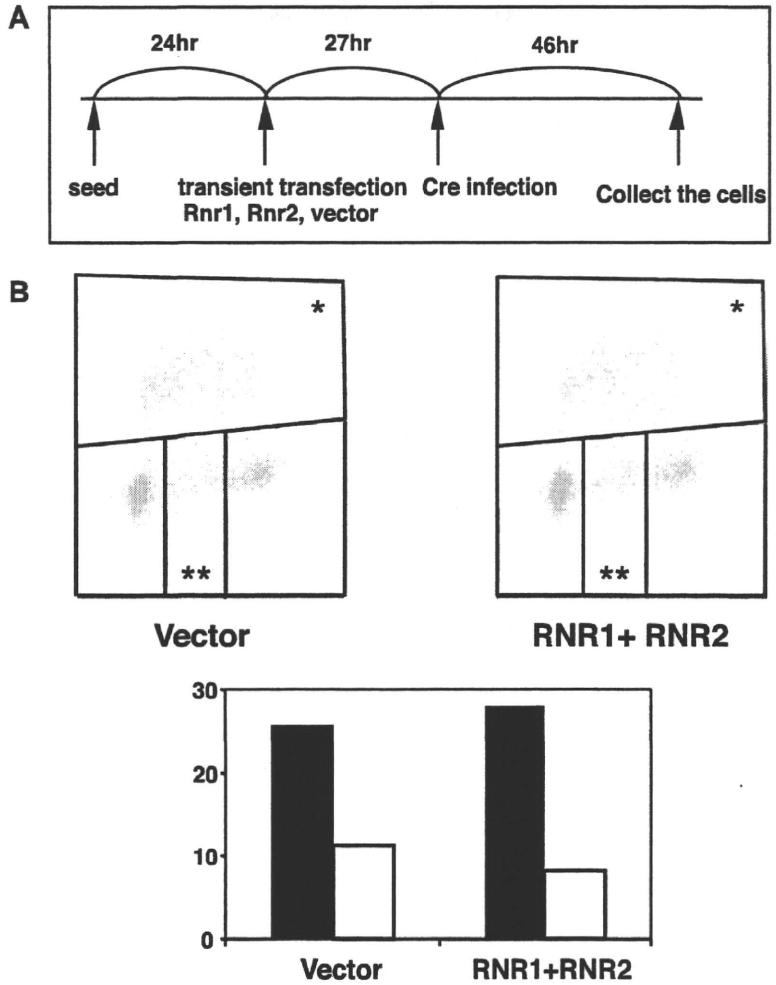

Fig. 4. Ectopic expression of RNR2 and RNR1 is insufficient for complement S phase arrest in Chk1 depleted MEFs. (A) Time course strategy for transient transfection of RNR2 and RNR1, and Chk1 depletion by Ade-Cre infection. (B) BrdU staining and FACS analysis were performed as indicated in Fig. 1. The percentages of each cell cycle distribution were also shown (black: BrdU positive; white: BrdU negative).

somatic cells by infection of adenoviruses expressing Cre reduced expression of more than 200 transcripts [10]. Therefore, increased S phase population in Chk1 depleted MEFs (Fig. 1A) are likely due to S phase arrest but not enhanced initiation of DNA replication. To address this question, we first examined BrdU incorporation in Chk1 depleted MEFs. After infection of adenoviruses expressing Cre, $Chk1^{del/-}$ MEFs were incubated with culture medium containing 10 μM BrdU for 30 min. Cells were then fixed, immunostained with anti-BrdU antibodies, and analyzed by FACScan. As shown in Fig. 1B, majority of S phase cells in control MEFs were BrdU positive and infection of adenoviruses expressing LacZ did not affect cell cycle progression. In contrast, although most of S phase cells in $Chk1^{del/-}$ MEFs were BrdU positive at 1 day after adenoviral infection, they became negative at two days or thereafter, indicating that increased S phase population is due to S phase arrest.

Transcriptional changes in genes involved in DNA replication after Chk1 depletion

We next examined the changes in the expression of genes involved in DNA replication after Chk1 depletion. Quantitative real time PCR revealed that the expression of RNR2, but not RNR1, was significantly reduced at two days after Chk1 depletion, whereas those of the other genes tested were almost constant during this experimental period (Fig. 2). Given that expression of many genes involved in RNR2, ASK, ORC1, cdc6, and cdc25c were dependent on the functional E2Fs [20,27–30], reduction in RNR2 expression was not likely due to the impairment of E2F functions. In addition, because RNR2 expression is cell cycle dependent, reaching at a maximum at S phase [20], decreased expression of RNR2 did not solely reflect the changes in cell cycle profile following Chk1 depletion (Fig. 1A).

The reduction in RNR2 protein as well as its mRNA was also confirmed by northern blotting, immunoblotting, and immunohistochemistry using specific antibodies to RNR2, respectively (Fig. 3A and B). The reduction in RNR2 protein was obvious at two days after Chk1 depletion, supporting the observation that S phase arrest in Chk1 depleted cells was detected at two days after infection. RNR2 was predominantly localized at cytoplasm and RNR1 was localized at both cytoplasm and nucleus, consistent with the previous reports [12], but signal of RNR2, but not RNR1, was almost disappeared after Chk1 depletion. Thus, S phase arrest following Cre infection appeared to be correlated well with the reduction in RNR2 protein.

Ectopic expression of RNR2 is not sufficient for complement S phase arrest in $Chk1^{del/-}$ MEFs

We finally asked whether ectopic expression could reverse the S phase arrest in $Chk1^{del/-}$ MEFs. Experimental protocols were

summarized in Fig. 4A. In brief, MEFs were transfected with both expression vectors for RNR1 and RNR2 and then infected with Cre. Forty six hours after infection, cells were incubated with the medium containing 10 μM BrdU and analyzed by FACScan. As shown in Fig. 4B, coexpression of RNR1 and RNR2 was not capable of reducing BrdU negative S phase population. These results suggests that ectopic expression of RNR1 and RNR2 is not sufficient for rescue the S phase arrest in Chk1 deficient MEFs and thus existence of unidentified transcriptional targets of Chk1 which is essential for completion of DNA replication. Clearly, additional work is necessary to clarify those Chk1 targets and their role in S phase progression.

Acknowledgments

We thank Dr. Yamada-Namikawa for discussion and technical assistance. This work was supported in part by the Ministry of Education, Science, Sports, and Culture of Japan through a Grant-in-Aid for Scientific Research (B) awarded to M.N.

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[Cell Cycle 7:11, 1555-1559; 1 June 2008]; ©2008 Landes Bioscience

Extra View

Checkpoints meet the transcription at a novel histone milestone (H3-T11)

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Abbreviations: PIKKs, phosphatidylinositol 3-kinase-related protein kinases; T, threonine; S, serine

Key words: DNA damage, transcription, checkpoint, Chk1, histone phosphorylation, GCN5

Eukaryotic cells are equipped with coordinated systems to contend with DNA damage, such as those which are used in cell cycle arrest, DNA repair and apoptosis, to maintain genomic integrity. These systems are regulated at least in part by transcriptional activation or repression. Although processes to activate transcription of specific genes have been characterized in the context of sequence-specific DNA binding factors, mechanisms of transcriptional repression have been largely unexplored. Recently, we identified phosphorylation of histone H3-threonine 11 (H3-T11), a novel chromatin modification for transcriptional activation, that was rapidly reduced after DNA damage. Intriguingly, checkpoint kinase 1 (Chk1) binds to chromatin and phosphorylates H3-T11 under unperturbed conditions. DNA-damage-induced Chk1 dissociation from chromatin closely correlates with decreased phosphorylation of H3-T11. Loss of H3-T11 phosphorylation results in decreased binding of GCN5 with H3, leading to reduced H3-K9 acetylation and transcriptional inhibition. From our results, we have begun to unravel the biological functions of H3-T11 phosphorylation and have uncovered a novel mechanism underlying transcriptional repression in response to DNA damage.

Cell Cycle Checkpoint and Chk1

DNA damage activates cell cycle checkpoints that transiently arrest the cell cycle to provide sufficient time for the damage to be repaired. Upon DNA damage, abnormal DNA structures are rapidly sensed and transduced by either or both the ATR and/or ATM phosphatidylinositol 3-kinase-related protein kinases (PIKKs). These kinases phosphorylate a wide variety of substrates to activate multiple responses, including cell cycle arrest, DNA repair, apoptosis and senescence. One such critical substrate is the effector kinase Chk1, which targets key regulators of the cell cycle, such as p53 and Cdc25 phosphatases. Chk1 is essential for checkpoint control, including intra-S and G_2/M DNA damage checkpoints[1-3] and replication checkpoints.[4,5] In addition to the checkpoint control, Chk1 is indispensable for cell viability and proliferation, suggesting the role of Chk1 in unperturbed conditions. During normal S phase, Chk1 is required to sustain high rates of replication fork progression and to suppress aberrantly increased initiation of DNA replication and subsequent spontaneous damage.[6,7] Chk1 is also thought to function as a key regulator of the initiation, progression and fidelity of unperturbed mitosis.[8] Taken together, Chk1 appears to play a central role in maintaining genome integrity. Indeed, Chk1 heterozygote mice exhibit a variety of abnormal cell cycle phenotypes that can contribute to tumorigenesis, suggesting that Chk1 may act as a haploinsufficient tumor suppressor.[9]

The regulation of Chk1 activity and its subcellular localization after DNA damage has recently been investigated. Chk1 is an active enzyme and a significant proportion of this enzyme is associated with chromatin in the absence of DNA damage. Following DNA damage, chromatin-bound Chk1 becomes phosphorylated at residues S317 and S345 by ATR or ATM, and rapidly dissociates from chromatin.[10] These phosphorylations appear not to regulate the kinase activity of Chk1 but rather its subcellular localization. Phosphorylated Chk1 tends to translocate to centrosomes[11,12] where it prevents premature activation of Cdk1/Cyclin B through inhibition of Cdc25.[13] Although the importance of the dissociation of Chk1 from chromatin and its translocation into centrosomes of Chk1 has been confirmed by immobilizing centrosomal Chk1,[10] the function of the chromatin-associated form of Chk1 remained to be identified. Because the chromatin-bound form of Chk1 possesses kinase activity, this enzyme likely has an additional substrate. Our recent figures resolved these issues, and demonstrated a new function for Chk1 in transcription by phosphorylation of H3-T11.

Transcriptional Repression and Histone Modifications

DNA damage alters global patterns of gene expression and causes a variety of cellular events. Among these, transcription of a variety of cell cycle regulatory genes such as *cyclin B1* and *cdk1* was repressed in a p53-independent manner after UV, X-ray and bleomycin treatment of mammalian cells. Given that histone acetylation is known to enhance gene transcription, we speculated that changes in histone modification may contribute to DNA-damage-induced transcriptional repression.

Covalent modifications of histone tails have been highly correlated with diverse biological processes. At least eight different types of modifications have been reported to date, and histone

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Submitted: 03/27/08; Accepted: 04/02/08

Previously published online as a *Cell Cycle* E-publication: http://www.landesbioscience.com/journals/cc/article/6062

Table 1 Summary of phosphorylation on H2AX and H3

Histones	Residues	Inducers	Kinases	Functions
H2AX	S139	DNA damage	ATR, ATM, DNAPK	Recruitment of DNA repair and checkpoint proteins to DSB sites
H3	T3	Mitosis	Haspin	Chromosome condensation
	S10	Mitosis	Aurora B	Chromosome condensation
		Interphase	MSK1, MSK2, RSK2, PKA, IKKα	Transcription
	T11	Mitosis	Dlk1/ZIP1	Chromosome condensation
		Interphase	Chk1, PRK1	Transcription
	S28	Mitosis	Aurora B, MSK1	Chromosome condensation

Residues, kinases, Inducers and functions of phosphorylation on histone H2AX and histone H3 are shown. The function of mitotic phosphorylation of H3-T11 has not been identified.

phosphorylation has been linked to chromosome condensation, segregation, activation of transcription, apoptosis and DNA damage repair. One of the most thoroughly analyzed histone phosphorylations associated with DNA damage is H2AX (Table 1). H2AX is rapidly phosphorylated after DNA damage at serine 139 (referred to as gamma-H2AX) with ATM, ATR and DNA-PK. In mammals, gamma-H2AX covers megabase regions around each double-strand break[14] and acts as a platform for the association of different repair proteins at the damaged sites.[15-18] Mitotic histone phosphorylation was also investigated in H3. Histone H3 is phosphorylated at at least four different sites, including serines 10 and 28 (S10, S28) and threonines 3 and 11 (T3, T11) (Table 1). Phosphorylation of S10 (pS10) is initiated in late G_2 in the pericentromere region, and then spreads throughout the chromatin during G_2/M until the end of mitosis.[19] During mitosis, phospho-S10 is catalyzed by Aurora B kinase and this phosphorylation is a prerequisite for chromosomal condensation and segregation.[20] S28 is also phosphorylated by Aurora B at mitosis and this phosphorylation is coupled with mitotic chromosome condensation.[21,22] Haspin phosphorylates H3-T3, and phospho-T3 is associated with mitotic chromosomal condensation. In mammals, phosphorylation of T11 is reported to occur at mitosis and to be most abundant in the centromere[23-25] and this phosphorylation is catalyzed by Dlk/ZIP kinase, a member of the DAP family.[25] However, it was readily detectable during interphase in several types of cells.[26] Phospho-S10 has also been observed during interphase and this phosphorylation activates transcription of several genes, including the serum-stimulated immediate early genes, *c-fos* and *c-jun*.[27]

We examined changes in histone modifications in response to DNA damage and found that pT11 is rapidly dephosphorylated. In addition, H3-K9 acetylation was modestly reduced in response to DNA damage. H3-K9 acetylation is mediated by GCN5 acetyltransferase and is essential for active transcription. One modification on histone tails affects other modifications at an inter-or intra-molecular level as well as protein binding specific to modified histones. For example, phosphorylation of H3-S10 inhibits the binding of HP1 to H3 methylated at K9.[28] Given that GCN5 recognizes H3 more effectively when it is phosphorylated at H3-S10,[29,30] it is speculated that phospho-T11, like pS10, may serve as a recognition site for recruitment of GCN5 and promote transcriptional activation. As expected, H3-T11 phosphorylation as well as pS10 significantly enhances the binding affinity between GCN5 and H3 peptides. ChIP analysis revealed an association of Chk1 and the subsequent phosphorylation of H3-T11 at promoters of both *cyclin B1* and *cdk1*. DNA damage reduced the association of Chk1 and pT11 at these promoters. Furthermore, H3-K9 acetylation was also reduced on *cyclin B1* and *cdk1* promoters after DNA damage or Chk1 depletion. Consistent with this observation, GCN5 specifically associates with *cyclin B* and *cdk1* promoter regions in unperturbed cells[31] and participates in the regulation of *cyclin B1* and *cdk1*. We propose a model in which Chk1 controls transcription through phosphorylation of H3-T11 (Fig. 1A). Dephosphorylation of H3-T11 in response to DNA damage likely suppresses GCN5 recruitment at *cyclin B1* and *cdk1* promoters and the transcription of GCN5-dependent genes.

The validity of our model was further confirmed. Caffeine treatment, ATR knockdown and expression of Chk1-S317A/S345A prevented dissociation of Chk1 from chromatin upon DNA damage. Intriguingly, the decrease in pT11 and transcriptional repression of *cyclin B1* and *cdk1* genes after DNA damage were suppressed, suggesting that Chk1 dissociation from chromatin directly regulates dephosphorylation of T11 and transcriptional repression following DNA damage. Acetylation of histone H3 at K9 is known to play an important role in transcriptional activation. In this regard, AcK9, but not AcK14, was significantly reduced in response to DNA damage or Chk1 depletion. Given that GCN5 is dispensable for acetylation of H3-K14,[32,33] it appears consistent that the reduced binding of GCN5 on *cyclin B1* and *cdk1* promoters did not affect AcK14. In addition to our findings, pT11 was reported to accelerate demethylation on K9 by recruitment of JMJD2C, leading to increased AcK9 and subsequent transcriptional activation on androgen receptor genes.[34] Thus, phosphorylation of T11 could influence the recruitment of GCN5 and demethylase JMJD2C, which would ultimately result in transcriptional activation through increased AcK9.

Modifications on histone tail likely serve as recognition markers that facilitate or prevent the binding of proteins (Fig. 1B). Heterochromatin protein HP1 binds methylated H3-K9[35] and Polycomb protein PC binds methylated H3-K27.[36,37] In addition, BPTF, CHD1 or ING2 interacts with methylated H3-K4. 14-3-3 proteins bind directly with pS10 that recruits them to nucleosomes at *c-fos* and *c-jun* promoters after mitogenic stimulation.[38] It was also demonstrated that methylation of H3-K4[39] and phosphorylation of H3-T3[40] inhibit the binding of NuRD and INHAT to H3 tail, respectively. pS10 is reported to block binding of the RSC chromatin remodeling factor to acetylated histone tails.[41] Therefore, identification and characterization of proteins or complexes binding to pT11 help us to better understand the mechanistic and biological links between pT11 and other modifications.

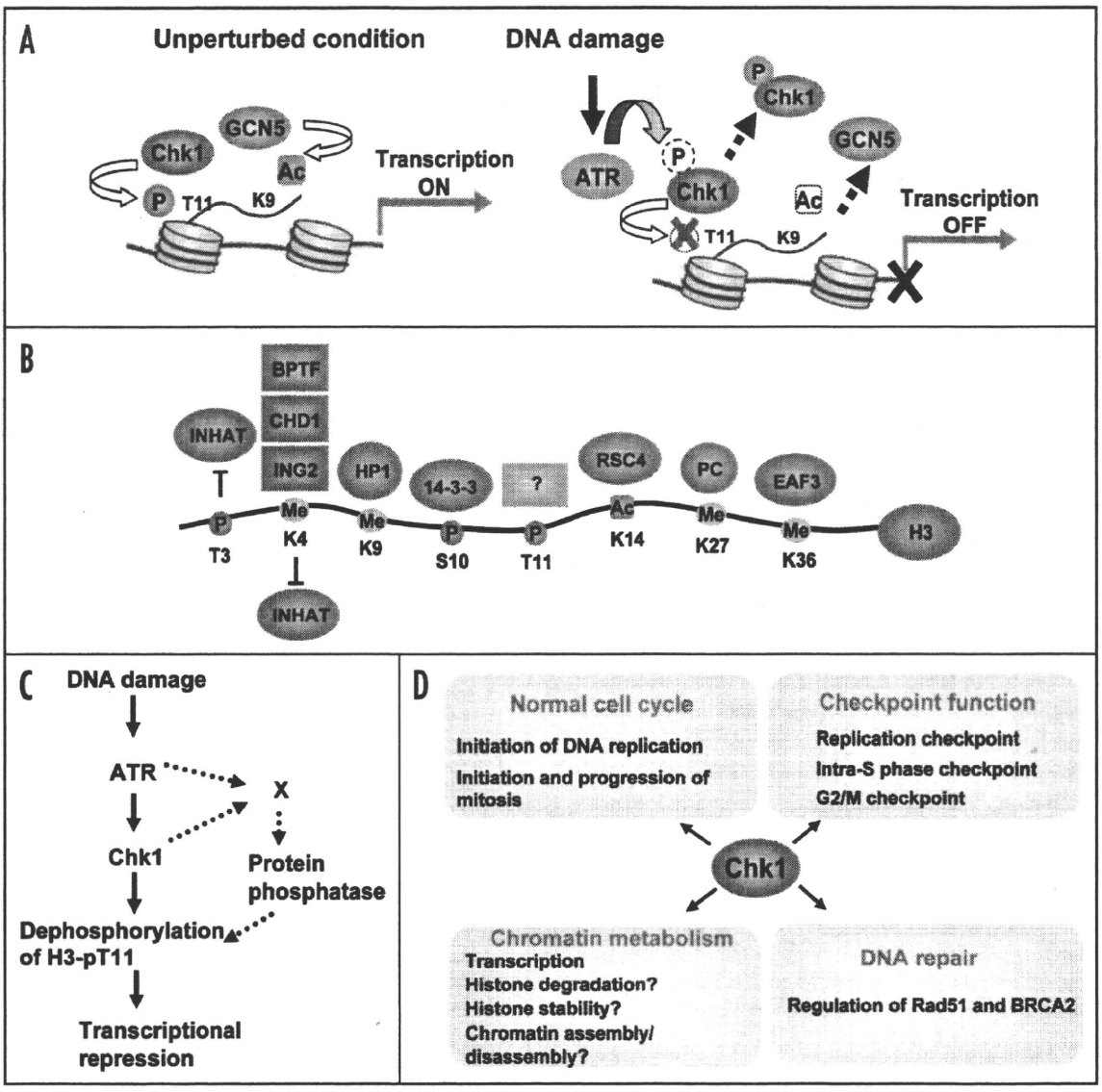

Figure 1. (A) Schematic model of Chk1-dependent transcriptional repression following DNA damage. In unperturbed cells, Chk1 localizes to chromatin and phosphorylates H3-T11, promoting recruitment of GCN5 to the promoter regions of specific genes. When DNA is damaged, Chk1 is phosphorylated by ATR, and rapidly dissociated from chromatin. Dephosphorylation of H3-T11 causes GCN5 release from the promoter region, resulting in a decrease in the acetylation of H3-K9 and transcriptional repression of the target genes. (B) Post-translational modifications in N-terminal tail of H3 and proteins binding to modified histone H3. The pT11 association factor(s) are unclear. (C) Two potential pathways for dephosphorylation of H3-pT11 following DNA damage. In addition to dissociation of Chk1 from chromatin, activation of protein phosphatase may also be involved. (D) Diverse biological functions of Chk1. Chk1 plays multiple roles in the normal cell cycle, checkpoints and DNA repair. Our studies uncover a new function for Chk1 as a histone kinase. The potential role of Chk1 in chromatin metabolism is also described.

Why Does Chk1 Regulate Two Distinct Pathways for Cdk1-Cyclin B1 Complexes?

Initiation of mitosis is controlled by Cdk1/Cyclin B1 kinase activity in many eukaryotes. Activation of Cdk1 depends on the dephosphorylation status of residues threonine 14 (T14) and tyrosine 15 (Y15) that is collaborately regulated by Cdc25 phosphatases and Wee1 and Myt1 kinases. In addition to the phosphorylation status of Y15, the availability of Cyclin B1 is also essential for Cdk1 kinase activity. In response to DNA damage, Cdc25 A is phosphorylated by Chk1 and which triggers its degradation, leading to subsequent inactivation of Cdk1/Cyclin B1. Therefore, inhibition of Cdk1-Cyclin B1 through its inhibitory phosphorylation might be sufficient for transient cell cycle arrest at G_2/M. Indeed, a loss of Chk1 leads to premature mitosis and apoptosis in embryonic cells[2,42,43] because of premature Cdk1/Cyclin B1 activation. What would be the physiological relevance of DNA damage-induced transcriptional repression of cell cycle regulatory genes by Chk1? Depletion of Chk1 in somatic cells resulted in permanent S phase arrest with a reduction more than 200 transcripts, suggesting that Chk1 has an essential function in transcription of the genes involved in cell cycle progression including *cyclin B1* and *cdk1*. Loss of Chk1-dependent H3-pT11 may affect H3-MeK9 at GCN5-dependent gene promoters. Hypermethylation of H3-K9 is known to be a chromatin marker for long term gene

suppression. Therefore, we propose that the Chk1-dependent repression of GCN5-dependent gene expression may serve as a long term (or permanent) checkpoint for DNA damage, leading cells with severe or unrepairable DNA damage to permanently exit from the cell cycle.

Perspectives

Our present results have revealed a novel mechanism underlying transcriptional repression in response to DNA damage through Chk1 redistribution. However, in addition to the regulation of Chk1 localization, it is tempting to speculate that phosphatases are also involved in dephosphorylation of H3-pT11 (Fig. 1C). There are several reports showing the some phosphatase activities are upregulated in response to DNA damage.[44,45] Therefore, identification of the phosphatase responsible for DNA-damage-induced H3-pT11 dephosphorylation and elucidation of the mechanisms by which DNA damage activates its phosphatase activity are critical for better understanding of transcriptional regulation upon DNA damage.

There are also unresolved issues regarding the role of Chk1 in histone phosphorylation. Chk1 phosphorylates not only H3 but also other core and linker histones in vitro.[26] (Shimada M, Nakanishi M, unpublished) The question remains as to which residues are phosphorylated by Chk1. There is also a need to determine the biological significance of these Chk1-associated histone phosphorylations and how they influence other histone modifications? Chk1 might have additional roles in chromatin metabolism such as histone degradation, histone stability and chromatin assembly and disassembly through phosphorylation of histones (Fig. 1D). Hopefully, the answers to these questions will help us understand how Chk1 coordinates biological processes. Furthermore, the information obtained from a series of future experiments will contribute to the understanding of changes in chromatin modifications and their function as cells transit from a normal to a specialized status, such as differentiated, malignant or senescent.

Acknowledgements

We thank our colleagues for discussions and comments on the manuscript and acknowledge the financial support of the Ministry of Education, Science, Sports and Culture of Japan. The authors apologize for any failures to cite relevant articles owing to space constraints.

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Int. J. Cancer: **122,** 1222–1228 (2008)

Susceptibility of p27^{kip1} knockout mice to urinary bladder carcinogenesis induced by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine may not simply be due to enhanced proliferation

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Deregulated proliferation is one of the fundamental characteristics of carcinogenesis. p27 is one of the most well characterized negative cell cycle regulator. In our previous study, expression of p27 protein was found to be dramatically suppressed on stimulation of cell proliferation by calculi in the rodent urinary bladder, withdrawal of the insult resulting in re-expression of p27 and regression of urothelial hyperplastic lesions. In the present study, to evaluate how loss of function impacts on urinary bladder carcinogenesis, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN), a bladder carcinogen was given to p27 knockout mice. Males and females with either null, hetero or wild-type p27 alleles were divided into 2 groups, one given drinking water containing 0.05% BBN for 10 weeks and the other receiving distilled water, then, killed at week 20. The experiment was repeated for confirmation of the outcome. In the second experiment, performed with a larger number of animals, the incidence of urinary bladder carcinomas was significantly higher in female p27-null mice than in their wild-type counterparts. p27 deficiency also resulted in their increase of relative weights of urinary bladders and section areas of carcinomas in BBN-treated mice. Interestingly, while BrdU labeling indices generally increased with progression of mucosal proliferative lesions, from normal epithelium, through hyperplasia to carcinoma, there was no significant variation with the p27 genotype, in tumors as well as normal urothelium. These findings suggest that p27 deficient mice have elevated susceptibility to BBN-induction of urinary bladder carcinogenesis through a mechanism which might be independent of acceleration of cell cycling.
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Key words: knockout mice; urinary bladder; p27; BBN

Introduction

Failure of cell cycle regulation at different checkpoints is a notable characteristic of carcinogenesis. Cyclins and cyclin-dependent kinases (Cdk) drive the cell cycle whereas Cdk inhibitors such as p27^{kip1}, p21^{waf1}, p16^{INK4a} and p15^{INK4b} act as negative regulators, for example controlling the *Rb* gene phosphorylation status which governs progression from the G1 to the S phase.

The inhibitor p27 belongs to the Cip/Kip family, negatively regulating cyclin D, E and A, and plays a pivotal role in the control of cell proliferation,[1,2] also mediating the growth arrest induced by transforming growth factor β, contact inhibition, growth in suspension, cyclic AMP agonists and other signals.[1,3,4] It is present abundantly and ubiquitously in quiescent cells but declines in proliferating cells in response to mitogenic stimulation by growth factors and cytokines. The concentration of p27 protein is thought to be predominantly regulated by the ubiquitin-proteasome pathway.[5] Cytoplasmic relocalization, also controlled by PKB/Akt-mediated p27 phosphorylation at threonine 157, is considered as an important pathway for p27-induced G1 arrest arrest.[6–8]

Although mutations in the *p27* gene are rare in human tumors,[9–11] decrease or loss of p27 protein correlates with a poor prognosis in breast,[12–14] stomach,[15] colon,[16] ovarian epithelial,[17–19] prostate[20,21] and urinary bladder[22–24] cancers.

In our previous study, protein levels of p27 were found to be slightly decreased in rat hyperplastic urothelial mucosa induced by calculi associated with cell proliferation, then becoming dramatically but reversibly increased after withdrawal of the insult at the same time that the hyperplastic urothelium returned to normal.[25] Similar down and up-regulation of p27 depending on the presence of cell-proliferative stimuli caused by uracil caliculi was also found in carcinogen-induced rat urothelial mucosal hyperplastic lesions, but not in neoplastic lesions such as carcinomas.[25] This fact suggests that disordered expression of p27 plays an important role in urinary bladder carcinogenesis. For further elucidation, we here evaluated alteration of susceptibility to *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN), a rodent urinary bladder carcinogen, in p27 knockout mice.

Material and methods

Animals

Male p27 heterozygous mice with a targeting construct designed to delete the entire protein-coding region of *p27* gene on one allele[26] were kindly provided by Nippon Roche Research Center (Kamakura, Japan). Following the provider's system, male p27$^{+/-}$ mice were then backcrossed to female C57BL/6 wild mice (Charles River Japan Inc., Atsugi, Japan) for maintenance of the genotype. Subsequently animals for the experiment were obtained by crossing male and female heterozygous p27 mice. They were genotyped with two primer sets: one for wild type *p27* detection; p27-5′, CCT GGA GCG GAT GGA CGC CAG ACA and p27-3′, GCC AGC ACC TTG CAG GCG CTC TTG; and the other for mutant type *p27* (insertion of phosphoglycerate kinase-1) detection; BK1, GGC TAT TGG CTC AAA ACG AAC CTC and MK4, ATG CTC CAG ACT GCC TTG GGA AAA on DNA extracted from tails. Then, they were divided into groups consisting of 6–8 individuals of the same genotype and sex per plastic cage with wooden chips for bedding in an air-conditioned room at

Grant sponsor: Grants-Aid for Cancer Research from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labour and Welfare. Grant sponsor: Grant-in-aid from the Ministry of Health, Labour and Welfare for the Second Term Comprehensive 10-Year Strategy for Cancer Control, Japan. Grant sponsor: Grant from the Society for Promotion of Toxicological Pathology of Nagoya, Japan.

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Received 2 June 2007; Accepted after revision 19 September 2007
DOI 10.1002/ijc.23249
Published online 20 November 2007 in Wiley InterScience (www.interscience.wiley.com).

Publication of the International Union Against Cancer

22 ± 2°C and 55% ± 5% humidity with a 12 hr light/dark cycle. Tap water and diet were available *ad libitum*.

Animal experiment

To elucidate the possible influence of mixed background strain during the establishment of the knockout mice, experiments were performed twice with different generations. Experiment 1 with 3–10 mice per group and the Experiment 2 with 11–20 mice per group were performed with the 4th and 10th (>90% inbreeding coefficients) generations, respectively, after we received parental animals.

Male and female mice of each p27 genotype, i.e. nullizygous ($p27^{-/-}$), heterozygous ($p27^{+/-}$) and wild ($p27^{+/+}$), at the age of 6–8 weeks were divided into one group given drinking water containing 0.05% BBN (Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan) for 10 weeks and the other group receiving distilled water (resulting in twelve groups in all). Food and water consumption was monitored once a month and body weights were recorded every week until the end of the experiment. At the 20th experimental week, all mice were killed under ether anesthesia and their urinary bladders, kidneys, livers and spleens were excised. They were weighed and bladders and kidneys were processed for histological examination. One hour before sacrifice in Experiment 2, bromodeoxyuridine (BrdU; Sigma Chemical Co., St. Louis, MO) was injected i.p. into six mice from each group at the dose of 100 mg/kg body weight for assessment of DNA synthesis. The genotype of all animals was confirmed at this point with a DNA sample extracted from the spleen.

Use of the animals in these carcinogenesis experiments was according to the Guidelines for the Care and Use of Laboratory Animals of Nagoya City University Graduate School of Medical Sciences, and was approved by the Institutional Animal Care and Use Committee.

Tissue processing

The urinary bladders were routinely filled with approximately 1 ml of 10% buffered formalin and immersed in the fixative with their necks closed. Those of five mice from each group in Experiment 2 were cut longitudinally into two halves, then one half was frozen in liquid nitrogen and stored at –80°C until DNA extraction and the other was fixed in formalin. Either before or after fixation, bladders were cut in half and weighed. Then, 4–8 longitudinal slices per bladder were made and embedded in paraffin for histological examination. Kidneys were sectioned transversely so that the renal pelvis was available for histological examination after routine processing.

Histo-pathological and immunohistochemical examination

Routine histological examination was performed with hematoxylin and eosin stained sections. Urothelial lesions were classified into simple, papillary or nodular (PN)-hyperplasia or carcinoma, and invasiveness of carcinomas was evaluated as Tis, T1, T2a, T2b and T3 for *in situ*, submucosal, inner half muscular, outer half muscular and extravesical invasion, respectively. In mice, urinary bladder carcinomas generally developed as non-papillary diffuse lesions[27] making it difficult to evaluate tumor multiplicity for the quantitative analysis. In slide sections, areas of carcinoma, recognized as severely atypical urothelium excluding mesenchymal tissue as much as possible were measured with the aid of an image analyzer (Image Processor for Analytical Pathology; Sumika Technoservice, Osaka, Japan). Then, relative values for percentage areas of lesion per total length of urothelium were calculated. For immunohistochemical analyses, anti-BrdU (DAKO Japan, Kyoto, Japan), anti-p27 (C-19; Santa-Cruz Biotechnology, Santa Cruz, CA), anti-p53 (CM-5; Novocastra Laboratories Ltd., Newcastle upon Tyne, UK), anti-p21 (M-19; Santa-Cruz), and anti-cyclin D1 (Immunobiological Laboratories, Gumma, Japan) antibodies were applied at dilutions of 1,000, 500, 800, 25 and 100, respectively. A Vectastain ABC Elite Kit (Vector Laboratories Inc., Burlingame, CA) and diaminobenzidine were used for visualization. For the evaluation of labeling indices of stained nuclei, more than 1,000 urothelial cells per corresponding lesion were counted and percentage values were generated.

Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analyses and direct sequencing

DNA was extracted from frozen bladder tissue specimens, dissolved in 100 µl of water and stored at –20°C. PCR-SSCP analysis was performed to detect mutations in exons 5–8 of the p53 gene, exon sequences being amplified with appropriate oligonucleotide primers as described previously.[28] PCR was carried out in a 10 µl reaction volume containing genomic DNA, 0.4 µM of each primer, 1.6 mM of dNTP mixture, 0.2 mCi of [^{32}P]dCTP (Amersham Biosciences UK Ltd., Buckinghamshire, UK), 2.5 mM of $MgCl_2$, and 10× LA PCR Buffer II (Takara Bio Inc., Otsu, Japan). TaKaRa LA Taq of 0.5 U (Takara Bio) was added and the samples were incubated at 95°C for 5 min followed by 35–40 cycles of amplification in a TaKaRa PCR Thermal Cycler (Takara Bio) with denaturation at 95°C for 45 sec, annealing at 58–62°C for 30 sec and extension at 72°C for 30 sec. Each PCR aliquot was subsequently mixed with 10 µl of stop buffer (98% formamide, 10 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol), heated at 95°C for 3 min, immediately placed on ice and loaded onto a 0.5× MDE gel (Cambrex Bio Science Rockland, Rockland, ME) with or without 5% glycerol. Electrophoresis was performed at 5–8 W for 8 hr. Then the gels were dried and used to expose X-ray film.

To sequence PCR products, the amplified fragments were cut out, eluted from the gels and re-amplified under the same conditions as described above. Electrophoresis of the amplified PCR products was performed in 3% agarose gels, from which DNA amplicons were purified with a QIAquick Gel Extraction Kit (QIAGEN K.K., Tokyo, Japan). Sequencing was performed with a BigDye Terminator Cycle Sequencing Kit and an ABI PRISM 310 Genetic Analyzer according to the supplier's instructions (Applied Biosystems, Foster City, CA).

Statistical analyses

Depending on the size of the group, the Kruskal–Wallis H-test or the Mann–Whitney U-test with the Bonferroni correction were performed for multiple comparisons and the Yates m × n chi-square test was applied for statistical analyses of incidence.

Results

The Experiment 1

Throughout the experimental period, body weight gain was slightly suppressed by BBN-treatment in the male mice of all p27 genotypes. Regardless of the treatment and sex, $p27^{-/-}$ mice tended to show higher body weights than $p27^{+/+}$ mice, with intermediate values obtained for $p27^{+/-}$ mice. The final body weights of $p27^{-/-}$ mice were significantly higher than those of $p27^{+/+}$ mice in untreated males (39.9 ± 1.7 vs. 33.9 ± 4.2 g; $p < 0.05$) and females (31.2 ± 1.6 vs. 22.7 ± 1.1 g; $p < 0.001$) and BBN-treated females (29.3 ± 2.1 vs. 23.6 ± 1.7 g; $p < 0.001$). Although relative urinary bladder weights (‰ of BW) varied greatly in BBN-treated groups averages for –/–, +/– and +/+ mice tended to be higher in BBN-treated groups (15.7 ± 13.6, 5.6 ± 7.5 and 3.4 ± 2.1 in males and 11.7 ± 20.1, 3.8 ± 1.1 and 2.2 ± 0.2 in females, respectively) compared with non-treated groups (1.6 ± 0.6, 1.6 ± 0.3 and 1.5 ± 0.3 in males and 1.8 ± 0.5, 2.1 ± 0.4 and 1.8 ± 0.5 in females, respectively) and $p27^{-/-}$ genotype mice than in non-treated groups and $p27^{+/+}$ genotype mice.

Histological analysis (Table I) showed almost all BBN-treated male mice to have developed urinary bladder carcinomas. In females, 7 out of 7 (100%) with the $p27^{-/-}$ genotype and 2 out of 7 (29%) with the $p27^{+/+}$ genotype developed carcinomas with BBN, these values being significantly different ($p < 0.05$). More-

TABLE I - HISTOPATHOLOGICAL FINDINGS OF THE URINARY BLADDER (THE EXPERIMENT 1)

Genotype of p27	BBN	No. of mice	Incidence (%)					Relative size of carcinoma (mm^2/mm mucosa)
			Hyperplasia		Carcinoma			
			Simple	PN	Non-Invasive	Invasive	Total	
Male								
−/−	+	5	5(100)[3]	5(100)[3]	1(20)	3(60)	4(80)[2]	2.00±2.11
+/−	+	10	10(100)[4]	10(100)[4]	0	9(90)[2]	9(90)[2]	0.25±0.34
+/+	+	7	7(100)[4]	7(100)[4]	1(14)	6(86)[2]	7(100)[2]	0.22±0.49
−/−	−	3	0	0	0	0	0	0
+/−	−	9	0	0	0	0	0	0
+/+	−	7	0	0	0	0	0	0
Female								
−/−	+	7	7(100)[3]	7(100)[3]	2(29)	5(71)[2]	7(100)[1,3]	0.59±1.20
+/−	+	8	8(100)[4]	8(100)[4]	2(25)	3(38)	5(63)	0.04±0.05
+/+	+	7	7(100)[3]	6(86)[3]	0	2(29)	2(29)	0.03±0.07
−/−	−	5	0	0	0	0	0	0
+/−	−	9	0	0	0	0	0	0
+/+	−	5	0	0	0	0	0	0

PN: Papillary or nodular.
[1]Significantly different from +/+ genotype of BBN-treated mice at $p < 0.05$.–[2,3,4]Significantly different from +/+ genotype of untreated mice at $p < 0.05$, 0.01, 0.001, respectively.

over, relative section areas of carcinomas (mm^2/mm mucosal length) tended to be larger with the $p27^{-/-}$ genotype than the $p27^{+/-}$ and $p27^{+/+}$ genotypes in males and females.

The Experiment 2

Body and relative organ weights and macroscopic observation. As shown in Table II, in line with Experiment 1 and the original report for this mouse,[26] increased body weights were found for $p27^{-/-}$ as compared with $p27^{+/+}$ genotype mice as well as elevated relative organ weights for the male liver, kidney and spleen and female spleen (data not shown). BBN-treatment tended to increase the relative urinary bladder weights, and values in the male $p27^{-/-}$ group were significantly higher than in the $p27^{+/+}$ ($p < 0.05$) and $p27^{+/-}$ ($p < 0.05$) cases (Table II). Macroscopically, urinary bladders in BBN-treated mice demonstrated thickening of the wall with mucosal proliferative lesions (Fig. 1). These changes were more obvious in males than in females and were particularly severe in $p27^{-/-}$ male mice.

Histological analysis of the urinary bladder and renal pelvis. Simple hyperplasia or papillary and nodular (PN) hyperplasia, putative premalignant lesions of murine urothelium,[29] occurred in all groups receiving BBN treatment (Table III). Carcinomas were observed in 100, 85 and 80% of male $p27^{-/-}$, $p27^{+/-}$ and $p27^{+/+}$ mice, respectively. The incidences in females were much lower at 38.9, 15.0 and 0%, respectively, with significant variation ($p < 0.05$) among the three p27 genotypes (Table III). Section areas of carcinomas corrected for mucosal length in male and female mice were greater with $p27^{-/-}$ than $p27^{+/+}$, with intermediate values with $p27^{+/-}$ (Table III). Histologically, most of the lesions were characterized as non-papillary type with frequent invasion. More than half of the carcinomas invaded into the muscle layer in male mice, regardless of the p27 genotype. In females, $p27^{-/-}$ mice tended to show a higher incidence of muscle-invading carcinomas than their $p27^{+/-}$ counterparts (Table III).

As shown in Table IV, similar urothelial lesions were also observed in the renal pelvis of BBN-treated mice, simple or PN hyperplasia being observed in 50, 15 and 15%. Moreover, carcinomas were evident in 15.8, 0 and 5.0% of the $p27^{-/-}$, $p27^{+/-}$ and $p27^{+/+}$ male mice, respectively. In females, the incidences were 44.4, 20.0 and 5.6% for simple or PN hyperplasia and 16.7, 5.0 and 0% for carcinomas in the $p27^{-/-}$, $p27^{+/-}$ and $p27^{+/+}$ mice, respectively.

Metastasis was not observed in any major organs in either study.

Immunohistological analysis of the cell cycle in the urinary bladder. BrdU labeling indices tended to be increased in BBN-treated urothelium and apparently reflected the grade of urothelial lesion (Table V). Even though sample number was limited, the indices in untreated normal urothelium, BBN-induced hyperplastic urothelium and carcinoma were similar across the p27 genotype for each category (Table V). The expression pattern of cyclin D1 was found to resemble that of BrdU labeling (data not shown). BrdU-indices were variable in areas of carcinoma, but were generally the same for all genotypes. Immunohistochemical staining for p27 confirmed its lack in $p27^{-/-}$ mice (Figs. 2*a* and 2*d*) and low expression in the urothelium of both $p27^{+/-}$ and $p27^{+/+}$ non-treated mice. In BBN-treated $p27^{+/-}$ and $p27^{+/+}$ mice, expression of p27 was elevated in hyperplastic lesions (Figs. 2*b* and 2*c*), but decreased numbers of positive cells were noted in carcinomas (Figs. 2*e* and 2*f*). p21 expression was apparent in the normal umbrella cells of all $p27^{-/-}$, $p27^{+/-}$ and $p27^{+/+}$ mice, but only a few in BBN-induced carcinoma cells with no compensatory up-regulation in $p27^{-/-}$ mice (Fig. 3). Nuclear expression of p53 was increased in BBN-treated urothelium and apparently reflected the grade of urothelial lesion. However, clear differences across the genotypes were not seen.

SSCP analysis of p53 in urinary bladder carcinomas. As summarized in Table VI, point mutations in the p53 gene (exon5-exon8) were detected by SSCP analysis in 2 of 5 (40%), 1 of 3 (33%) and 1 of 3 (33%) urothelial carcinomas in BBN-treated male mice with the $p27^{-/-}$, $p27^{+/-}$ and $p27^{+/+}$ genotypes, respec-

TABLE II - BODY AND RELATIVE BLADDER WEIGHTS

Genotype of p27	BBN	No. of mice	Final body weight (g)	Relative bladder weight (‰)
Male				
−/−	+	19	34.1 ± 2.5[1,3]	16.3 ± 22.6[2,4]
+/−	+	20	31.1 ± 1.6	4.5 ± 5.1
+/+	+	20	30.6 ± 2.8	3.0 ± 1.5
−/−	−	14	34.7 ± 2.1[2,4]	1.7 ± 0.5
+/−	−	13	31.9 ± 2.7	1.9 ± 0.5
+/+	−	15	31.8 ± 3.1	1.5 ± 0.6
Female				
−/−	+	18	29.5 ± 3.2[1,3]	2.7 ± 1.5
+/−	+	20	26.5 ± 2.2	2.4 ± 0.8
+/+	+	18	24.2 ± 1.8	2.1 ± 0.5
−/−	−	11	31.3 ± 2.3[1,3]	1.5 ± 0.6
+/−	−	15	26.3 ± 2.0[2]	1.9 ± 0.6
+/+	−	15	24.0 ± 2.6	2.0 ± 0.6

Data are means ± SD values.
[1,2]$p < 0.01$, 0.05 vs +/+ group (of the same treatment), respectively.–[3,4]$p < 0.01$, 0.05 vs +/− group (of the same treatment), respectively.

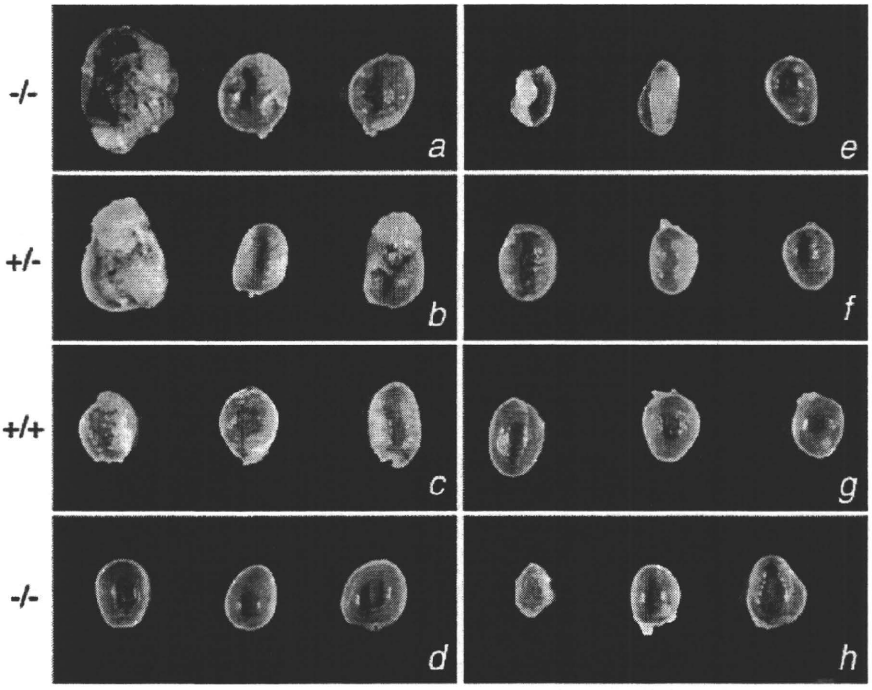

FIGURE 1 – Representative macroscopic appearance of urinary bladders in Experiment 2. BBN-treated urinary bladders in males (*a–c*) and females (*e–g*) are rough in surface, compared with the non-treated male (*d*) and female (*h*) controls. Larger tumors are evident in −/− (*a* and *e*) than +/+ (*c* and *g*) in both sexes. Animals with the +/− genotype (*b* and *f*) also have relatively large tumors.

TABLE III – HISTOPATHOLOGICAL FINDINGS OF THE URINARY BLADDER (THE EXPERIMENT 2)

Genotype of p27	No. of mice	Simple or PN hyperplasia[1] (%)	Carcinoma						Relative size of carcinoma (mm^2/mm mucosa)
			Tis	T1	T2a	T2b	T3	Total (%)	
Male									
−/−	19	11 (100)[2]	0	7	3	5	4	19 (100)	0.64 ± 0.72
+/−	20	15 (100)	2	6	4	4	1	17 (85.0)	0.37 ± 0.62
+/+	20	15 (100)	2	5	4	2	3	16 (80.0)	0.13 ± 0.15
Female									
−/−	18	13 (100)	1	1	3	2	0	7 (38.9)[3]	0.26 ± 0.20
+/−	20	15 (100)	0	2	1	0	0	3 (15.0)[3]	0.03 ± 0.02
+/+	18	13 (100)	0	0	0	0	0	0[3]	0

Tis: carcinoma in situ, T1: submucosal invasion, T2a: inner half muscular invasion, T2b: outer half muscular invasion, T3: extravesical invasion, PN: papillary or nodular.
[1]5 cases for frozen specimens were excluded in each group.–[2]3 dead cases were additionally excluded.–[3]Significantly different among genotypes at $p < 0.05$.

tively. One was a silent mutation and no particular pattern was evident. Mutations were not detected in PN hyperplasia of BBN-treated male mice or in either carcinomas or PN hyperplasia of BBN-treated female mice.

Discussion

The present duplicated experiment revealed that p27 deficiency enhances susceptibility to BBN-induction of urinary bladder carcinogenesis in mice. Significant genotype differences were thus noted in the incidences of carcinoma in the females or relative section areas of carcinoma/bladder weight in the males. It has been reported that p27 is haplo-insufficient for tumor suppression.[30] The values in heterozygous animals of our study were also between wild and deficient genotypes, but, relatively close to those for the wild type.

Interestingly, while BrdU labeling indices were generally elevated with progression of mucosal proliferative lesions, from normal epithelium through hyperplasia to carcinoma, there was no significant variation among the p27 genotypes. A similar tendency was seen for cyclin D1-labeling indices (data not shown). These facts suggest that p27 deficiency does not simply increase cell pro-

TABLE IV – INCIDENCE OF RENAL PELVIC NEOPLASIA

Genotype of p27	No. of mice	Simple or PN hyperplasia[1] (%)	Carcinoma				
			Tis	T1	T2	T3	Total(%)
Male							
−/−	16[1]	8 (50.0)	0	1	1	1	3 (15.8)
+/−	20	3 (15.0)	0	0	0	0	0
+/+	20	3 (15.0)	0	1	0	0	1 (5.0)
Female							
−/−	18	8 (44.4)	0	1	1	1	3 (16.7)
+/−	20	4 (20.0)	0	0	0	1	1 (5.0)
+/+	18	1 (5.6)	0	0	0	0	0

Tis: carcinoma in situ, T1: submucosal invasion, T2: muscular invasion, T3: renal parenchymal invasion, PN: papillary or nodular.
[1]Dead cases were excluded.

TABLE V – BRDU LABELING INDICES OF URINARY BLADDER LESION(%)

Genotype of p27	BBN	Normal	Simple hyperplasia	PN hyperplasia	Carcinoma
Male					
−/−	+		(4) 1.85 ± 0.65	(4) 4.56 ± 1.71	(6) 6.37 ± 5.40
+/−	+	(1) 0.29	(4) 1.56 ± 1.64	(6) 5.02 ± 2.19	(6) 10.9 ± 4.66
+/+	+		(3) 1.02 ± 0.90	(5) 5.38 ± 1.99	(6) 11.3 ± 2.48
−/−	−	(6) 0.10 ± 0.09			
+/−	−	(6) 0.08 ± 0.12			
+/+	−	(6) 0.10 ± 0.11			
Female					
−/−	+	(3) 0.30 ± 0.30	(4) 0.63 ± 0.70	(3) 1.46 ± 0.68	(1) 11.97
+/−	+	(3) 0.36 ± 0.34	(5) 0.98 ± 0.55	(4) 3.82 ± 2.99	(1) 6.77
+/+	+	(2) 0 ± 0	(5) 0.22 ± 0.07	(3) 2.43 ± 1.47	(1) 8.93
−/−	−	(6) 0.09 ± 0.10			
+/−	−	(6) 0.11 ± 0.10			
+/+	−	(6) 0.03 ± 0.05			

( ): Effective number of mice for each lesion. PN: papillary or nodular.

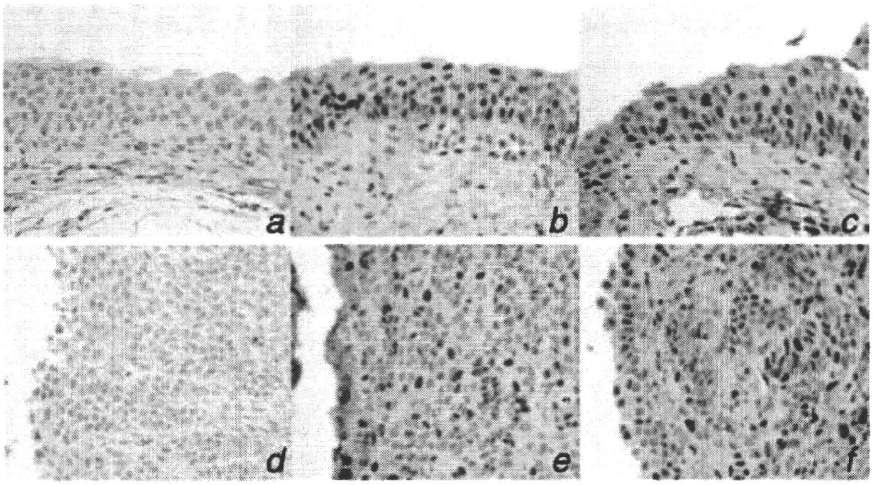

FIGURE 2 – Representative immunohistochemical staining for p27 in BBN-induced urinary bladder hyperplasia (*a–c*) and carcinomas (*d–f*) of male mice. p27 null (−/−: *a*, *d*) mice lack p27 as expected. p27 hetero (+/−: *b*, *e*) and wild (+/+: *c*, *f*) mice express p27 in a relatively high proportion of urothelial nuclei in hyperplasia and to a lesser extent in carcinomas. Slightly weaker staining was observed in hetero than in wild type mice.

liferation and that other parameters pertinent to regulation of cell cycle and tumor growth might be involved.

p27 inactivation has been reported as an independent prognostic marker in various human cancers, correlating with higher tumor grade and poor survival.[12–24] Detailed evaluation of clinical cases of urinary bladder carcinomas revealed a correlation of low p27 expression with high tumor grade and reduced disease-free survival in primary superficial cancers[22] as well as increased muscle invasion and lymph node metastasis.[23,24] On the other hand, Doganay *et al.*, provided evidence that expression of p27 is generally low in urinary bladder carcinoma but is not related to tumor grade, stage, PCNA index, recurrence rate and prognosis. They concluded that p27 expression may not be an independent predictor of outcome with transitional cell carcinoma of the urinary bladder.[31]

In a previous study using a chemically induced skin carcinogenesis model in p27 knockout mice, the mean number of papillomas per mice did not differ between the p27 genotypes, but the incidence for papillomas > 5 mm in size in p27 null mice was 3.3 times higher than in the wild-type,[32] pointing to rapid clonal expansion of initiated cells during promotion. A similar result was reported in the tumors of salivary and mammary gland in $p27^{-/-}$ mice compared with in $p27^{+/+}$ mice carrying the MMTV/v-Ha-*ras* transgene.[33]

In our experiment, due to the morphological characteristics of non-papillary and invasive BBN-induced mouse urinary bladder carcinomas,[27] we could not evaluate the numbers of tumors per mice. Even though the degree of muscle invasion or histology of carcinomas did not statistically differ with the p27 genotype status, the tumor size tended to be larger with the $p27^{-/-}$ genotype. p27 expression in both $p27^{+/-}$ and $p27^{+/+}$ mice was also decreased in BBN-induced urinary bladder carcinomas as compared with premalignant hyperplastic lesions, similar to the decrease observed earlier in F344 rats.[25] This fact points to the cross-species broad importance of p27 in the urinary bladder carcinogenesis.

Combined with the fact that p27 deficiency does not cause significant cell cycle enhancement and does increase tumor volume, our data suggest that the onset of the tumor might be earlier in $p27^{-/-}$ mice than $p27^{+/+}$ littermate. Thus, p27 may act mainly as a tumor suppressor in the early period of carcinogenesis.

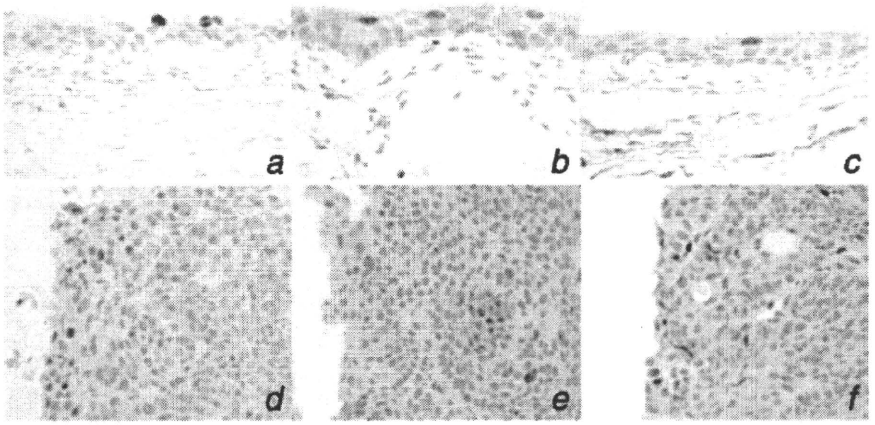

FIGURE 3 – Representative immunohistochemical staining for p21 in non-treated control urinary bladder (*a–c*) and BBN-induced carcinomas (*d–f*) of male mice. Note positive nuclear staining in normal umbrella cells and a few carcinoma cells with no obvious difference among p27 genotypes; null (–/–: *a*, *d*), hetero (+/–: *b*, *e*) and wild-type (+/+: *c*, *f*).

TABLE VI – INCIDENCE OF P53 MUTATION IN BBN-TREATED MICE URINARY BLADDER TISSUE AND MUTATION STATUS

Genotype of p27	Incidence (%)		Mutation status				
	Ca	PN	Case	Lesion	Exon	Codon	Base (amino acid) change
Male							
–/–	2/5 (40)	0	101	Ca (T2b)	6	213	GTG (Leu)→GCG (Ala)
					7	252	ATC (Ile)→AGC (Ser)
			105	Ca (T2b)	8	297	CCC (Pro)→CCT (Pro)
+/–	1/3 (33.3)	0/2	505	Ca (T2b)	7	245	CGC (Arg)→TGC (Cys)
					8	275	CCT (Pro)→ACT (Thr)
					8	301	GCA (Ala)→GGA (Gly)
+/+	1/3 (33.3)	0/2	903	Ca (T2a)	5	132	TGC (Cys)→TGG (Trp)
Female							
–/–	0/3	0/2					
+/–	0/1	0/1					
+/+	0	0					

Ca: carcinoma, PN: papillary or nodular hyperplasia, T2a: inner half muscular invasion, T2b: outer half muscular invasion.

p27 deficiency has been reported to be a key event in the pathogenesis of prostate cancer as indicated by the short latency and complete penetrance in $Pten^{+/-}$ mice.[34] Furthermore, in mice expressing the SV40 large T antigen under control of the androgen receptor dependent-probasin promoter, p27 deficiency did not increase the cell proliferation index or reduce the apoptotic index, but was associated with progression of prostate tumors from high-grade prostatic intraepithelial neoplasia to poorly differentiated carcinomas.[35] Germ-line mutations in p27 have been identified as responsible for a multiple endocrine neoplasia syndrome in rats and humans.[36] Altered expression of p21, another member of Cip/Kip family or its upstream factor, p53, were not observed in either $p27^{+/-}$ or $p27^{-/-}$ mice in the present study. It was also reported that knockdown of p27 with siRNA p21 in epithelial cells did not effect p21 expression, the BrdU-labeling indices or the percentage of cells entering S phase.[37] The rate for p53 mutations in urothelial carcinomas was not increased in our $p27^{-/-}$ mice, although loss of nuclear p27 in human colorectal cancer is reported to be correlated with microsatellite instability and CpG island methylator phenotype.[38] Therefore p27 may have tumor suppressive functions besides its role in cell cycle regulation and p53-p21 pathway. In the mouse urothelium, p27 is normally expressed in the nucleus, but there is accumulating evidence that cytoplasmic relocalization of p27 protein due to PKB/Akt-mediated phosphorylation, an alternative regulation pathway of p27 inactivation, plays an important role in human tumors, such as breast cancers[6–8] and acute myelogenous leukemia.[39] Recent study using $p27^{CK-}$ mice, which carries two point mutations both in the cyclin-binding domain and the CDK-binding domain, showed cytoplasmic localization of p27 was potentially oncogenic through a cyclin-CDK-independent function.[40] Thus we hypothesize that loss of nuclear p27 accelerates mice urinary bladder carcinogenesis, but the mechanistic pathway and impact of p27 expression may differ with the organ or depend on the status of other factors.

In conclusion, p27 deficiency appears to promote BBN-induced urinary bladder carcinogenesis in p27 knockout mice, in line with the poor prognosis of $p27^{-/-}$ clinical bladder carcinomas and this promotion may not simply be related to cell cycle acceleration.

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Cell

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

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DOI 10.1016/j.cell.2007.12.013

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

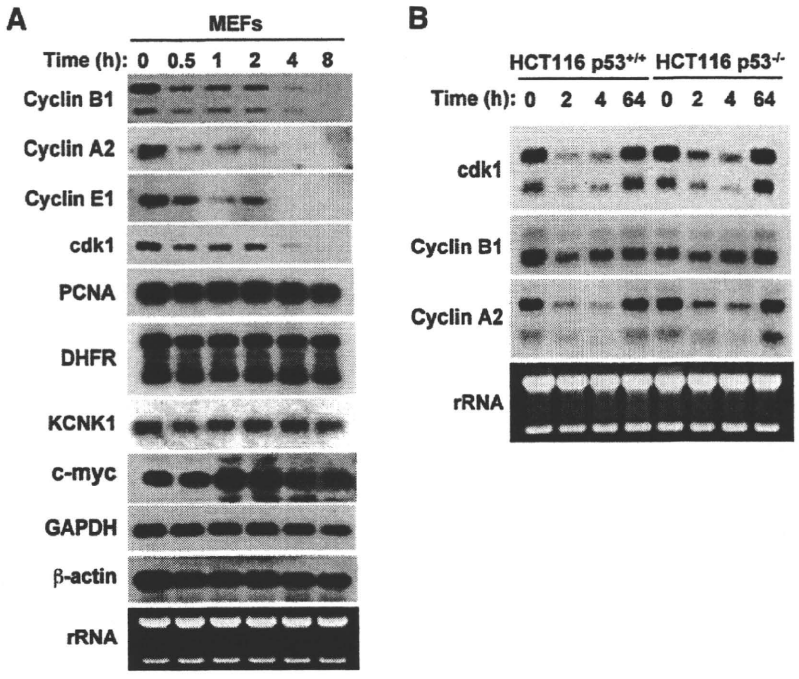

Figure 1. Transcriptional Repression of Cell-Cycle Regulatory Genes following UV Irradiation

(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

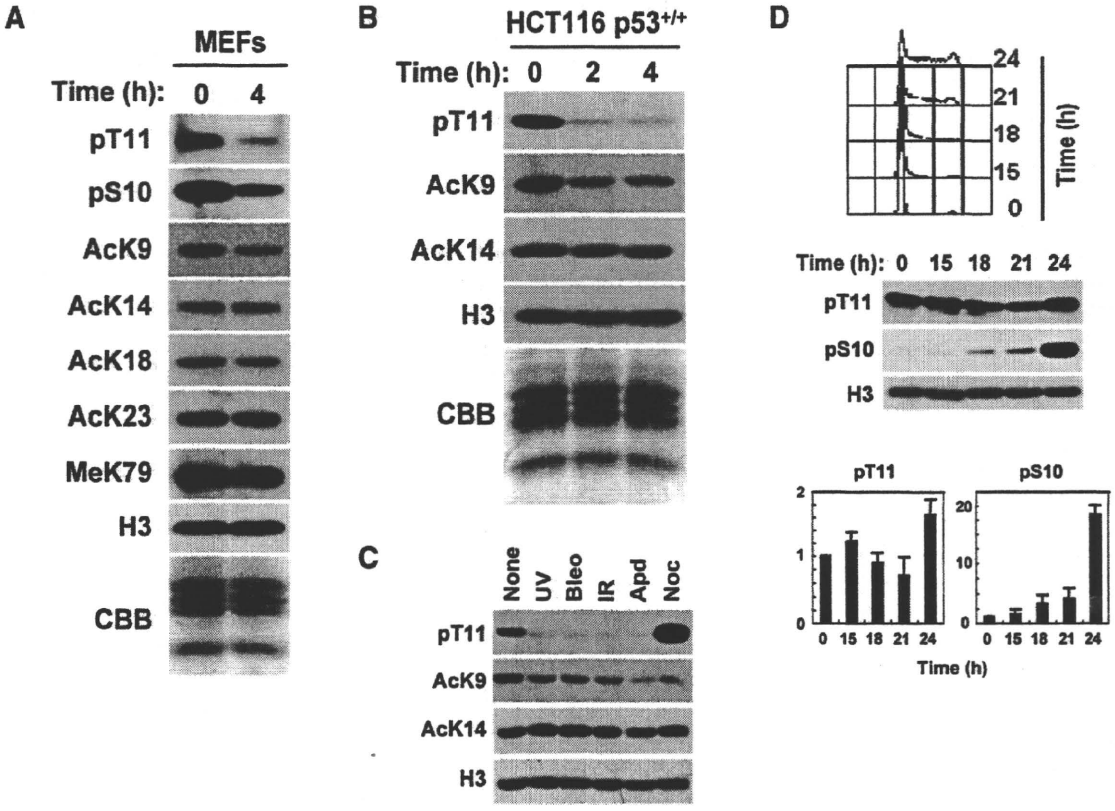

Figure 2. Changes to Core Histone Modifications following DNA Damage

(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

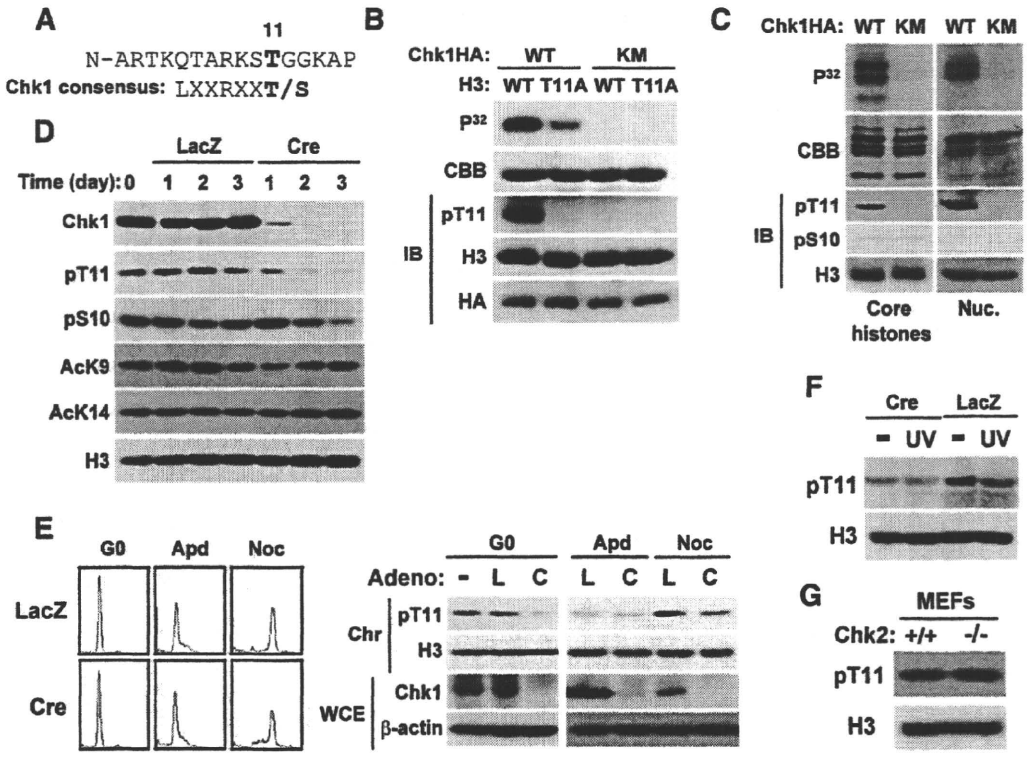

Figure 3. Chk1 Phosphorylates Histone H3-T11 Both In Vitro and In Vivo

(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.

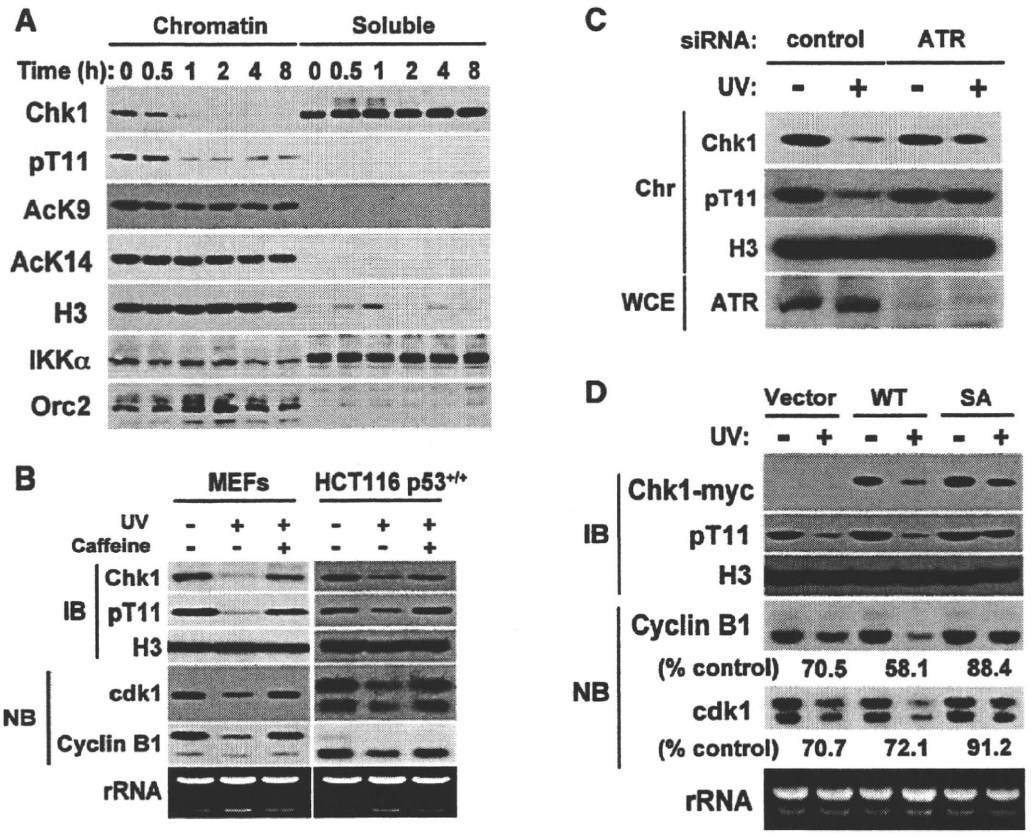

Figure 4. Release of Chk1 from Chromatin following DNA Damage Correlates with Decreased H3-T11 Phosphorylation

(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