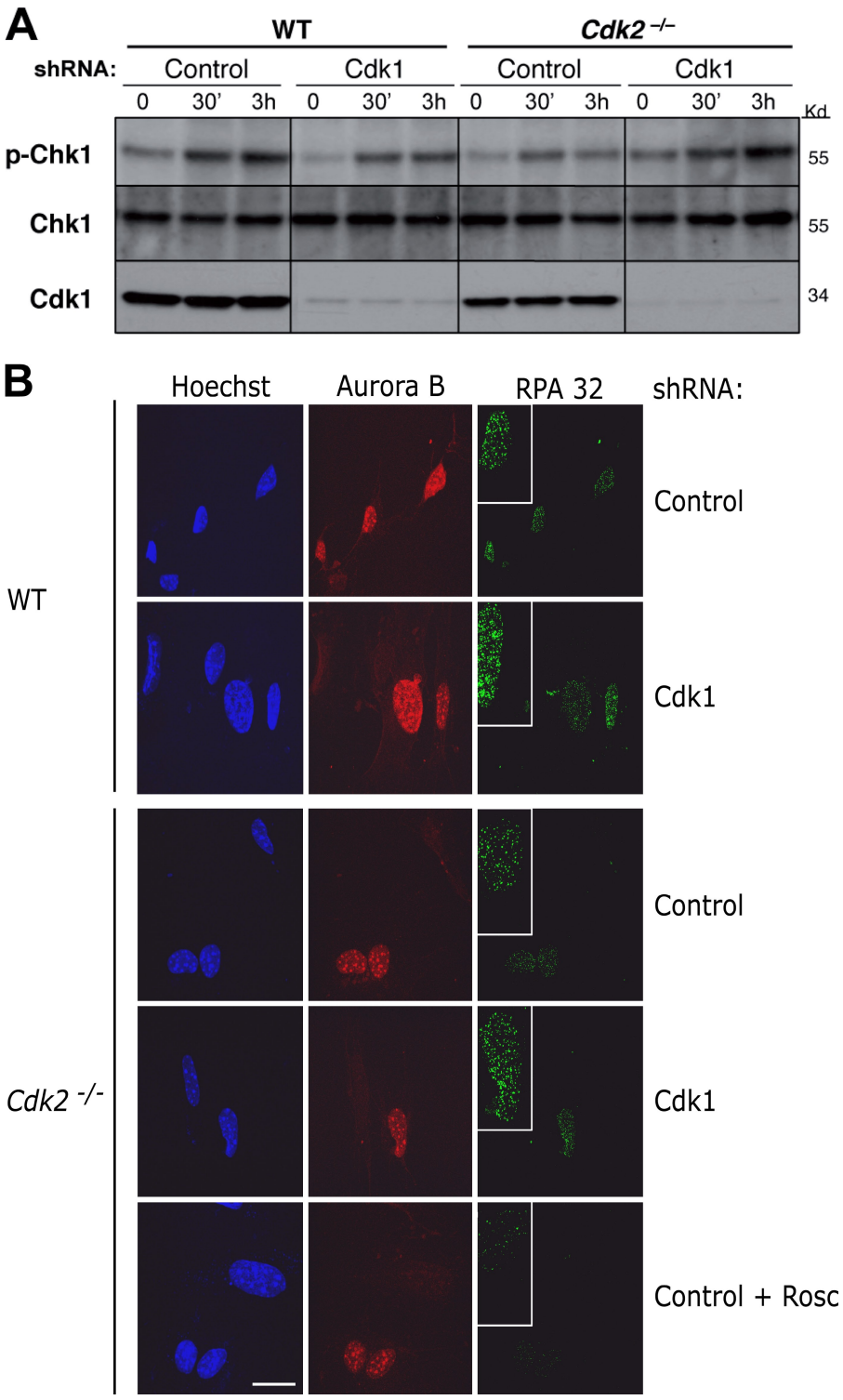

Figure 4. **Cdk1 and Cdk2 are dispensable for the onset of the DDR.** (A) Wild-type (WT) and *Cdk2*$^{-/-}$ MEFs were infected with lentiviral vectors expressing a scramble control or an shRNA against *Cdk1*. Extracts were prepared at the indicated time points after IR and blotted with antibodies against Chk1-Ser^{345} or Chk1 as indicated in Materials and methods. Cdk1 is shown as depletion control. Black lines indicate that intervening lanes have been spliced out. (B) MEFs were infected with the indicated shRNAs, subjected to IR, maintained in culture for 3 h, and preextracted before incubation with the indicated antibodies and confocal analysis. Insets on RPA32 fields show magnified views of a positive cell. (bottom) *Cdk2*$^{-/-}$ MEFs were preincubated for 3 h before IR with 50 μM of the Cdk inhibitor roscovitine (Rosc). Bar, 15 μM.

Cdk1 could be the master regulator of this process. To this end, we infected primary MEFs with lentiviral vectors encoding a short hairpin RNA (shRNA) against Cdk1 or a scramble control. Unexpectedly, depletion of Cdk1 to levels undetectable by Western blotting had a negligible impact on the onset of the DDR upon IR as measured by Chk1 phosphorylation (Fig. 4 A). Cdk2 is also active during the G2 phase and displays partially redundant activities with Cdk1 (Aleem et al., 2005; Hochegger et al., 2007). Thus, to determine whether these observations were the result of a putative compensatory activity exerted by Cdk2, we performed a similar experiment using primary *Cdk2*$^{-/-}$ MEFs. Phosphorylation of Chk1 on Ser^{345} followed normal kinetics in MEFs knocked down for Cdk1 in spite of lacking Cdk2 expression (Fig. 4 A). Moreover, all AurB-positive cells showed conspicuous nuclear staining of chromatin-bound RPA foci independently of their Cdk1 and Cdk2 status (Fig. 4 B).

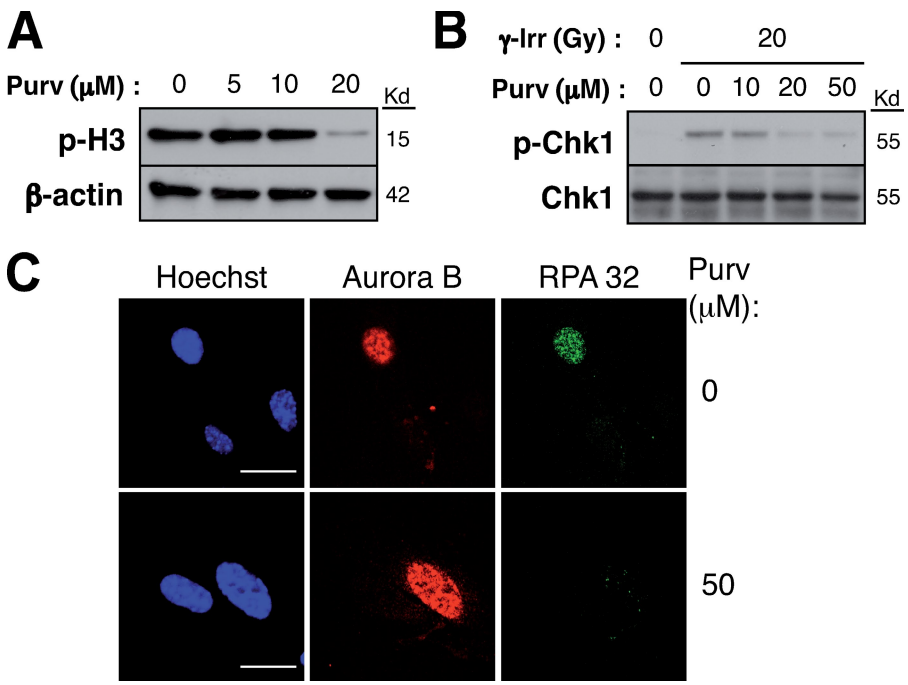

Figure 5. **The Cdk inhibitor purvalanol abrogates DSB end processing.** (A) TKO MEFs were treated for 2 h with the indicated concentrations of the Cdk inhibitor purvalanol (Purv). Extracts were blotted with a phosphospecific antibody against histone H3–Ser[10]. (B) Purvalanol-treated cells were subjected to IR, and extracts were prepared after 30 min. Extracts were blotted with antibodies against Chk1-Ser[345] or Chk1 as indicated in Materials and methods. (C) Purvalanol-treated cells were subjected to IR. After 30 min, the cells were preextracted followed by confocal analysis with the indicated antibodies. Bars, 20 μM.

Finally, we treated *Cdk2*$^{-/-}$ MEFs with chemical inhibitors. As illustrated in Fig. 4 B (bottom), preincubation of these MEFs with the general Cdk inhibitor roscovitine prevented the appearance of RPA foci after IR (Yu and Chen, 2004; Jazayeri et al., 2006). To further demonstrate that a minimum threshold of Cdk activity was required for implementing the DDR, we also infected TKO MEFs with the shRNA against Cdk1. Total depletion of Cdk activity eliminated the appearance of chromatin-bound RPA after IR (unpublished data). Unfortunately, knockdown of Cdk1 in TKO cells also eliminated AurB staining, precluding a proper estimation of HR in S and G2. Therefore, we treated TKO MEFs with purvalanol, a more selective Cdk1 inhibitor. Purvalanol treatment prevented entry into mitosis as measured by the elimination of Ser[10] phosphorylation on histone H3. This treatment did not interfere with the phosphorylation of γ-H2AX after IR (Fig. S3 A). Yet it eliminated the formation of RPA foci in all AurB-positive cells and the phosphorylation of Chk1-Ser[345] after IR (Fig. 5). In addition, the mobilization of ectopic Rad52-GFP into IR-induced foci was also abolished (Fig. S3 B). Similar results were obtained with U2OS cells (Fig. S3 C).

Altogether, these observations suggest that DSB end resection and DDR activation during S and G2 is controlled by total Cdk activity rather than by individual Cdks. Moreover, the lack of HR-mediated repair in G1 cannot be explained by a putative dependency on canonical S and G2 Cdks because Cdk4 and Cdk6 are able to efficiently induce this process (Fig. 4). Although in vivo experimental evidence is limited, in vitro data suggest that there is considerable overlap among Cdks in terms of substrate specificity (Hochegger et al., 2008). Our data imply that this is also extensive to factors controlling HR-mediated repair because it can be efficiently promoted by Cdk4/6. Understanding whether these kinases also contribute to this process in a wild-type background will require additional work.

In yeast, Cdk activity modulates the responses to DNA damage (Aylon et al., 2004; Ira et al., 2004). In mammalian cells, the increased number of Cdks has made it difficult to ascertain what particular Cdk is responsible for this activity. Availability of MEFs lacking all interphase Cdks has allowed us to interrogate the effect of Cdk activity on the activation of the DDR. In contrast to previous studies, our data demonstrate that activation of the DDR is controlled by the overall level of Cdk activity rather than by activation of specific cell cycle Cdks.

Materials and methods

Cell culture and cell cycle checkpoints

MEFs were isolated from embryonic day (E) 13.5 embryos of the indicated genotypes and cultured in DME supplemented with 2 mM glutamine, 1% penicillin/streptomycin, and 10% FBS. For the G1/S checkpoint, MEFs were subjected to IR, and their DNA content was analyzed by propidium iodide staining after 10 h. To analyze S phase entry, MEFs (10^6 cells/10-cm dish) were cultivated for 72 h in DME + 0.1% FBS and restimulated with 10% FBS at the time of IR. Cells were pulse labeled for 2 h with 50 μM BrdU (Sigma-Aldrich), harvested at the indicated times, and stained with anti-BrdU fluorescent antibodies (BD). For the G2/M checkpoint, MEFs were analyzed 45 min after addition of NCS. For DNA damage hypersensitivity assays, MEFs were grown for 5 d in the presence of aphidicolin (Sigma-Aldrich), MMS (Sigma-Aldrich), or NCS (Sigma-Aldrich), and their relative growth was compared with those of untreated cells. Retro- and lentiviral infections were performed as described previously (Santamaría et al., 2007). The Cdk inhibitors roscovitine and purvalanol were purchased from Sigma-Aldrich.

FACS analysis

Phosphorylated histone H3 staining was performed with specific antibodies (1:50 dilution; Millipore). Positive cells were quantified using a cytometer (FACSAria; BD).

Protein analysis

Protein extracts were prepared by incubating cell pellets in NP-40 buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% NP-40, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and Complete protease inhibitor cocktail [Roche]) on ice for 20 min. Lysates were cleared by centrifugation at 14,000 rpm for 15 min. Duplicate samples were electrophoresed, blotted,

and incubated in parallel with antibodies against the phosphorylated and total protein. Blots were subsequently incubated with antibodies against β-actin as a loading control. Antibodies included those against Cdk1 (Santa Cruz Biotechnology, Inc.), Chk1 (Novocastra), S345-Chk1 (Cell Signaling Technology), ATM (Novus Biologicals), S1981-ATM (Rockland Immunochemicals), SV40-T (EMD), AurB (BD), S4/8-RPA32 (Bethyl Laboratories, Inc.), and β-actin (Sigma-Aldrich). Peroxidase-conjugated IgG (Dako) were used as secondary antibodies followed by ECL detection (GE Healthcare).

Immunofluorescence

For confocal microscopy, MEFs were grown on poly-D-lysine coverslips (BD). For the analysis of AurB and chromatin-bound RPA, soluble proteins were preextracted (4 min on ice) by mild detergent permeabilization with CSKI buffer (10 mM Pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, and 0.5% Triton X-100) before fixation. Images were acquired at room temperature with an Acousto Optical Beam Splitter unit (TCS-SP5; Leica) using oil as immersion media and a 63× Plan Apo 1.4 NA objective (HCX; Leica). For high throughput microscopy, MEFs were grown on MicroClear-bottom 96-well dishes (Greiner Bio One) and analyzed with a bioimager (Pathway 855; BD). Image acquisition (ORCA 1394; Hamamatsu Photonics) was performed at room temperature using oil as an immersion media and a 40× 0.75 NA Plan Apo objective (HCX). For both confocal and high throughput microscopy, image analysis was performed with imaging software (Altovision; BD). All images for quantitative analyses were acquired under nonsaturating exposure conditions. Primary antibodies included those elicited against AurB (BD), RPA32 (Cell Signaling Technology), and γ-H2AX (Millipore). Secondary antibodies were conjugated with Alexa Fluor 598 or 488 (Invitrogen). Hoechst 33342 (Invitrogen) was used for DNA staining. The Rad52-GFP retroviral construct was provided by E. Brown (University of Pennsylvania, Philadelphia, PA).

Online supplemental material

Fig. S1 shows the absence of constitutive DDR in unperturbed TKO MEFs. Fig. S2 shows that the DDR and activation of the G2/M checkpoint are not enhanced by increased Cdk activity. Fig. S3 contains additional data regarding the effect of purvalanol on DDR activation and DSB end processing in TKO MEFs and U2OS cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200903033/DC1.

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