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Chk1 Is a Histone H3 Threonine 11 Kinase that Regulates DNA Damage-Induced Transcriptional Repression

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SUMMARY

DNA damage results in activation or suppression of transcription of a large number of genes. Transcriptional activation has been well characterized in the context of sequence-specific DNA-bound activators, whereas mechanisms of transcriptional suppression are largely unexplored. We show here that DNA damage rapidly reduces histone H3 Threonine 11 (T11) phosphorylation. This correlates with repression of genes, including *cyclin B1* and *cdk1*. H3-T11 phosphorylation occurs throughout the cell cycle and is Chk1 dependent in vivo. Following DNA damage, Chk1 undergoes rapid chromatin dissociation, concomitant with reduced H3-T11 phosphorylation. Furthermore, we find that loss of H3-T11 phosphorylation correlates with reduced binding of the histone acetyltransferase GCN5 at *cyclin B1* and *cdk1* promoters and reduced H3-K9 acetylation. We propose a mechanism for Chk1 as a histone kinase, responsible for DNA-damage-induced transcriptional repression by loss of histone acetylation.

INTRODUCTION

In eukaryotes, DNA damage alters the global patterns of gene expression to orchestrate a variety of cellular events including growth arrest, apoptosis, and DNA repair. Following ultraviolet (UV)-induced DNA damage ~4% of transcripts show >3 fold-changes. Approximately 90% represent downregulation (Gentile et al., 2003). Unlike transcriptional activation, transcriptional repression after DNA damage has been largely unexplored.

Targeted covalent modification of the amino-terminal tails of core histones is an important mechanism regulating RNA polymerase II-dependent transcription. The enzymes that catalyze histone lysine acetylation are known as histone acetyltransferases (HATs). The GCN5 family represent the first identified nuclear HATs (Brownell et al., 1996), and GCN5 is present in at least four high-molecular-weight complexes in vivo, the SAGA, ADA, SALSA, and SLIK complexes (Grant et al., 1997, 1998; Sterner et al., 1999). These interact with transcriptional activators during promoter targeting and with TATA box-binding protein (TBP) during the regulation of basal factor activity (Barlev et al., 1995). The emerging model is that these complexes are recruited to promoters through interactions with sequence-specific activator proteins. GCN5 mediates H3-K9 and H3-K14 acetylation within the basal promoter and subsequently general transcription factors are recruited and transcription is elevated. This general principle is complicated by the fact that multiple modifications occur simultaneously within a single histone tail and on the histone tails contained in a single nucleosome. Coupled with the observation that one histone modification can modulate other modifications, a complex series of events and interactions are proposed to mediate the exact level of transcriptional activity from a single promoter (Kouzarides, 2007).

One such link between these histone modifications is exemplified by MAP kinase-dependent mitogen-stimulated gene expression. This is mediated via H3-S10 phosphorylation and the subsequent recruitment of GCN5 to immediate early gene promoters, such as c-*fos* (Cheung et al., 2000). Consistent with this, the GCN5 HAT domain exhibits ~10-fold higher substrate specificity for an S10-phosphorylated H3 peptide when compared to the corresponding nonphosphorylated control (Cheung et al., 2000). The T11 residue of H3 is also phosphorylated (Preuss et al., 2003). Immunohistochemical analysis revealed H3-T11 phosphorylation to be predominant on mitotic chromosomes and to be enriched at centromeres. Conversely, a structure-function analysis using H3-phosphopeptides and yeast mutants indicated that the T11 residue is essential for GCN5 recruitment and is required for optimal transcription at GCN5-dependent promoters (Clements et al., 2003). Thus, it has been suggested that H3-T11 phosphorylation, in addition to H3-S10 phosphorylation, may play an important role in transcriptional regulation in response to unknown signaling pathways.

In response to DNA damage, DNA structures are rapidly sensed and transduced by either the ATR or ATM phosphatidylinositol 3-kinase-related protein kinases (PIKKs). These phosphorylate a wide variety of substrates including Chk1 and Chk2. While

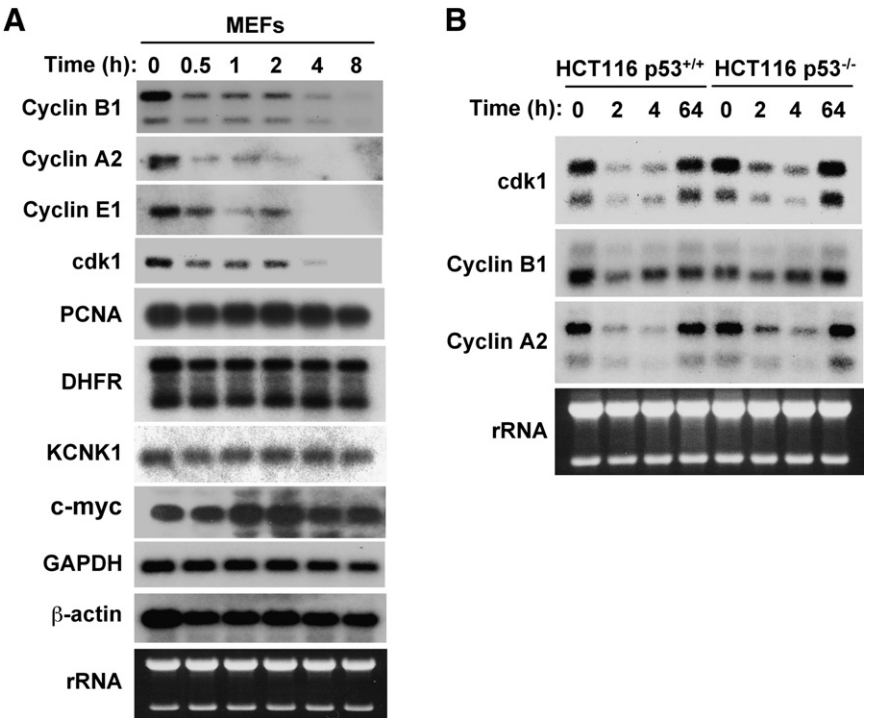

Figure 1. Transcriptional Repression of Cell-Cycle Regulatory Genes following UV Irradiation

(A) Chk1$^{flox/-}$ MEFs were exposed to UV irradiation and harvested at the times indicated. Steady-state transcript levels were detected by northern blotting. 28S and 18S rRNAs are shown stained with ethidium bromide as loading controls. (B) HCT116 p53$^{+/+}$ and HCT116 p53$^{-/-}$ cells were exposed to UV irradiation and transcript levels and rRNAs were detected as in (A).

Chk1 phosphorylation by PIKKs is required for its role in cell-cycle arrest and apoptosis, Chk1 is a constitutively active enzyme and the PIKK-dependent phosphorylation appears not to regulate its kinase activity but rather its subcellular localization. For example, following PIKK-dependent phosphorylation, Chk1 is targeted to centrosomes (Kramer et al., 2004; Niida et al., 2007), where cyclin B1-cdk1 is first activated at the onset of mitosis (Jackman et al., 2003). In undamaged cells, a significant proportion of Chk1 is chromatin associated. Following DNA damage, PIKK-dependent Chk1 phosphorylation results in rapid Chk1 dissociation from chromatin (Smits et al., 2006). Therefore, the possibility exists that chromatin-associated and kinase-active Chk1 mediates unknown functions via phopshorylation of unknown targets.

In this study, we show that DNA damage induces the rapid transcriptional repression of a variety of genes related to cell-cycle progression. This is concomitant with dephosphorylation of H3-T11 and a modest deacetylation of H3-K9. We identify Chk1 as the kinase responsible for H3-T11 phosphorylation and show that Chk1 dissociation from chromatin upon DNA damage closely correlates with decreased H3-T11 phosphorylation. We also demonstrate that, in vitro, GCN5 binds to the H3-pT11 peptide far more efficiently than to unphosphorylated peptide. Taken together, our results suggest a novel mechanism underlying the repression of transcription following DNA damage.

RESULTS

Transcriptional Repression of Cell-Cycle Regulatory Genes in Response to DNA Damage

To investigate the molecular mechanisms underlying the repression of transcription following DNA damage, we used mouse embryonic fibroblasts (MEFs) to examine the steady levels of a variety of cell-cycle regulatory genes after treatment with UV (Figure 1A). Northern blot analysis revealed a decrease in transcript levels for *cyclin B1*, *cyclin A2*, *cyclin E1*, and *cdk1* as early as 0.5 hr after treatment. Levels remained low for the duration of the experiment, being essentially undetectable at the 8 hr time point. These genes are known targets of E2F, raising the possibility that transcriptional repression acts via global effects on E2F activity. To eliminate this possibility, we examined transcript levels of the additional E2F targets, *PCNA*, *DHFR*, and *KCNK1*. *GAPDH* and *β-actin* were used as loading controls. Transcript levels from all five genes remained constant during the course of the experiments. Thus, the repression of transcription of several cell-cycle regulatory genes following DNA damage is independent of both E2F and global effects on transcription. DNA damage-dependent transcriptional repression did not correlate with transcript half life (HL) since c-*myc* transcript (HL 1.2 hr) was constant during the experimental period (*cyclin B1*: HL 2.0 hr, *cdk1*: HL 5.1 hr, *PCNA*: HL 11.7 hr, *KCNK1*: HL 11.7 hr, *GAPDH*: HL 29.5 hr).

p53 is known to function as a negative regulator of *cyclin B1* and *cdk1* transcription (Flatt et al., 2000). We exposed HCT116 p53$^{+/+}$ and an isogenetic derivative, HCT116 p53$^{-/-}$ cells, to UV and examined the levels of *cdk1*, *cyclin B1*, and *cyclin A2* expression (Figure 1B). Expression levels were repressed in a similar manner as previously seen in MEFs and recovered efficiently 64 hr postirradiation. There was no significant difference dependent on the p53 status. Thus, these results indicate that transcriptional repression of a subset of cell-cycle genes is p53 independent.

To establish if changes to the expression of cell-cycle regulatory genes was observed following treatment with other DNA damage agents, we treated MEFs or HCT116 p53$^{+/+}$ cells with either X-ray or bleomycin and examined *cyclin B1* and *cdk1* expression (Figure S1). Both X-ray and bleomycin treatment suppressed *cyclin B1* and *cdk1* expression in a dose- and time-dependent fashion.

Reduction in H3-T11 Phosphorylation after DNA Damage

We examined the known histone H3 modifications (H3-S10 and H3-T11 phosphorylation, H3-AcK9, -AcK14, -AcK18, -AcK23, and H3-MeK79), H2A modification (H2A-AcK5), and H4

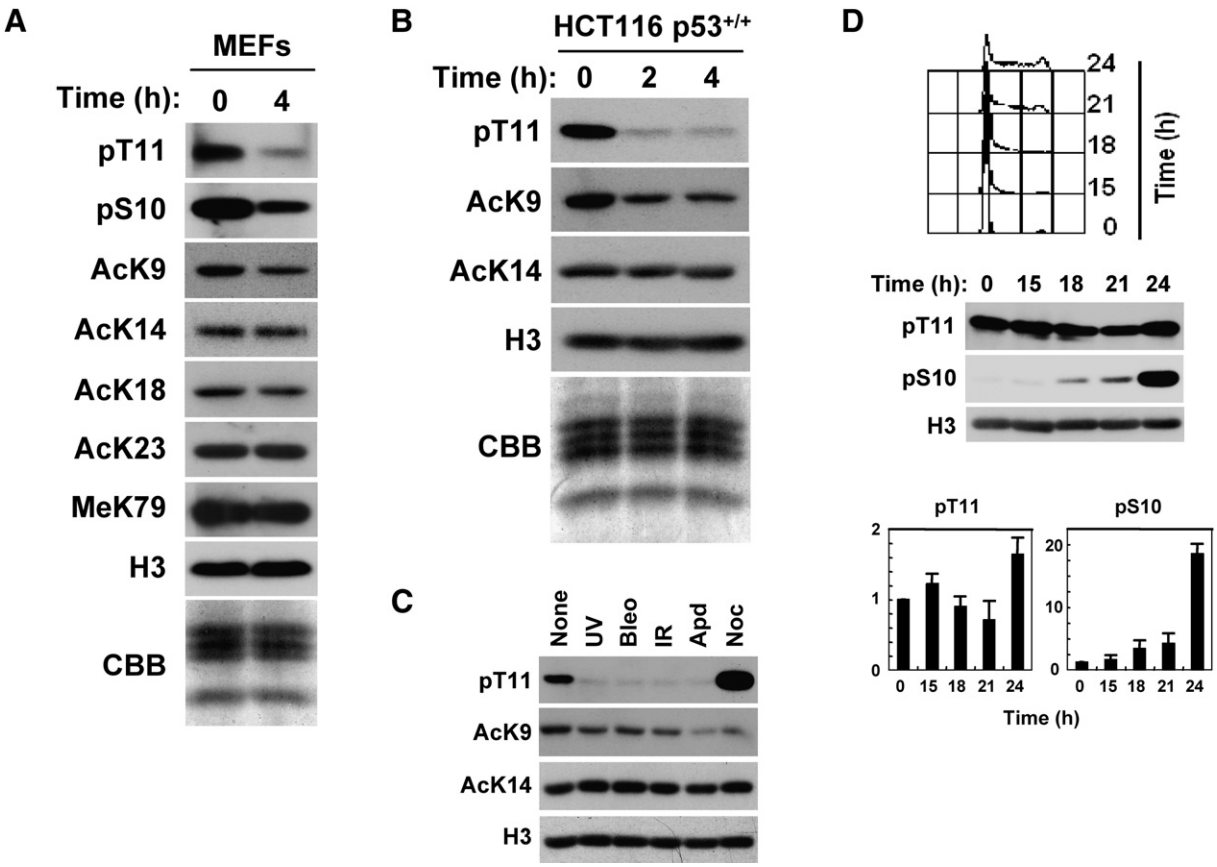

Figure 2. Changes to Core Histone Modifications following DNA Damage

(A) Chk1$^{flox/-}$ MEFs were exposed to UV irradiation. After 4 hr cells were harvested and chromatin fractions prepared and analyzed by immunoblotting for the presence of the indicated histone modifications using modification-specific antibodies. CBB staining of chromatin fractions is shown as a loading control.

(B) HCT116 p53$^{+/+}$ cells were exposed to UV irradiation and analyzed for histone modifications as described for (A).

(C) Chk1$^{flox/-}$ MEF cells were treated with UV, bleomycin (Bleo), X-ray irradiation (IR), aphidicolin (Apd), or nocodazole (Noc). Following exposure (UV: 4 hr, Bleo: 16 hr, IR: 2 hr, Apd: 16 hr, or Noc: 16 hr), changes in histone modifications of the indicated residues were analyzed as in (A). Phosphorylation of H3-T11 and acetylation of H3-K9 were reduced in response to all DNA-damaging agents.

(D) Chk1$^{flox/-}$ MEF cells rendered quiescent by serum starvation (4 days) and growth were restored by the addition of 15% FBS. Cells were harvested at the indicated times and subjected to FACS (top), and chromatin fractions prepared and analyzed by immunoblotting using the indicated antibodies (middle). Changes in H3-T11 or -S10 phosphorylation obtained from three independent experiments are shown as mean ± SD (Bottom). Data were normalized for amount of H3.

modification (H4-AcK8) for global changes. Antibodies specific to the histone modification were used to probe chromatin fractions derived from MEFs either before or 4 hr after UV irradiation (Figure 2A). A significant decrease in both H3-T11 and H3-S10 phosphorylation and a modest decrease in H3-K9 acetylation were observed following DNA damage. In addition, H2A-K5 and H4-K8 acetylations were moderately decreased following DNA damage (data not shown). To establish that this was not a cell line-specific effect, we performed a similar analysis on HCT116 p53$^{+/+}$ cells. We observed similar reductions in H3-T11 phosphorylation plus H3-K9 acetylation (Figure 2B).

To establish if changes to the modifications of histones were observed following treatment with other DNA damage agents, we treated MEFs with either UV, bleomycin, X-rays, or aphidicolin (Figure 2C) and examined levels of H3-T11 phosphorylation and H3-K9 and -K14 acetylation. After verifying the specificity of the anti-phospho-H3-T11 antibody (Figure S2), we observed that each DNA-damaging treatment reduced H3-T11 phosphorylation and H3-K9 acetylation but did not affect H3-K14 acetylation. In contrast, treating cells with nocodazole for 16 hr strongly enhanced H3-T11 phosphorylation but reduced H3-K9 acetylation. This most likely results from an increase in the population of mitotic cells.

Structural analysis revealed that when GCN5 binds to the N-terminal tail of H3, the side chain of H3-T11 is deeply buried in the peptide-binding cleft (Clements et al., 2003). Thus, the increased negative charge associated with H3-T11 phosphorylation may enhance H3-GCN5 interactions. To address this question, we examined the recently solved structure of *Tetrahymena* GCN5 cocrystallized with H3 peptide. By computer modeling, we found that H3-T11 phosphorylation could make substantial van der Waals interactions with the side chains of GCN5-R113, E122, I189, K190, and Y192 without H3-S10 phosphorylation (Figure S3). Thus H3-T11 phosphorylation may have a similar effect on GCN5 interaction as that shown for H3-S10 phosphorylation.

Therefore, we focused our attention on the physiological relevance of H3-T11 phosphorylation during DNA-damage-induced transcriptional repression. It has been reported that H3-T11 phosphorylation is mitosis specific and may facilitate centromere function (Preuss et al., 2003). Mitotic specificity would be inconsistent with our model and therefore we re-examined H3-S10 and H3-T11 phosphorylation during interphase. We synchronized MEFs at quiescence and restarted the cell cycle. H3-S10 phosphorylation was absent in arrested cells and only accumulated later in the time course when FACS profiles suggested that cells were entering mitosis (Figure 2D). In contrast, H3-T11 phosphorylation was readily detectable in extracts from quiescent cells and the level did not vary greatly during the experiment. A maximum (<2-fold increase) was noted at 24 hr. Thus, unlike H3-S10 phosphorylation, H3-T11 phosphorylation is not specific to mitosis. Therefore, the decrease we observed in H3-T11 phosphorylation following DNA damage cannot solely be due to a reduction in the population of mitotic cells.

To further confirm the H3-T11 phosphorylation during interphase, we synchronized HCT116 and Tera-1 cells in quiescence by serum starvation and tsFT210 cells (temperature-sensitive cdk1 mutant) in G2/M phase by culturing at 39°C (Figure S4). Cells were subsequently released into the cell cycle and subjected to FACS analysis and immunoblotting with anti-pT11 antibodies. While H3-T11 phosphorylation was enhanced at mitosis, it was readily detectable during interphase. We conclude that H3-T11 phosphorylation during interphase is not cell type specific.

Chk1 Mediates Phosphorylation of H3-T11 in Interphase

The amino acid sequence surrounding the T11 residue was examined for kinase consensus sequences. A minimum consensus for Chk1-dependent phosphorylation was identified (Figure 3A). Chk1 preferentially phosphorylates its substrates at serine or threonine residues with Lys or Arg located at the −3 position (O'Neill et al., 2002). To establish if Chk1 could phosphorylate H3-T11 in vitro, we performed an in vitro kinase assay using purified H3 or a T11A mutant as substrate. Chk1, but not a kinase-deficient Chk1 (Chk1-K38M), incorporated radiolabeled phosphorous into H3. Substitution of T11 with alanine significantly reduced incorporation, indicating T11 as the main target residue of Chk1 (Figure 3B). Using antibodies specific to H3 phospho-T11, we established that Chk1-phosphorylated H3 contained molecules in which T11 was phosphorylated. H3-pT11 antibodies failed to recognize Chk1-phosphorylated H3-T11A, further confirming its specificity (Figure 3B). In addition to phosphorylating purified H3, we also demonstrated that Chk1 could phosphorylate H3 present either in nucleosomes or mixed with core histones (Figure 3C). This is strong evidence that Chk1 is a physiological histone kinase. We also used a yeast two-hybrid assay (data not shown) to establish that Chk1 could interact with H3. Taken together, our results are consistent with Chk1 being able to phosphorylate H3-T11 in vivo.

To establish if Chk1 is responsible for the in vivo H3-T11 phosphorylation that we have identified in MEFs, $Chk1^{flox/-}$ MEF cells were infected with Cre-adenovirus or LacZ expressing control adenovirus and the status of H3 modifications was examined (Figure 3D). Loss of H3-T11 phosphorylation was modest 1 day after adenovirus infection and pronounced at 2 days. This is consistent with chromatin-bound Chk1 having a half life of 1.2 days (data not shown). A modest reduction in H3-S10 phosphorylation and H3-K9 acetylation was also observed. H3-K14 acetylation was not affected.

Chk1 loss leads to replication fork collapse and subsequent DNA damage during S phase (Syljuasen et al., 2005) and concomitant PIKK activation and Chk2 signaling. Therefore, it remained possible that the decrease in H3-T11 phosphorylation is indirect and caused by responses to the DNA damage induced by Chk1 depletion. To exclude this possibility, we arrested cells by serum starvation before infecting with Ade-Cre virus to eliminate Chk1 in quiescent cells (Figure 3E). Expression of Cre, but not LacZ, in quiescent cells resulted in decreased H3-T11 phosphorylation. The fact that Chk1 depletion did not cause increased single- or double-strand breaks in quiescent cells (Figure S5) strongly indicates that Chk1 is necessary for normal H3-T11 phosphorylation and that loss of Chk1 rapidly results in decreased levels of phosphorylated H3-T11. We also observed that cells synchronized in early S phase by aphidicolin treatment show reduced H3-T11 phosphorylation independent of Chk1 status (Figure 3E). One possible explanation for this is that the aphidicolin-treated cells activate Chk1, mimicking the conditions of DNA damage. Indeed, when Chk1-depleted cells were treated with UV no further reduction in H3-T11 phosphorylation signal was observed (Figure 3F). Our results indicate that Chk1 is required for the in vivo phosphorylation of H3-T11. Interestingly, Chk1 depletion did not result in a pronounced decrease in H3-T11 phosphorylation when MEFs were synchronized by nocodazole treatment (Figure 3E). This is consistent with the existence of a mitotic-specific H3-T11 kinase. Because Chk1 and Chk2 have a similar consensus sequence for phospho-targets, we also examined H3-T11 phosphorylation in $Chk2^{-/-}$ MEFs (Figure 3G). Chk2 was dispensable for in vivo H3-T11 phosphorylation.

Chk1 Chromatin Dissociation after DNA Damage Correlates with H3-T11 Dephosphorylation and Transcript Levels of *cdk1* and *cyclin B1*

Chk1 release from chromatin is triggered by PIKK-dependent phosphorylation and is essential for G2 checkpoint arrest. We observed that Chk1 was largely dissociated from chromatin between 0.5 and 1 hr after DNA damage (Figure 4A). The kinetics of the reduction in H3-T11 phosphorylation were virtually identical. Similarly, the reduction in H3-K9 acetylation followed similar kinetics. H3-K14 acetylation remained unchanged as expected. The decreased phosphorylation of H3-T11 is likely to be due to dephosphorylation because we did not observe release of phosphorylated H3 into the soluble fraction. We next examined UV dose-dependent changes in Chk1 dissociation, γ-H2AX induction, H3-T11 phosphorylation, and *cyclin B1* and *cdk1* expression (Figure S6). A correlation between γ-H2AX induction and Chk1 chromatin dissociation was observed and the kinetics of Chk1 dissociation and the loss of H3-T11 phosphorylation were virtually identical. Importantly, suppression of *cyclin B1* and *cdk1* expression showed similar kinetics.

If Chk1 chromatin dissociation is a causative event for reduced H3-T11 phosphorylation, we would predict that the response should be dependent on the PIKKs. Treatment with caffeine, an

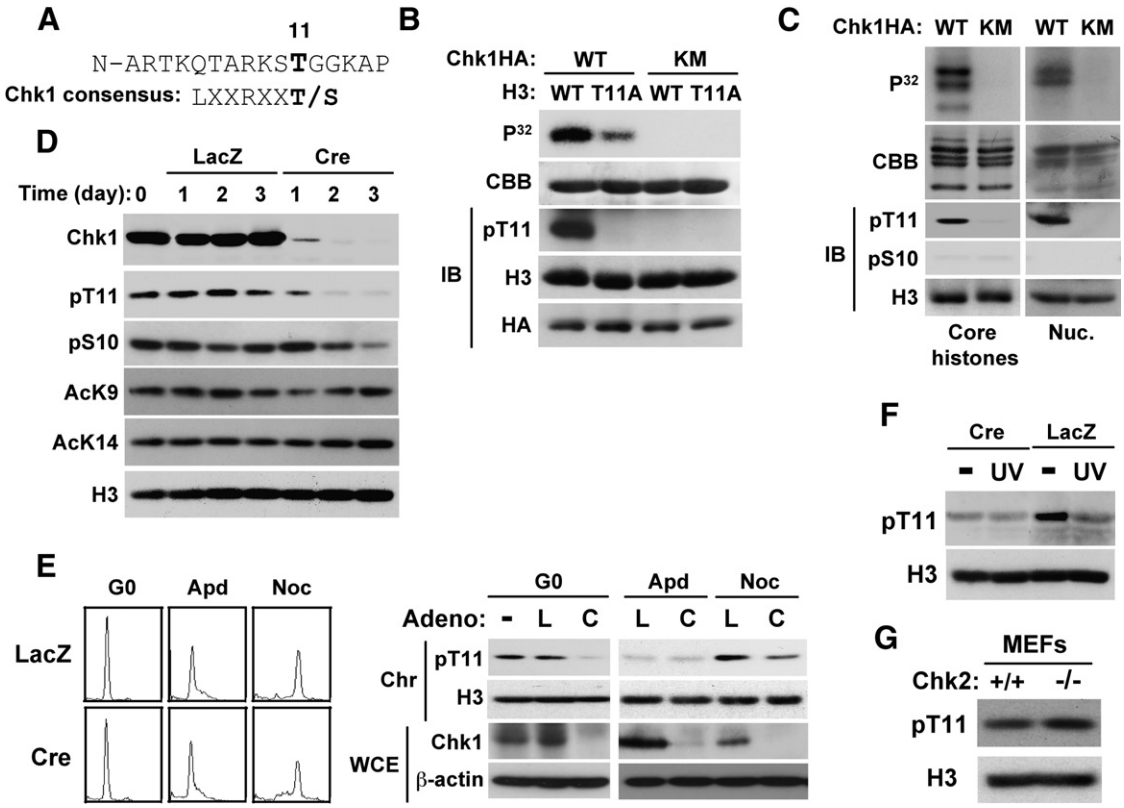

Figure 3. Chk1 Phosphorylates Histone H3-T11 Both In Vitro and In Vivo

(A) Alignment of the H3 N-terminal tail and the Chk1 phosphorylation consensus motif. The residues of H3 presented in bold fit the minimum consensus motif.

(B) Immunopurified Chk1-HA (WT) and Chk1-K38M-HA kinase-deficient mutant (KM) were used for in vitro kinase assays on purified wild-type H3 (WT) or H3-T11A (T11 substituted with A) as substrate. Top two panels: products were separated by SDS-PAGE (15%) and visualized by autoradiography (P^{32}) and Coomassie-brilliant blue (CBB). Bottom three panels: a reaction without P^{32}ATP was subjected to immunoblotting using the indicated antibodies to phospho-T11 (pT11) or H3 (H3). Immunoprecipitated Chk1-HA (WT) and Chk1-KM-HA (KM) were detected using anti-HA antibodies.

(C) Chk1 kinase assay was performed as in (B) using core histones (core histones) or nucleosomes (nuc) as a substrate. Top two panels: results of autoradiography (P^{32}) and CBB staining (CBB). Bottom three panels: the reaction without P^{32}ATP subjected to immunoblotting using the indicated antibodies.

(D) Chk1$^{flox/-}$ MEF cells were infected with either Ade-LacZ (LacZ) or Ade-Cre (Cre) and harvested at the indicated times after infection. Chromatin fractions were prepared and analyzed by immunoblotting using the indicated antibodies.

(E) (G0) Chk1$^{flox/-}$ MEFs were rendered quiescent by serum starvation and infected with Ade-LacZ (L) or Ade-Cre (C). Cells were cultured for an additional 2 days under serum-starved condition. Chk1$^{flox/-}$ MEFs were infected with Ade-LacZ or Ade-Cre and cultured in medium containing aphidicolin (Apd) for 2.5 days. Chk1$^{flox/-}$ MEFs were infected with Ade-LacZ or Ade-Cre and cultured in medium containing nocodazole (Noc) for 2.5 days. Cell-cycle distributions were determined by FACS analysis (left), H3-T11 phosphorylation status and H3 were analyzed by immunoblotting using chromatin fractions (Chr), and Chk1 and β-actin were done using whole-cell extracts (WCE) (right).

(F) Chk1$^{flox/-}$ MEFs were infected with Ade-LacZ (LacZ) or Ade-Cre (Cre), irradiated with UV 2 days after infection and harvested 4 hr after irradiation. Chromatin fractions were prepared and H3-T11 phosphorylation status analyzed by immunoblotting.

(G) H3-T11 phosphorylation was analyzed as in (D) using wild-type or Chk2$^{-/-}$ MEFs.

inhibitor of the ATM and ATR kinases, inhibited Chk1 release from chromatin and maintained H3-T11 phosphorylation in response to DNA damage (Figure 4B). We also observe that caffeine prevents the DNA damage-dependent loss of *cdk1* and *cyclin B1* transcripts. Thus, the reduction of H3-T11 phosphorylation is PIKK dependent and correlates with Chk1 chromatin dissociation and with the transcriptional status of *cdk1* and *cyclin B1*.

UV-induced Chk1 phosphorylation is dependent on ATR but not ATM (Brown and Baltimore, 2003), and UV-induced Chk1 dissociation requires ATR (Smits et al., 2006). ATR-specific but not control siRNA significantly reduced ATR protein level, UV-induced Chk1 dissociation, and H3-pT11 dephosphorylation (Figure 4C). UV-induced Chk1 chromatin dissociation is known to require ATR-dependent phosphorylation at Chk1-S317 and -S345. A Chk1-S317A/S345A mutant (Chk1-SA) is retained on chromatin in the presence of DNA damage (Niida et al., 2007; Smits et al., 2006). HCT116 cells expressing Chk1-SA-myc, but not cells expressing Chk1-myc, retained significant Chk1-SA-myc on chromatin following UV treatment (Figure 4D). Furthermore, H3-T11 phosphorylation and *cyclin B1* plus *cdk1* expression were partially restored only in cells expressing Chk1-SA-myc. These results confirm that H3-T11 dephosphorylation and suppression of *cyclin B1* plus *cdk1* expression are dependent on the Chk1 chromatin dissociation.

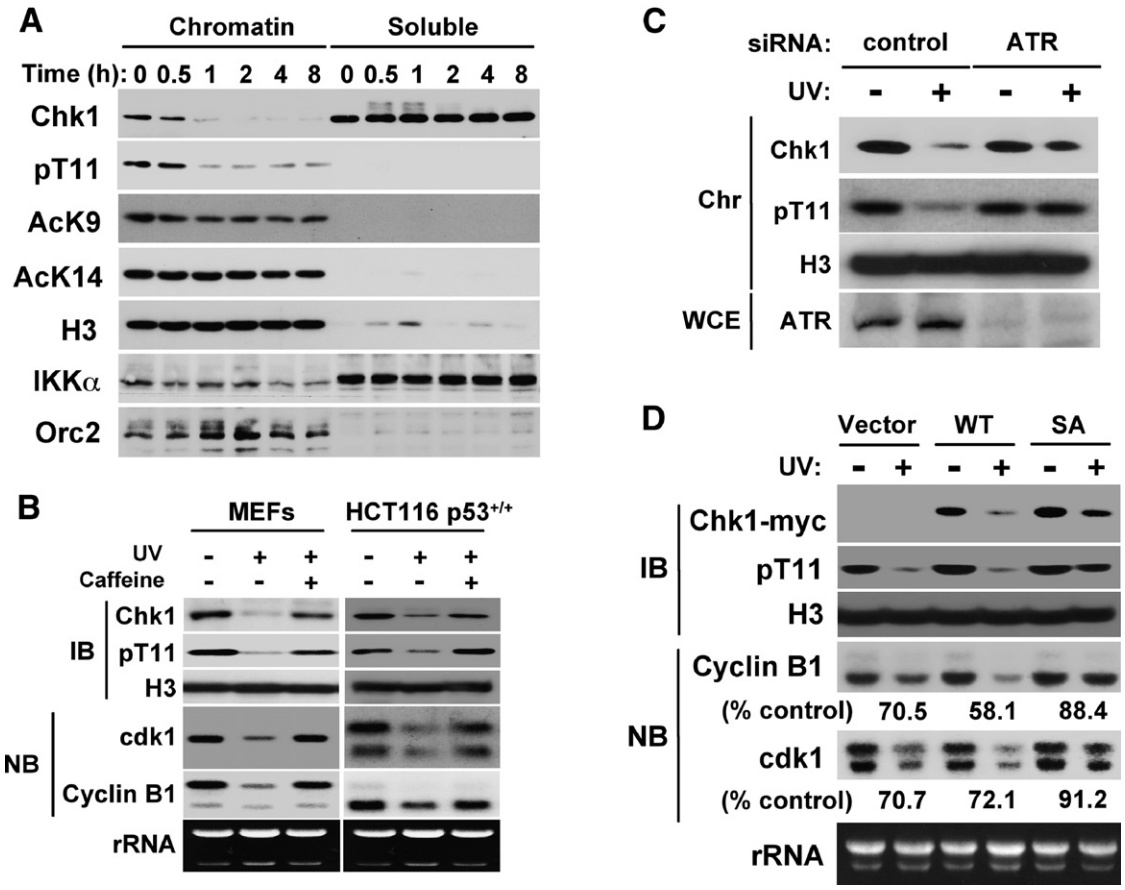

Figure 4. Release of Chk1 from Chromatin following DNA Damage Correlates with Decreased H3-T11 Phosphorylation

(A) Chk1$^{flox/-}$ MEF cells were exposed to UV irradiation (500 J/m^2) and harvested at the indicated times, and chromatin fractions and soluble material were prepared for analysis by immunoblotting with the indicated antibodies.

(B) Chk1$^{flox/-}$ MEFs or HCT116 p53$^{+/+}$ cells were preincubated in the presence (+) or absence (−) of 20 mM caffeine for 10 min and either exposed or not to UV irradiation (500 J/m^2). Cells were harvested after 2 hr, chromatin fractions were prepared and immunoblotted as described in (A), or RNA levels examined by northern blot analysis with the indicated probes. rRNA is shown as a control.

(C) HCT116 p53$^{+/+}$ cells were transfected with control or ATR siRNA. After 72 hr, cells were treated with (+) or without (−) UV (180 J/m^2). After 1 hr incubation, cells were harvested and immunoblotting analysis was performed on chromatin fractions for Chk1, H3-pT11, and H3 and on whole-cell extract for ATR.

(D) HCT116 p53$^{+/+}$ cells were transfected with empty vector or expression vector encoding Chk1 wild-type (WT) or Chk1-S317A/S345A (SA) protein. After 48 hr, the cells were treated with (+) or without (−) UV (250 J/m^2). Chromatin fractions were prepared for immunoblot analysis after 1 hr incubation or RNA was extracted after 4 hr incubation. Northern blot analysis was performed with the indicated probes as described above. Intensity is presented as a % of −UVcontrol.

Transcriptional Reduction of Cell-Cycle Regulators after Chk1 Depletion in Somatic Cells

To test whether Chk1 depletion should result in transcriptional repression of the same genes repressed in response to DNA damage, we assayed the consequences of Chk1 depletion in MEFs. Three days after Chk1 depletion by Ade-Cre virus transfection, Chk1$^{-/-}$ MEFs did not undergo premature mitosis but arrested in S phase (Figure 5A). Loss of the inhibitory Y15 phosphorylation of cdk1 occurred after 2 days in our Chk1$^{-/-}$ MEFs, in much the same way as has been reported for ES cells. However, cyclin B1 protein was significantly reduced (Figure 5B) and at 3 days both cdk1 and cyclin B1 protein levels were very low. This presumably explains why Chk1-depleted MEFs do not prematurely enter mitosis. Important for our work, the reduction in cyclin B1 and cdk1 proteins correlates with decreased levels of the corresponding mRNA (Figure 5C). The steady-state levels of *PCNA*, *DHFR*, and *KCNK1* mRNA were not significantly affected by Chk1 depletion. Thus, after DNA damage or Chk1 depletion in MEFs, *cdk1* and *cyclin B1* (but not other E2F-target genes such as *PCNA* and *DHFR*) are transcriptionally repressed. This indicates that Chk1 is required for the correct expression of *cyclin B1* and *cdk1*.

Although Chk1-SA-myc does not restore cell-cycle checkpoint functions (Niida et al., 2007), it did partly suppress the cell-cycle arrest defect (Figure 5D), prevent H3-T11 dephosphorylation, and partially stabilize *cyclin B1* plus *cdk1* expression (Figure 5E). Interestingly, unlike expression of wild-type Chk1-myc, Chk1-SA-myc failed to prevent DNA damage-dependent γ-H2AX accumulation as a consequence of the endogenous Chk1 depletion, presumably because it is compromised for checkpoint functions. Thus, Chk1-SA-myc, which is not released

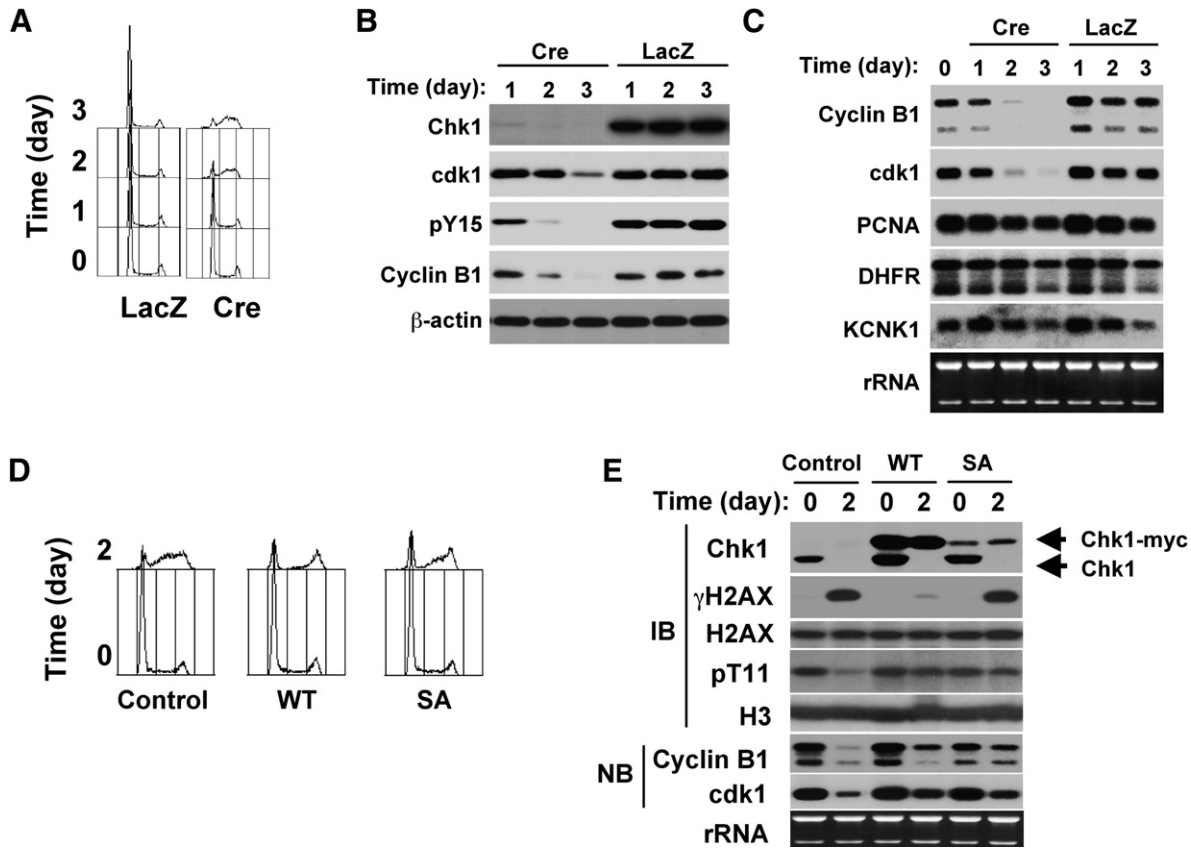

Figure 5. Chk1 Depletion in MEFs Results in S Phase Arrest But Not Mitotic Catastrophe

Chk1$^{flox/-}$ MEFs were infected with Ade-LacZ or Ade-Cre and harvested at the indicated days after infection. (A) Cell-cycle distribution was determined by FACS analysis. (B) Soluble extracts were prepared and subjected to immunoblotting with the antibodies indicated. (C) Northern blot analysis used the probes indicated. (D) Chk1$^{flox/-}$ MEFs expressing Chk1 wild-type (WT) or Chk1-S317A/S345A (SA) were infected with Ade-Cre. After 2 days of infection, the cells were prepared for FACS analysis. (E) Chromatin was analyzed by immunoblot and RNA was analyzed by northern blotting.

from chromatin after DNA damage, results in maintenance of H3-T11 phosphorylation and expression of *cyclin B1* plus *cdk1* in the presence of DNA damage.

To establish the extent of Chk1-dependent transcriptional maintenance, we conducted microarray experiments to investigate the global requirement for Chk1 in gene expression. Cells rendered quiescent by serum starvation were infected with Ade-Cre or the control Ade-LacZ viruses and held for 2 days, and mRNA were prepared and analyzed by hybridization to microarrays (Filgen Array mouse 32K, oligo DNA microarray). We observed that 214 transcripts were downregulated following Chk1 depletion (Table S1). Thus, Chk1 is required for the correct expression of many genes in vivo. We chose three genes, *pctk2*, *trib2*, and *HMGA2* and confirmed that their expression was repressed in response to DNA damage, further linking the function of Chk1 to DNA-damage-induced transcriptional repression.

GCN5 HAT Preferentially Binds to Phosphorylated H3 Peptide In Vitro

Computer modeling suggested that H3-T11 phosphorylation could enhance binding of the GCN5 HAT to nucleosomes (Figure S3). We therefore examined whether H3-T11 phosphorylation influences the ability of GCN5 to bind to an H3 tail peptide. Four synthetic H3 peptides, S10/T11, pS10/T11, S10/pT11, and pS10/pT11, differing only by the presence or absence of a phosphate group at the S10 and T11 residues were used as substrates in SPR Biosensor Binding Analysis with yeast GCN5 (aa 100–255) purified from insect cells (Figure 6).

In the presence of CoA (0.15 mM), GCN5 bound to H3-pT11, H3-pS10, and H3-pS10/pT11 with similar dissociation constants (Figure 6C). Binding to unphosphorylated H3 peptide showed ~20-fold lower affinity. Intriguingly, in the absence of CoA, binding affinities between GCN5 and two peptides were significantly reduced. CoA-dependent binding of GCN5 to the H3 peptide was predicted by structural analysis of GCN5/CoA/histone ternary complex, in which CoA is required to reorient the enzyme for histone binding (Rojas et al., 1999). Our results show that either H3-T11 or H3-S10 phosphorylation dramatically enhances the preference of GCN5 for H3.

H3-T11 Phosphorylation Is Involved in Recruitment of GCN5 at the Sites of *cdk1* and the *cyclin B1* Promoter

As we see a modest decrease in H3-K9 acetylation, an attractive model is that H3-T11 phosphorylation by Chk1 results in GCN5

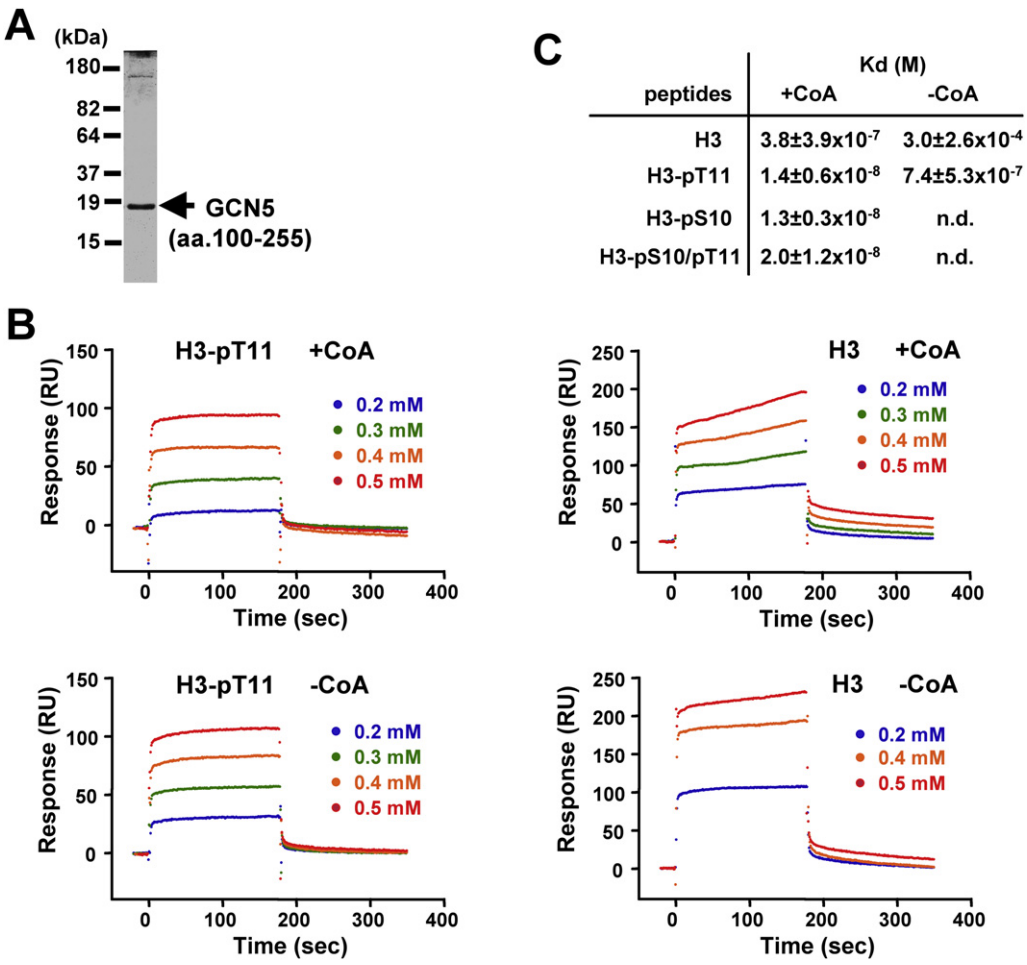

Figure 6. GCN5 Has Increased Affinity for H3-pT11 Peptide

(A) CBB staining of purified yeast GCN5 HAT domain (aa 100–255).

(B) BIAcore analysis of the interaction between GCN5 and H3 peptides. The purified GCN5 from (A) was immobilized on the surface of biosensor Chips.

(C) Binding affinities (Kd [M]) between GCN5 HAT and H3 peptides. Data were collected in the presence or absence of CoA. Values from three independent experiments are shown as means ± SD.

recruitment and transcriptional activation via lysine acetylation. To explore the possibility that GCN5 is mechanistically involved in Chk1-dependent transcriptional repression following DNA damage, we used chromatin immunoprecipitation (ChIP) to test for the presence of H3-T11 phosphorylation, H3-K9 and -K14 acetylation, and Chk1 and GCN5 proteins at the promoters of *cdk1* and *cyclin B1* (transcriptionally repressed following DNA damage) and at the promoter region of *GAPDH* (transcription unaffected by DNA damage).

We established that Chk1 was present at all three promoter regions in unperturbed cells. Following DNA damage (Figure 7A) or Chk1 depletion (Figure 7B) by Ade-Cre infection, Chk1 was barely detectable at any of the three promoter regions. As predicted, the level of H3-T11 phosphorylation was concomitantly reduced at all promoters following both treatments. Neither Chk1 nor H3-T11 phosphorylation was observed at subtelomeric regions or in the intergenic regions upstream or downstream of *cyclin B1* (Figure 7C). Because H3-T11 phosphorylation was particularly enriched at centromeres during mitosis (Preuss et al., 2003), H3-T11 phosphorylation, but not Chk1, was detected at the centromeric region. Of particular interest, we found that H3-K9 acetylation strongly correlated with H3-T11 phosphorylation on the promoter regions of both *cdk1* and *cyclin B1* but did not correlate with H3-T11 phosphorylation at the *GAPDH* promoter region. *GAPDH* transcription is not repressed by either DNA damage or Chk1 depletion and thus does not correlate with Chk1 status. We observed that GCN5 was present at the promoter regions of *cdk1* and *cyclin B1* in unperturbed cells but was not detected at the promoter region of *GAPDH*. Furthermore, GCN5 levels at *cdk1* and *cyclin B1* promoters were significantly reduced in response to either DNA damage or Chk1 depletion. These results are consistent with a model whereby Chk1-mediated transcriptional repression is mediated by the loss of GCN5 recruitment to promoter regions of target genes when Chk1 dissociates from the chromatin and H3-T11 becomes dephosphorylated. The subsequent reduction in acetylation of K9 presumably reflects the associated change in promoter-bound HAT activities.

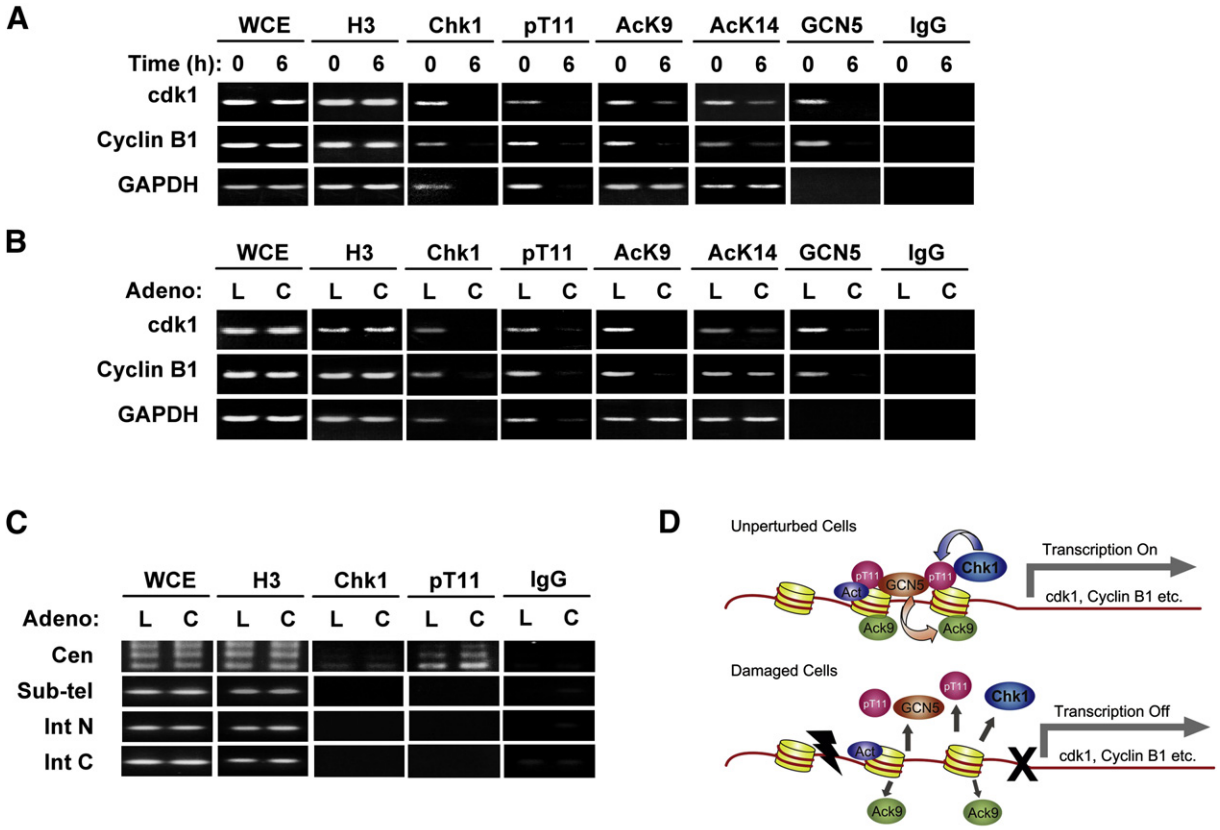

Figure 7. DNA Damage or Chk1 Depletion Results in Decreased H3-T11 Phosphorylation and Decreased H3-K9 Acetylation, which Correlates with GCN5 Recruitment to *cdk1* and *cyclin B1* Promoters

(A) Chk1$^{flox/-}$ MEF cells were left untreated or exposed to UV irradiation.

(B and C) The Chk1$^{flox/-}$ MEFs were infected with Ade-LacZ (L) or Ade-Cre (C) for 3 days. In all experiments cells were harvested at the indicated times, chromatin fractions prepared and subjected to ChIP analysis of (A and B) the indicated promoter regions or (C) centromere (Cen), subtelomere (Sub-tel), Int N (intergenic regions between *cyclin B1* and *slc30a5*), or Int C (intergenic region between *cyclin B1* and *cenph*). Following precipitation with indicated antibodies, semiquantitative PCR (from 30 to 35 cycles) was used to determine the extent of DNA enrichment.

(D) Model of Chk1-dependent transcriptional repression in response to DNA damage. Under unperturbed conditions, Chk1 associates with chromatin and phosphoylates H3-T11. Phosphorylation of T11 enhances GCN5 recruitment to promoters of relevant genes (*cyclin B1* and *cdk1*) leading to H3-K9 acetylation. In response to DNA damage, Chk1 dissociates from chromatin and H3-T11 becomes dephosphorylated. The decreased phosphorylation at T11 impairs GCN5 recruitment to the promoter, leading to the deacetylation of H3-K9 and reduced transcription.

DISCUSSION

Taken together, the data presented here demonstrate that Chk1 is a histone H3 kinase that regulates transcriptional repression in response to DNA damage. First, Chk1 is capable of phosphorylating nucleosomal histone H3 specifically on residue T11. This demonstrates that Chk1 can efficiently recognize a nucleosome, the subunit structure of chromatin, as a substrate (Figure 3C). Second, Chk1 is chromatin associated under unperturbed conditions (Figure 4A), and the PIKK-mediated DNA-damage-dependent Chk1 chromatin dissociation was prerequisite for concomitant H3-T11 dephosphorylation (Figure 4B). Third, Chk1 depletion resulted in a decrease in phosphorylation of H3-T11 in vivo (Figure 3D).

We used antibodies specific to individual histone modifications to reveal that H3-K9 acetylation was modestly reduced concomitant with the reduction in H3-T11 phosphorylation, in response to both DNA damage (Figure 2) and Chk1 ablation (Figure 3D). H3-K9 acetylation is mediated by GCN5 acetyltransferase and is essential for active transcription. Phosphorylation of H3-T11 was first demonstrated in Rat-1 cells and was reported to be predominantly associated with mitotic chromosomes (Preuss et al., 2003). In the context of our observation that H3-T11 phosphorylation can be readily detected during interphase, this suggested that H3-T11 phosphorylation in addition to H3-S10 phosphorylation has a function in transcriptional regulation. Consistent with this, we clearly demonstrate that H3-T11 phosphorylation significantly enhances the binding affinity between GCN5 HAT and H3 peptides. Thus, changes in the phosphorylation status of H3-T11 in response to DNA damage likely influence GCN5 recruitment at promoters and thus transcription of GCN5-dependent genes.

Although GCN5 was initially reported to acetylate both H3-K9 and H3-K14 (Kuo et al., 1996), GCN5 depletion in mouse and chick cells revealed that while H3-K9 acetylation was significantly reduced, acetylation of H3-K14 remained constant (Bu et al.,

2007; Kikuchi et al., 2005). Here we observed that H3-K9, but not H3-K14, acetylation was always reduced in response to either DNA damage or Chk1 depletion. This was consistent with reduced GCN5 recruitment in these circumstances and the presence of redundant acetyltransferases for H3-K14 as previously suggested (Kouzarides, 2007). An involvement of GCN5 in the mechanism of DNA-damage-dependent transcriptional repression is supported by our ChIP analysis examining the *cyclin B1* and *cdk1* promoter regions (Figure 6). Here we demonstrate that association of Chk1 and the subsequent phosphorylation of H3-T11 were observed at the *GAPDH* promoter region as well as at the promoters of *cyclin B1* and *cdk1*. However, we found that H3-K9 acetylation was only reduced at the *cyclin B1* and *cdk1* promoters after DNA damage or Chk1 depletion but was not changed at the *GAPDH* promoter region. Consistent with this difference, we could not detect GCN5 at the *GAPDH* promoter region under unperturbed conditions but could detect GCN5 at both the *cyclin B1* and *cdk1* promoter regions in unperturbed cells. This suggests that GCN5 is not required for *GAPDH* gene expression (Bu et al., 2007; Kikuchi et al., 2005) but participates in the regulation of *cyclin B1* and *cdk1*. These results suggest that Chk1-dependent changes in H3-K9 acetylation and gene transcription may be dependent on specific DNA-bound transcription factors.

Surprisingly, although Chk1 loss resulted in premature mitosis and a rapid increase in apoptosis in embryonic cells (Liu et al., 2000; Niida et al., 2005; Takai et al., 2000), Chk1 loss resulted in cell-cycle arrest within S phase in somatic MEFs (Figure 5A). In ES cells, Chk1 depletion resulted in loss of the inhibitory Y15 phosphorylation on cdk1 and premature cyclin B1-cdk1 activation. In MEFs, Chk1 depletion also caused loss of inhibitory Y15 phosphorylation on cdk1. The failure to activate cyclin B1-cdk1 and enter premature mitosis is presumably a result of concomitant loss of cyclin B1 and cdk1 proteins (Figure 5B). Irrespective of cell fate, our data clearly demonstrate that Chk1 is required for expression of cyclin B1 and cdk1 and thus for mitotic transitions in MEFs. The rapid proliferation arrest we observed in Chk1-depleted MEFs is consistent with a basal function for Chk1, promoting the expression of a subset of cell-cycle control genes in unperturbed cells. Interestingly, ATR-deficient MEFs divided normally for 1–2 cell cycles after ATR depletion (Brown and Baltimore, 2003). Our results demonstrate that ATR knockdown using specific siRNA prevents the decrease in H3-T11 phosphorylation caused by UV treatment. This would be consistent with our model where the requirement for Chk1 in basal transcription would be independent of ATR function.

The molecular events that underlie the cell-cycle arrest with incomplete DNA replication when Chk1 is depleted remain unknown. It is possible that a number of genes required for DNA replication are transcriptionally repressed when Chk1 is depleted in MEFs. In this regard, Chk1-SA, in which ATR-dependent phosphorylation sites are mutated, can partially suppress several phenotypes observed in Chk1-depleted cells, including *cyclin B1* and *cdk1* expression (even in the presence of DNA damage) and cell-cycle arrest, lending support to this suggestion. We note that GCN5 is utilized as an accessory acetyltransferase for E2Fs, which regulate the expression of the many genes involved in DNA replication (Lang et al., 2001). Furthermore, GCN5 is recruited to the promoters of many cell-cycle genes (Caretti et al., 2003), and GCN5-deficient cells have been shown to exhibit a significant decrease in their growth capability, which is associated with a reduction in the expression of many cell-cycle regulatory genes (Kikuchi et al., 2005). These observations are consistent with GCN5 having a mechanistic role in the repression of transcription following DNA damage and suggest that Chk1 and GCN5 function in the same pathway to regulate the transcription of cell-cycle genes and thus promote cell-cycle progression.

What is the physiological relevance of transcriptional repression in response to DNA damage? We propose that the Chk1-dependent repression of GCN5-dependent gene expression serves as an alternative checkpoint mechanism to promote cell-cycle delay or arrest, in addition to the regulation of inhibitory Y15 phosphorylation of cdk1. The observation that MEFs respond differently from ES cells following Chk1 depletion may suggest that the relative importance of the different Chk1-dependent pathways plays a role in "fine tuning" the DNA-damage response in different cell types. Previous observations that the ectopic expression of both cyclin B1 and cdk1AF can cooperate to abrogate the DNA-damage checkpoint (Jin et al., 1998) remain consistent with our model and observations.

In summary, we propose a model for transcriptional repression in response to DNA damage (Figure 7D). In the absence of DNA damage, Chk1 localizes to chromatin and phosphorylates the T11 residue of histone H3, promoting recruitment of GCN5 to the promoter regions of specific genes. When DNA is damaged or when replication forks are stalled, Chk1 is phosphorylated by the PIKKs, ATR, and ATM, resulting in rapid dissociation from the chromatin. Subsequent dephosphorylation of H3-T11 then results in GCN5 dissociating from the promoter region, leading to a decrease in the acetylation of H3-K9 and subsequent repression of transcription of the target genes. We also observed that Chk1 can phosphorylate other nucleosomal core histone (Figure 3C and data not shown), but the target residue and the physiological role of this phosphorylation remain elusive. Our present results uncover a novel mechanism underlying transcriptional repression in response to DNA damage through phosphorylation of H3 at residue T11.

EXPERIMENTAL PROCEDURES

Establishment of Chk1$^{flox/-}$ MEFs, Treatment with Genotoxic Agents

Chk1$^{flox/-}$ ES cells were generated as described previously (Niida et al., 2005). ES cells were injected into an embryonic day (E) 3.5 blast cyst and transplanted into the uterus of a surrogate mother. The chimeric embryo was harvested at day E13.5 and MEFs from the embryo were prepared. Chimeric MEFs were cultured in G418-containing DMEM with 10% FBS. Immortalized conditional Chk1-deficient MEFs were established by three-month subculture. HCT116 p53$^{-/-}$ cells were cultured in McCoy's 5a medium containing 10% FBS. All cells were cultured at 37°C under 5% CO_2 and treated with either UV (500 J/m^2), IR (10 Gy), bleomycin (40 μg/ml), aphidicolin (5 μM), or nocodazole (0.5 μg/ml).

Immunoblotting

Chromatin fractionation was performed as previously described (Niida et al., 2007). For preparation of whole-cell extracts, cells were lysed in IP kinase buffer (50 mM HEPES, pH 8.0, 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA 1 mM DTT, 0.1% Tween20, 10% glycerol) containing a cocktail of protease and phosphatase inhibitors. The antibodies used for immunoblotting are shown in the Supplemental Experimental Procedures.

Kinase Assay

Chk1-HA and Chk1-K38M-HA were immunopurified using anti-HA antibodies from infected Sf9 cells. Kinase reactions were performed as described (Niida et al., 2005). Substrates were purified histone H3, core histones prepared as described previously (Tachibana et al., 2002) or nucleosomes prepared with Micrococcus endonuclease as described previously (Sassone-Corsi et al., 1999). For cold reactions, kinase assays were performed without [γ-^{32}P]ATP.

Chromatin Immunoprecipitation Assay

ChIP assays were performed essentially as described (Tachibana et al., 2002). 1×10^7 cells, either irradiated or not with UV or infected with Ade-LacZ or Ade-Cre, were used for the chromatin preparation. Primers used for PCR are shown in the Supplemental Experimental Procedures. Antibodies specific to H3 (ab1791; Abcam), Chk1 (sc-8408; Santa Cruz), pT11 (ab5168; Abcam), AcK9 (ab4441; Abcam), AcK14 (07-353; Upstate), and GCN5 (sc-20698; Santa Cruz) were used for immunoprecipitation.

Knockdown Experiments by siRNA Transfection

HCT116 cells were transfected with a control siRNA (Silencer Negative Control number 1, Ambion 4611) or siRNA for ATR (CCUCCGUGAUGUUGCUUGAtt) using Lipofectamine 2000 (Invitrogen).

Establishing Chk1$^{flox/-}$ MEFs Expressing Chk1 Wild-Type or Chk1-S317A/S345A

Twenty micrograms of pCAGGS-Chk1 wild-type and Chk1-S317A/S345A and 5 μg of PGK-Hyg vector were linearized with SalI and KpnI, respectively. Linearized DNA was electroporated into Chk1$^{flox/-}$ MEFs with a Gene Pulser II. Cells were selected with 0.15 mg/ml hygromycin B for 7 days. Single colonies were screened by immunoblotting with anti-Chk1.

Protein Expression and Purification

Baculoviruses expressing Myc- and His-tagged HAT domains of *S. cerevisiae* GCN5 (residues 100–255) were generated as described previously (Tojima et al., 2000). Human histone H3.1 wild-type or T11 substituted with A was cloned into pET3a, expressed in *E. coli*, and purified as described previously with minor modification (Luger et al., 1999).

Peptide Binding Assay

SPR measurements were performed at 25°C using a Biacore 2000 instrument (Biacore, Inc., Uppsala, Sweden). The HAT domain of *S. cerevisiae* GCN5 was immobilized on the sensor chip CM5 using the amine coupling method according to the manufacturer's instructions. For kinetic measurements, 90 μl of each peptide sample was passed over the sensor surface in the presence or absence of CoA (0.15 mM) at a flow rate of 30 μl/min. The resultant sensor-grams were analyzed using BIAevaluation software (version 4.1).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at http://www.cell.com/cgi/content/full/132/2/221/DC1/.

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