

# ***GADD45*-induced cell cycle G2-M arrest associates with altered subcellular distribution of cyclin B1 and is independent of p38 kinase activity**

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**In response to DNA damage, the cell cycle checkpoint is an important biological event in maintaining genomic fidelity. *Gadd45*, a p53-regulated and DNA damage inducible protein, has recently been demonstrated to play a role in the G2-M checkpoint in response to DNA damage. In the current study, we further investigated the biochemical mechanism(s) involved in the *GADD45*-activated cell cycle G2-M arrest. Using the tetracycline-controlled system (tet-off), we established *GADD45*-inducible lines in HCT116 (wild-type p53) and Hela (inactivated p53 status) cells. Following inducible expression of the *Gadd45* protein, cell growth was strongly suppressed in both HCT116 and Hela cells. Interestingly, HCT116 cells revealed a significant G2-M arrest but Hela cells failed to arrest at the G2-M phases, indicating that the *GADD45*-activated G2-M arrest requires normal p53 function. The *GADD45*-induced G2-M arrest was observed independent of p38 kinase activity. Importantly, induction of *Gadd45* protein resulted in a reduction of nuclear cyclin B1 protein, whose nuclear localization is critical for the completion of G2-M transition. The reduced nuclear cyclin B1 levels correlated with inhibition of Cdc2/cyclin B1 kinase activity. Additionally, overexpression of cyclin B1 substantially abrogated the *GADD45*-induced cell growth suppression. Therefore, *GADD45* inhibition of Cdc2 kinase activity through alteration of cyclin B1 subcellular localization may be an essential step in the *GADD45*-induced cell cycle G2-M arrest and growth suppression. *Oncogene* (2002) 21, 8696–8704. doi:10.1038/sj.onc.1206034**

**Keywords:** p53; *GADD45*; G2-M arrest; cyclin B1

## **Introduction**

In response to DNA damage, mammalian cells arrest at the transition from G1 to S phase (G1-S checkpoint) and G2 to M phase (G2-M checkpoint) (Hartwell and Weinert, 1989). Cell cycle arrest at these checkpoints prevents DNA replication and mitosis in the presence of DNA damage. Inactivation of those cell cycle checkpoints results in genomic instability, which is closely associated with cell transformation and tumorigenesis. In addition, disruption of normal cell cycle controlling machinery often has dramatic consequences on therapeutic sensitivity (Elledge, 1996; Hartwell and Kastan, 1994; Kohn *et al.*, 1994; O'Connor and Kohn, 1992; Paulovich *et al.*, 1997).

Currently, the mechanism(s) by which DNA damaging agents activate cell cycle G1-S checkpoint is well understood. The tumor suppressor p53 gene plays a critical role in the control of G1-S arrest. Following DNA damage, p53 transcriptionally up-regulates p21 (el-Deiry *et al.*, 1993), one of the p53-downstream genes and a potent cell cycle-dependent kinase inhibitor. Subsequently, induced p21 forms complexes with Cdk-cyclin and inhibits the activity of cdk4-cyclin D, Cdk6-cyclin D, Cdk2-cyclin E, and Cdk2-cyclin A, and in turn transiently arrest cells at the G1-S transition (Harper *et al.*, 1993; Sherr and Roberts, 1995; Xiong *et al.*, 1993; Zhang *et al.*, 1994). It has been demonstrated that the disruption of endogenous p21 abrogates the G1-S checkpoint after cell exposure to DNA damage (Waldman *et al.*, 1995). P53 has also been implicated in the control of the G2-M checkpoint. Introduction of p53 into p53-deficient human fibroblasts results in both G1-S and G2-M arrest (Agarwal *et al.*, 1995; Stewart *et al.*, 1995) and the HPV-16 E6 viral oncoprotein, which blocks p53 function, has been shown to decrease the stringency of the mitotic checkpoint (Thompson *et al.*, 1997). Recent evidence indicates that p53 and p21 are required for maintaining the G2 checkpoint in human HCT116 cells (Bunz *et al.*, 1998). In addition, 14-3-3, which blocks Cdc25 activity and arrests cells at the G2-M transition, is demonstrated as one of the p53 downstream genes

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Received 3 June 2002; revised 30 August 2002; accepted 5 September 2002

(Hermeking *et al.*, 1997). Most recently, *GADD45*, a p53-regulated and DNA damage-inducible gene, is found to play an important role in the G2-M checkpoint in response to certain types of DNA damaging agents (Jin *et al.*, 2000; Wang *et al.*, 1999; Zhan *et al.*, 1999).

However, the G2-M checkpoint is complex and may involve redundant controls including both p53-independent and p53-dependent mechanisms. It has been well accepted that many of the G2-M regulators appear to ultimately target Cdc2, a protein kinase required for the mitotic entry in mammalian cells (Elledge, 1996; O'Connor, 1997). Activation of Cdc2 kinase requires its association with mitotic cyclins (cyclin B1 and cyclin A), and phosphorylation at Thr-161 and dephosphorylation at Thr-14/Tyr-15 cells (Ducommun *et al.*, 1991; Elledge, 1996; O'Connor, 1997). After DNA damage, several G2-M regulators, including Chk1, Chk2, 14-3-3 and ATM, alter Cdc2 activity by inhibiting dephosphorylation of Cdc25C phosphatase. The inhibition of Cdc25C activity prevents the removal of inhibitory phosphorylations from Thr-14 and Tyr-15 of Cdc2 (Elledge, 1996; O'Connor and Fan, 1996; Paulovich *et al.*, 1997). In addition, DNA damage is able to suppress Cdc2 activity by inhibiting the accumulation of cyclin B1 mRNA and protein (Bernhard *et al.*, 1995; Muschel *et al.*, 1991, 1992). Delayed entry into mitosis following DNA damage also correlates with nuclear exclusion of cyclin B1 protein (Toyoshima *et al.*, 1998).

The *GADD45* gene is induced by a variety of DNA damaging agents, including ionizing radiation (IR), methyl methanesulfonate (MMS), UV radiation (UV), hydroxyurea and medium starvation (Fornace *et al.*, 1988, 1989; Papathanasiou *et al.*, 1991). The IR-induction of *GADD45* is transcriptionally regulated by p53 via a p53-binding site in the third intron (Kastan *et al.*, 1992; Zhan *et al.*, 1994a). In contrast, *GADD45* induction by UV radiation or MMS treatment is detected in all mammalian cells regardless of p53 status. However, recent evidence shows that p53 can still contribute to cellular responses to UV, MMS and medium starvation although it is not required (Zhan *et al.*, 1996, 1998). Gadd45 is a nuclear protein and binds to multiple important cellular proteins such as proliferating cell nuclear antigen (PCNA) (Hall *et al.*, 1995; Smith *et al.*, 1994), p21 protein (Chen *et al.*, 1996; Kearsey *et al.*, 1995; Zhao *et al.*, 2000), core histone protein (Carrier *et al.*, 1999), MTK/MEKK4 (Takekawa and Saito, 1998), an upstream activator of the JNK pathway, and Cdc2 protein kinase. The presence of Gadd45 in these complexes indicates that Gadd45 may be an important player in cell cycle control, DNA repair and the regulation of signaling pathway. The role of *GADD45* in maintaining genomic stability has been demonstrated by the recent finding that the mouse embryonic fibroblasts (MEF), derived from *gadd45*-null mice exhibit aneuploidy, chromosomal aberrations, gene amplification and centrosome amplification. Additionally, *gadd45*-knockout mice

display increased radiation carcinogenesis (Hollander *et al.*, 1999). In this study, we have further investigated the role of *GADD45* in the G2-M checkpoint and demonstrated that the *GADD45*-induced G2-M arrest depends on normal cellular p53 function, but is independent of p38 kinase activity, which is reported to be required for the initiation of the G2-M checkpoint after UV radiation. In addition, inducible expression of Gadd45 protein has been shown to result in alterations of cyclin B1 subcellular distribution, which might be a consequence of the interaction of Gadd45 with Cdc2 proteins.

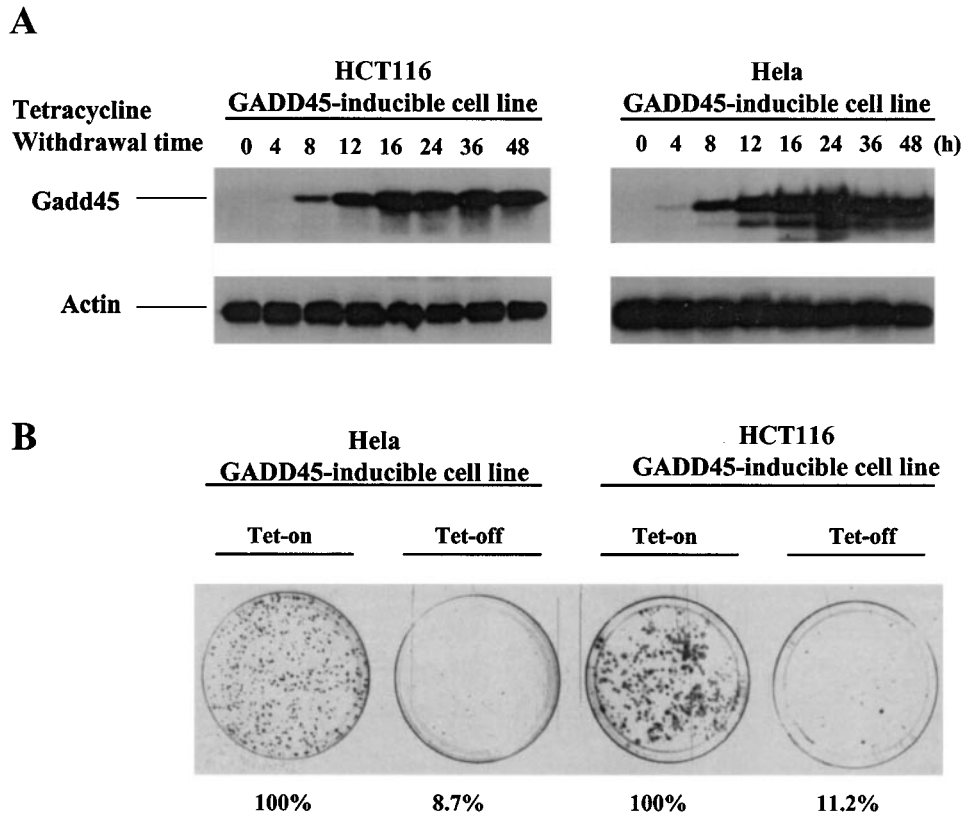
## Results

### *Inducible expression of Gadd45 protein suppresses human cell growth*

To further investigate the biological mechanism(s) by which *GADD45* plays a role in the control of cell cycle regulation, we established tetracycline-regulated *GADD45*-inducible cell lines in human cervical cancer HeLa cells (see Materials and methods), where cellular p53 function is inactivated, and human colorectal carcinoma HCT116 cells, which has wild-type p53 and normal p53 function. As shown in Figure 1a, both HeLa *GADD45*-inducible cells and HCT116 *GADD45*-inducible cells exhibited extremely low basal levels of the endogenous Gadd45 protein. Following withdrawal of tetracycline, Gadd45 protein was greatly induced and presented more than 10-fold induction in both cell lines. Next, the effect of Gadd45 protein on growth suppression was examined in these two *GADD45*-inducible cell lines. To perform this experiment, 500, 1000 or 2000 cells were seeded and grown in DMEM medium at 100-cm dishes 16 h prior to tetracycline withdrawal. After removing tetracycline, cells continued to grow for 14 days and then were fixed, scored for colonies containing more than 50 cells. Similar to our previous finding that overexpression of *GADD45* protein via transient transfection inhibits tumor cell growth (Zhan *et al.*, 1994b), inducible expression of Gadd45 protein in both HeLa and HCT116 lines strongly suppressed colony formation (Figure. 1B), indicating a suppressive role of *GADD45* in cell growth regardless of p53 status. In agreement with this observation, both HeLa and HCT116 lines with inducible expression of Gadd45 protein exhibited a substantial slow growth rate (results not shown). Taken together, these results indicate that *GADD45* plays a negative role in the control of cell progression.

### *GADD45 induced cell cycle G2-M arrest depends on normal cellular p53 function*

In order to further determine the role of *GADD45* in the control of cell cycle G2-M arrest, cell cycle distribution analyses were conducted in both HCT116 and HeLa *GADD45*-inducible cell lines. Following removal of tetracycline, *GADD45*-inducible cells were collected at 24 h or 36 h and subject to flow cytometry



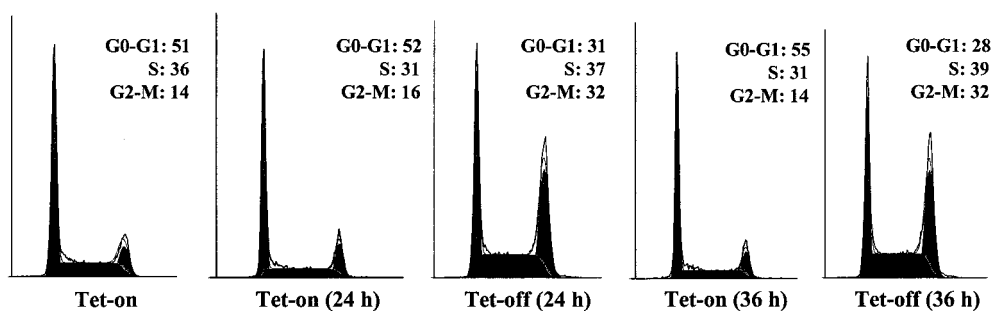
**Figure 1** Tumor cell growth suppression by Gadd45 protein. (a) Induction of Gadd45 protein in HCT116 and Hela cells controlled by the Tet-off system. HCT116 *GADD45*-inducible lines and Hela *GADD45*-inducible cell lines were established as described in Materials and methods. Cells were placed in 100 mm dishes at a density of  $4 \times 10^5$  and grown in DMEM medium containing tetracycline at a concentration of  $2 \mu\text{g/ml}$ . After withdrawal of tetracycline, cells were collected at the indicated time points for preparation of cellular protein.  $100 \mu\text{g}$  of whole cell protein was used for immunoblotting analysis with anti-*GADD45* antibody. As a loading control, anti actin antibody was included. (b) Induction of Gadd45 protein suppresses cell growth. HCT116 and Hela *GADD45*-inducible cells were seeded at a density of 1000 cells per 100 mm dish and grown in medium containing  $2 \mu\text{g/ml}$  of tetracycline. After 16 h, medium was removed and plates were washed three times with PBS, then fresh medium containing no tetracycline was added into plates. The cells were fixed and stained at 14 days and scored for colonies containing at least 50 cells. The experiments were performed four times and only representative results were shown here

analysis. The results presented in Figure 2 depict a representative profile of cell cycle distribution in those cells. In the HCT116 *GADD45*-inducible line (wt p53 status), cells grown in the presence of tetracycline presented 14–16% population in G2-M phase. However, inducible expression of Gadd45 protein resulted in a clear accumulation of the G2-M fraction. Approximately 32% of the cells were arrested at the G2-M phase of the cell cycle in the absence of tetracycline, indicating that *GADD45* expression alone is able to halt cells in G2-M phase. In contrast, after inducible expression of Gadd45, Hela cells (inactivated p53 status) did not exhibit any evident changes of cell cycle distribution. In consistence with these results, introduction of *GADD45* expression vector into HCT116 via transient transfection resulted in increased G2-M population in HCT116 but not in HCT116 p53<sup>-/-</sup>, where p53 alleles were knocked out by homologous recombination approach (result not shown). These observations further demonstrate that *GADD45*-mediated G2-M arrest requires normal cellular p53 function.

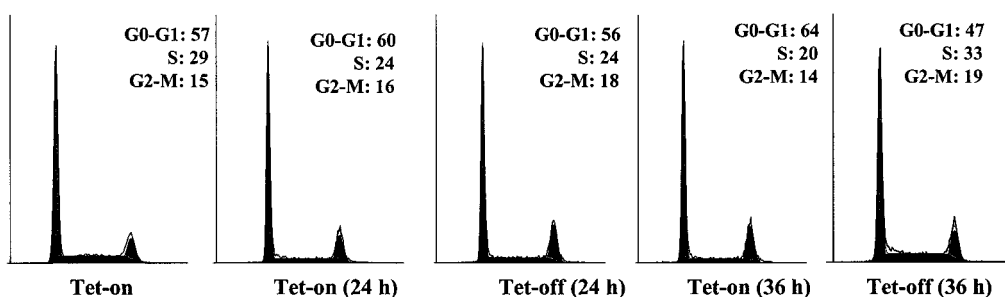
#### *GADD45* induced cell cycle G2-M arrest is not affected by inhibitors of p38

The mitogen-activated protein kinase p38 has recently been reported to play a critical role in cell cycle G2-M checkpoint in response to UV radiation (Bulavin *et al.*, 2001). To understand whether p38 kinase activation contributes to the *GADD45*-induced G2-M arrest, the mitotic index was measured in *GADD45* inducible cells in the presence of p38 kinase inhibitor, SB202190. In Figure 3a, high mitotic indices were observed in HCT116 cells treated with nocodazole. In response to UV radiation, mitotic indices substantially decreased, indicating that UV treatment arrests cells in the G-M transition. Addition of p38 inhibitor SB202190 at a concentration of  $10 \mu\text{M}$  was shown to greatly attenuate the UV-induced G2-M arrest. In Figure 3b, inducible expression of Gadd45 protein exhibited low mitotic indices, which reflects a significant G2-M arrest by Gadd45. However, p38 inhibitor SB202190 ( $10 \mu\text{M}$ ) showed little effect on the Gadd45-induced G2-M arrest. These results suggest that the *GADD45*

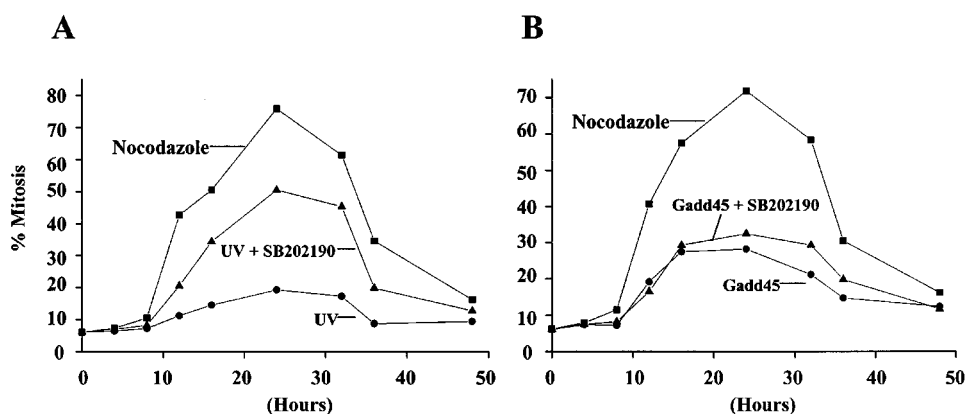
### HCT116 Gadd45-inducible cell line



### Hela Gadd45-inducible cell line



**Figure 2** Cell cycle G2-M arrest following inducible expression of Gadd45 protein in both HCT116 and Hela cells. HCT116 and Hela *GADD45*-inducible cells were grown in DMEM medium with 10% fetal bovine serum in the presence of tetracycline at a concentration of 2  $\mu\text{g/ml}$ . After withdrawal of tetracycline, cells were collected at the indicated time points and subject to flow cytometric analysis as described in Materials and methods



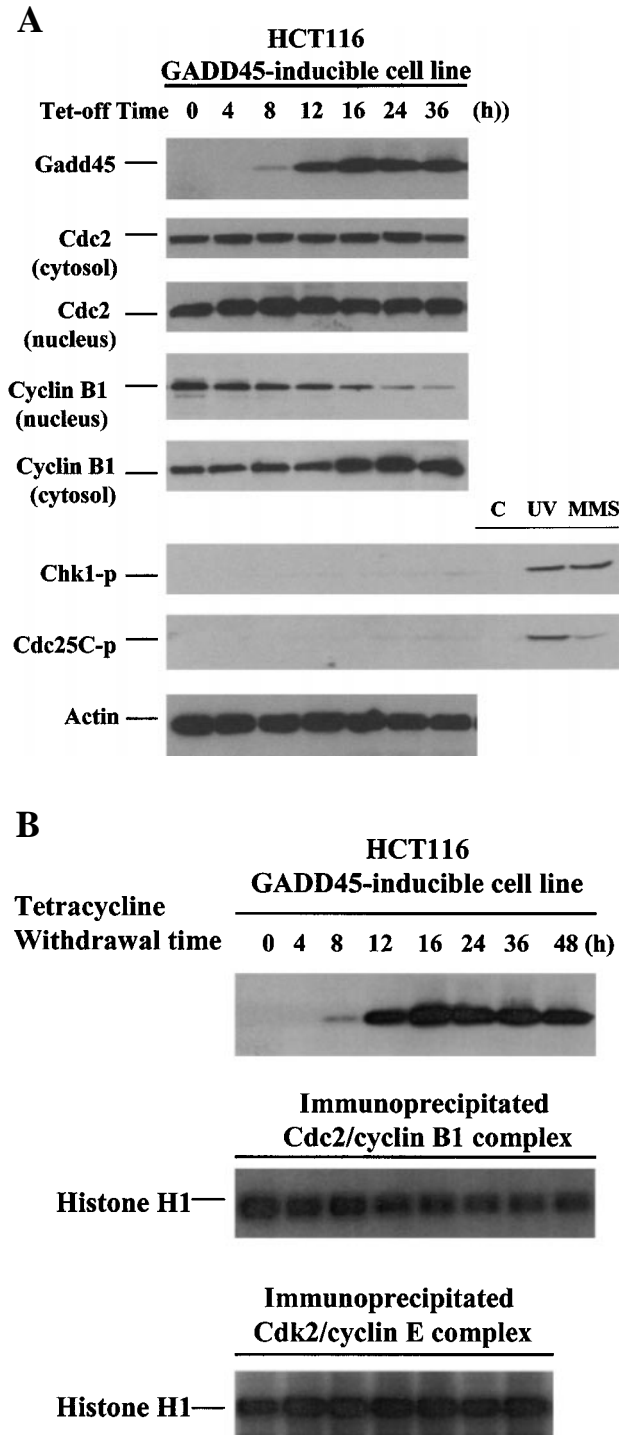
**Figure 3** Mitotic entry after UV radiation or inducible expression of Gadd45 protein. (a) HCT116 cells were UV irradiated in the presence of 10  $\mu\text{M}$  p38 kinase inhibitor SB203580 and mitotic indices were determined as described in Materials and methods. (b) HCT116 *GADD45*-inducible cells were grown in medium with tetracycline (2  $\mu\text{g/ml}$ ). Upon the withdrawal of tetracycline, cells were exposed to 10  $\mu\text{M}$  p38 kinase inhibitor SB203580 and followed by determination of mitotic indices at the indicated time points

induction of cell cycle G2-M checkpoint does not require activation of p38 kinase.

*Expression of Gadd45 protein alters the level of nuclear cyclin B1 but does not affect phosphorylation statuses of Cdc25C or Chk1*

In our previous report, we have demonstrated that Gadd45 protein physically interacts with Cdc2 kinase,

dissociates Cdc2/cyclin B1 complexes and in turn inhibits Cdc2 kinase activity, but does not alter Cdc2 phosphorylation status (Zhan *et al.*, 1999). However, the biochemical consequence of the interaction between Gadd45 and Cdc2 remains to be further defined. Since nuclear localization of cyclin B1 protein is thought to be critical for the completion of G2-M transition, we further examined cyclin B1 protein distributions in both the nucleus and cytoplasm. As shown in Figure

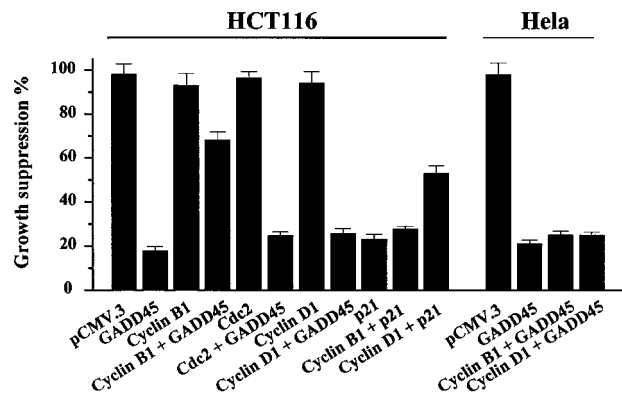


**Figure 4** Subcellular localization of cyclin B1 protein and inhibition of Cdc2 kinase activity following inducible expression of Gadd45. (a) Cellular proteins were prepared from HCT116 *GADD45*-inducible cells after withdrawal of tetracycline at the indicated time points. 100  $\mu$ g of proteins were loaded onto SDS-PAGE gel for detection of subcellular distribution of cyclin B1 protein and phosphorylations of CHK1 or Cdc25C. (b) One mg of cellular proteins isolated from HCT116 *GADD45*-inducible cells at the indicated time points was immunoprecipitated with anti-cyclin B1 or cyclin E antibodies, and histone H1 kinase assays were performed as described in Materials and methods. Labeled histone H1 was detected by autoradiography following size separation on a SDS-PAGE gel

4a, following inducible expression of Gadd45 protein in HCT116 cells, there were no evident alterations of Cdc2 protein in both the cytosol and nuclear compartments. Interestingly, nuclear cyclin B1 protein exhibited a significant reduction in response to induction of Gadd45. In support of this observation, cytosol cyclin B1 appeared to increase after Gadd45 induction. These results indicate that Gadd45 induction caused subcellular redistribution of cyclin B1 protein. In the same experiment, Chk1 and Cdc25C phosphorylation statuses were also examined, but no phosphorylations of Chk2 or Cdc25C were detected following Gadd45 induction. However, cells treated with UV and MMS displayed increased phosphorylations for Chk2 and Cdc25C. Additionally, we analysed Cdc2 and Cdk2 kinase activity following Gadd45 protein expression and found Cdc2 was inhibited by Gadd45 but Cdk2 kinase activity remained at the similar levels after Gadd45 expression (Figure 4b). Taken together, Gadd45 protein is able to alter cyclin B1 nuclear localization and in turn inhibits Cdc2 kinase activity.

*Cyclin B1 abrogates the GADD45-induced cell growth suppression*

We have previously demonstrated that *GADD45*-induced growth suppression (Zhan *et al.*, 1994b), in a great content, correlates with its inhibition of Cdc2/cyclin B1 kinase activity. It is assumed that interaction of Gadd45 with Cdc2 causes dissociation of the Cdc2/cyclin B1 complex, and in turn alters subcellular localization of cyclin B1, which contributes to the loss of Cdc2 kinase activity. Therefore, we examined whether introduction of cyclin B1 into cells can rescue *GADD45*-induced growth-suppression. To do this, a *GADD45* expression vector was co-transfected with expression vectors for cyclin B1, Cdc2, or cyclin D1 into HCT116 cells (p53 wt line). In Figure 5, expression of *GADD45* in HCT116 cells



**Figure 5** Effect of cyclin B1 expression on the *GADD45*-induced cell growth suppression. Human colorectal carcinoma HCT116 cells were transfected with the indicated expression vectors. Following selection with G418 for 2 weeks, cells were fixed and the colonies that contained at least 50 cells were counted. Quantitative results represent the average of three individual experiments

suppressed 80% cell growth. While in the presence of cyclin B1 overexpression, *GADD45* only generated 30% growth suppression, indicating that cyclin B1 is able to abrogate *GADD45*-induced growth inhibition. In contrast, overexpression of both Cdc2 and cyclin D1 failed to rescue the *GADD45*-induced growth suppression. Additionally, cyclin B1 had little effect on p21, suggesting that p21-induced cell growth might not be mainly through its role in the G2-M arrest. Interestingly, *GADD45* was able to suppress cell growth in Hela cells, which contains inactivated p53 and does not exhibit *GADD45*-induced G2-M arrest. However, both cyclin B1 and cyclin D1 were incapable of abrogating *GADD45*-induced cell growth, indicating *GADD45* inhibits cell growth in cells with abnormal p53 probably through a different mechanism distinct from the inhibition of Cdc2 kinase activity in p53 wt cell lines.

## Discussion

In this report, we further investigated the role of Gadd45, a p53-regulated and stress-inducible protein, in the control of cell cycle G2-M checkpoint. Using tetracycline-controlling system (tet-off), we established *GADD45*-inducible lines in both HCT116 (wt p53) and Hela (negative p53 status) cells. Therefore, induction of Gadd45 protein was nicely manipulated by the withdrawal of tetracycline. Following inducible expression of Gadd45, cell growth was strongly inhibited in both HCT116 and Hela lines. In consistence with our previous finding that the introduction of *GADD45* expression vector into human normal fibroblast via microinjection approach causes cells to arrest at the G2-M transition, induction of Gadd45 protein in the HCT116 *GADD45*-inducible line greatly increased cell population in G2-M phase, but Gadd45 expression was unable to induce G2-M arrest in Hela cells, which contain inactivated p53. The *GADD45*-induced G2-M arrest appeared independent of p38 kinase activity, as employment of p38 kinase inhibitor (SB202190) did not abrogate *GADD45*-induced G2-M arrest. More importantly, overexpression of Gadd45 protein was shown to result in reduction of nuclear cyclin B1 protein and inhibited Cdc2 kinase activity, but had no effect on Chk1, Cdc25C phosphorylation and Cdk2 activity. In addition, co-introduction of cyclin B1 expression vector was able to substantially disrupt the *GADD45*-induced growth suppression.

The tumor suppressor p53 gene has been implicated in the control of cell cycle checkpoint in response to genotoxic stress (Bunz *et al.*, 1998, 1999; Kastan *et al.*, 1991, 1992). The role for p53 in G1-S arrest is clearly shown to be mediated through p21 (Harper *et al.*, 1993; Sherr and Roberts, 1995; Xiong *et al.*, 1993; Zhang *et al.*, 1994). However, the role of p53 in the control of the G2-M arrest is under debate and remains to be further elucidated. It is postulated that as one of the p53-targeted genes (Kastan *et al.*, 1992; Zhan *et al.*, 1994a), *GADD45* might be a strong player in mediating

p53-regulated cell cycle G2-M checkpoint. Previous studies have shown that Gadd45 protein interacts with Cdc2 and dissociates the Cdc2/cyclin B1 complex (Jin *et al.*, 2000; Zhan *et al.*, 1999). Subsequently, 'free' cyclin B1 protein dissociated from the Cdc2 complex is more likely pumped out from the nucleus, probably by the nuclear transport system. As a result of exclusion of cyclin B1 protein from the nucleus, Cdc2 kinase activity is inhibited and followed up by the cell cycle G2-M arrest. This goes along with the finding by Toyoshima *et al.* (1998) that DNA damage causes increased nuclear export of cyclin B1 and in turn arrests cells at the G2-M transition. Our observations that inducible expression of *GADD45* protein alters cyclin B1 nuclear localization (Figure 4) have suggested that exclusion of nuclear cyclin B1 protein by Gadd45 might be an essential step for the *GADD45*-induced G2-M arrest. Therefore, the findings in this work have further presented the precise evidence that the p53-*GADD45* pathway is well involved in the control of G2-M arrest.

The mechanism(s) for p53 dependence of the *GADD45*-induced cell cycle G2-M arrest is not clear at the present time. Bunz *et al.* (1998) has reported that cells with disrupted p53 display an impaired G2-M checkpoint after DNA damage, and suggested that the role for p53 in sustaining G2-M arrest after DNA damage might be mediated through p21. However, our previous investigations have already demonstrated that p21 is not required for *GADD45*-induced G2-M arrest, since introduction of *GADD45* expression vector into p21 deficient cells, where endogenous p21 has been disrupted, is able to generate G2-M arrest (Wang *et al.*, 1999). We have also not found any alterations of MDM2 protein level following Gadd45 induction and no physical interactions between Gadd45 and MDM2 proteins (result not shown). Therefore, both p21 and MDM2 appear not to be the candidates to mediate the role for p53 in *GADD45*-induced G2-M arrest. Future investigation is required to explore the mechanism by which p53 is required for the *GADD45*-induced G2-M arrest.

The mitogen-activated kinase p38 is required for initiating the G2-M checkpoint after UV radiation, probably through phosphorylating Cdc25B at serines 309 and 361 (Bulavin *et al.*, 2001). However, the *GADD45*-induced G2-M arrest is independent of p38 kinase activity. These results have further confirmed that Gadd45 acts at the late G2-M transition or early mitotic phase, instead of at the initiation of G2-M transition. In addition, the inhibitory effect of the Gadd45 protein appears to be specifically localized on Cdc2/cyclin B1 complex, as induction of Gadd45 protein does not alter phosphorylations of Chk1 and Cdc25C. Overexpression of cyclin B1 protein has been found in certain types of human tumors although the biological function of this overexpressed protein in tumorigenesis remains unclear (Soria *et al.*, 2000). Interestingly, Overexpression of cyclin B1 is closely associated with loss of a p53 function (Yu *et al.*, 2002). In Figure 5, co-expression of cyclin B1 with Gadd45

protein abrogated the Gadd45-induced cell growth suppression. This evidence has provided a new insight into understanding on the role of cyclin B1 in development of genomic instability and tumorigenesis.

*GADD45* was shown to suppress cell growth in both HCT116 (wt p53) and HeLa (inactivated p53) cells, regardless of p53 status (Figure 5). However, *GADD45* only generated G2-M arrest in HCT116 cells, but not in HeLa cells, suggesting that the *GADD45*-induced growth suppression is complex and might involve the biological events distinct from the G2-M arrest. In fact, Takekawa and Saito (1998) have previously reported that *GADD45* interacts with MTK1/MEKK4, an upstream activator of the JNK pathways, and induced apoptosis in HeLa cells. Therefore, *GADD45* is able to play a negative role in cell growth probably through both cell cycle arrest and apoptosis. The importance of *GADD45* in maintenance of genomic fidelity has been presented by the evidence that gadd45-null mice generated by gene targeting exhibit aneuploidy, chromosome aberrations, gene amplification and centrosome amplification, and increased tumorigenesis after DNA damaging agents (Hollander *et al.*, 1999). Therefore, the current studies have further demonstrated the mechanism(s) by which *GADD45* plays a role in maintaining genomic stability and provides insight into understanding the p53-*GADD45* pathway in cellular response to genotoxic stress.

## Materials and methods

### *Establishment of the GADD45 inducible cell line and cell culture*

To establish *GADD45* tet-off inducible cell lines, human colorectal carcinoma HCT116 cells were initially transfected with pTet-Off plasmid (Clontech, Palo Alto, CA, USA), which is commercially available and expresses the tTA regulator proteins, and the G418-resistant colonies were selected and amplified. Next, the cells expressing tTA proteins were subject to second round transfection with pTRE-*GADD45* construct, where the *GADD45* gene was inserted into *Bam*H1/*Hind*III sites of pTRE plasmid (Clontech). The cells transfected with pTRE-*GADD45* plasmid were selected by hygromycin at 200  $\mu$ g/ml for 14 days, and each hygromycin-resistant colony was separately collected for detection of Gadd45 protein expression under tet-off system. In the case of HeLa *GADD45* inducible lines, HeLa cells expressing tTA were commercially obtained from Clontech and transfected with pTRE-*GADD45* construct and the hygromycin-resistant cells were selected as described in HCT116 cells. *GADD45*-inducible cells were grown in DMEM medium supplemented with 10% fetal bovine in the presence of tetracycline at a concentration of 2  $\mu$ g/ml. To induce expression of Gadd45 protein, DMEM containing tetracycline was removed and the plates were washed four times with PBS, and fresh DMEM medium containing no tetracycline was then added to cells. Cells were collected at the indicated time points for examination of induced Gadd45 protein.

### *Antibodies and immunoblotting analysis*

The following antibodies were used in the experiments; *GADD45*, Cdc2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), cyclin B1 (Pharmingen, San Diego, CA, USA)

and Cdc25C and Chk1 phosphorylation-sites specific antibodies (Cell Signaling Technology Inc, Beverly, MA, USA). *GADD45*-inducible cells were exponentially grown in DMEM medium containing tetracycline at a concentration of 2  $\mu$ g/ml. After withdrawal of tetracycline, cells were collected at the indicated time. For preparation of cellular protein, plates were rinsed with PBS and cells were lysed in PBS containing 100  $\mu$ g/ml phenyl-methylsulfonyl fluoride, 2  $\mu$ g/ml aprotinin, 2  $\mu$ g/ml leupeptin and 1% NP-40 (lysis buffer). Lysates were collected by scraping and cleared by centrifugation at 4°C. 100  $\mu$ g of cellular protein was loaded onto 12% SDS-PAGE gel and transferred to Protran membranes. Membranes were blocked for 1 h at room temperature in 5% milk, washed with PBST (PBS with 0.1% Tween-20), and incubated with indicated antibodies for 2 h. Membranes were washed four times in PBST and HRP-conjugated anti-mouse antibody was added at 1:4000 in 5% milk. After 1 h, membranes were washed and detected by ECL (Amersham, Arlington Height, IL, USA) and exposed to X-ray film (Kodak, Rochester, NY, USA).

### *Growth suppression assay*

Five hundred, 1000, or 2000 cells from HeLa or HCT116 *GADD45*-inducible lines were seeded in 100-cm dishes and grown in DMEM medium containing 2  $\mu$ g/ml tetracycline for 16 h. Following withdrawal of tetracycline, cells were fixed at 14 days and scored for colonies containing at least 50 cells (Zhan *et al.*, 1994b).

### *Cdc2 and Cdk2 kinase assays*

Cellular lysates isolated from the *GADD45*-inducible cells were incubated with 10  $\mu$ l of cyclin B1 antibody (Pharmingen) or 20  $\mu$ l of Cdk2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and 20  $\mu$ l of protein A/G agarose beads (Santa Cruz Biotechnology) at 4°C for 6 h. Immuno-complexes were washed four times with lysis buffer and followed by kinase buffer. Histone H1 kinase assays were then performed in the presence of 10  $\mu$ g of histone H1 (Upstate Biotechnology, Lake Placid, NY, USA), 15 mM MgCl<sub>2</sub>, 7 mM  $\beta$ -glycerol phosphate, 1.5 mM EDTA, 0.25 mM sodium orthovanadate, 0.25 mM DTT and 10  $\mu$ Ci of  $\gamma$ -<sup>32</sup>P ATP in 30  $\mu$ l volume. After 15 min at 30°C, the reactions were mixed with an equal amount of standard 2 $\times$ SDS protein denature loading buffer, sized-separated on a 12% SDS-PAGE gel (Zhan *et al.*, 1999).

### *Flow cytometry analysis*

HCT116 and HeLa *GADD45*-inducible cells were plated into 100-mm dishes at a density of  $6 \times 10^5$  and grown in DMEM containing 2  $\mu$ g/ml of tetracycline. Sixteen hours later, medium was removed and plates were washed four times followed by addition of fresh medium. After incubation for 36 h, cells were collected, washed with PBS, fixed with 70% ethanol for 2 h at 4°C. Cells were then incubated with RNase (10  $\mu$ g/ml) for 30 min and stained with propidium iodine (Sigma; 50  $\mu$ g/ml). Cell cycle analysis was performed using Becton Dickson fluorescence-activated cell analyzer. At least 10,000 FITC positive cells were analysed using CellQuest and Modfit programs (Wang *et al.*, 1999).

### *Analysis of mitotic index in HCT116 GADD45-inducible cells*

HCT116 *GADD45*-inducible cells were seeded at a density of  $6 \times 10^5$  in DMEM containing 2  $\mu$ g/ml of tetracycline.

Following withdrawal of tetracycline, cells were grown in the presence of 10  $\mu$ M p38 kinase inhibitor SB203580 and harvested at the indicated time points, fixed in methanol:acetic acid (3:1), spread on glass microscope slides, air-dried and stained with 5% Giemsa. Nuclei exhibiting condensed, evenly staining chromosomes were scored as mitotic. At least 1000 cells were counted in each determination. Meanwhile, HCT116 cells treated with p38 kinase inhibitor SB203580

were exposed to UV radiation and subjected to analysis of mitotic index.

#### Acknowledgments

This study was supported in part by National Institutes of Health Grant R01 CA-83874.

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