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ORIGINAL ARTICLE

CDC20, a potential cancer therapeutic target, is negatively regulated by p53

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The p53 protein inhibits malignant transformation through direct and indirect regulation of transcription of many genes related to cell cycle, apoptosis and cellular senescence. A number of genes induced by p53 have been well characterized, but biological significance of genes whose expression was suppressed by p53 is still largely undisclosed. To clarify the roles of p53-suppressive genes in carcinogenesis, we analysed two data sets of whole-genome expression profiles, one for cells in which wild-type p53 was exogenously introduced and the other for a large number of clinical cancer tissues. Here, we identified CDC20 that was frequently upregulated in many types of malignancies and remarkably suppressed by ectopic introduction of p53. CDC20 expression was suppressed by genotoxic stresses in p53- and p21-dependent manners through CDE-CHR elements in the *CDC20* promoter. Furthermore, small interference RNA (siRNA)-mediated silencing of p53 induced CDC20 expression in normal human dermal fibroblast cells. As we expected, treatment of cancer cells with siRNA against *CDC20* induced G_2/M arrest and suppressed cell growth. Our results indicate that p53 inhibits tumor cell growth through the indirect regulation of CDC20 and that CDC20 might be a good potential therapeutic target for a broad spectrum of human cancer.
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Introduction

The p53 tumor suppressor gene is mutated or inactivated in a majority of human cancers (Hollstein *et al*., 1994; Soussi *et al*., 2006). The p53 protein is induced and activated at nucleus by a variety of genotoxic stresses such as DNA damage, hypoxia, oxidative stress and heat shock (Vogelstein *et al*., 2000). Activated p53 protein directly and indirectly regulates transcription of many genes that are involved in cell cycle, apoptosis and cellular senescence, and subsequently inhibits malignant transformation and tumor progression. Therefore isolation of genes that are regulated by p53 provides critical information central to the biological and physiological functions of the p53 (Nakamura, 2004). We have screened through various approaches and identified a number of p53-inducible genes such as p53R2, p53AIP1 and p53RDL1, and revealed the mechanisms how p53 regulates cell death as well as cell survival by balancing the expressions of these downstream target genes (Oda *et al*., 2000; Tanaka *et al*., 2000; Tanikawa *et al*., 2003). Dozens of genes, including those identified by our group, that are induced by p53 have been isolated and well characterized so far. In addition, many genes that are negatively regulated by p53 such as Survivin, CDC2 and CDC25C have been reported (Taylor *et al*., 2001; Hoffman *et al*., 2002). However, the biological significance of genes repressed by p53 is still largely undisclosed.

Systematic analysis of thousands of genes by means of cDNA microarray is an effective approach to identify genes that are involved in p53-related pathways. Our previous microarray analysis identified approximately 100 genes were upregulated or downregulated by p53. Among them, we considered those showing altered expressions in clinical cancer tissues likely to play significant roles in development/progression of human cancer. For example, one of p53-target genes, MDM2, is upregulated in cancer tissues and exhibits oncogenic function through regulation of p53 degradation (Zhou *et al*., 1995). Therefore, the expression analysis of candidate p53-target genes in cancer tissues offers valuable information to narrow down a subset of genes playing significant roles in human carcinogenesis.

Genes that were inactivated by p53 and were frequently upregulated in human cancer were considered to be good molecular targets for drug development. In fact, Survivin that is suppressed by p53 is highly expressed in various cancer tissues and specific inhibitors for it are developed for clinical application (Zaffaroni *et al*., 2005). Since we have been analysing gene expression profiles of various cancer tissues using

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cDNA microarray, we compared the gene expression profile data for cells in which p53 gene was exogenously introduced and those for various clinical cancers. We here report CDC20 that was remarkably suppressed by p53 introduction and was upregulated in a wide variety of human cancer tissues. CDC20, a homolog of *Saccharomyces cerevisiae* cell division cycle 20 protein, is one of the most important factors that control the spindle checkpoint (Fang *et al*., 1998). In this study we clarified the mechanism that p53 suppresses cell growth by regulation of CDC20 expression. Our findings indicate that CDC20 may be a promising cancer therapeutic target for development of a novel cancer therapy.

Results

Increased expression of CDC20 in various cancer tissues

We have analysed expression profiles of a total of 36 864 cDNAs using mRNA isolated from U373MG glioblastoma cells that were infected with adenovirus designed to express wild-type p53 (Ad-p53) or LacZ (Ad-LacZ) (Tanikawa *et al*., 2003) and identified 67 genes that were significantly downregulated by ectopic introduction of wild-type p53 (data not shown). Then we examined their expression levels in cancer tissues using the gene expression profile database established in our laboratory that consists of the data for various cancer tissues (Ono *et al*., 2000; Kitahara *et al*., 2001), and selected candidate genes that might play significant roles in the p53-dependent tumor suppressive pathway. Among them, we here selected CDC20 for further analysis, because in our microarray CDC20 was significantly downregulated by introduction of wild-type p53 and was overexpressed in a large subset of malignancies such as colorectal, breast, lung and bladder cancers (Figure 1a).

The expression of CDC20 was increased to more than threefolds than its corresponding normal tissues in about 44% of all cancer tissues examined (Table 1). To verify the results of microarray analysis, we carried out quantitative real-time PCR and confirmed that the expression of CDC20 was increased more than fivefolds in 10 of 13 colorectal cancers tissues and in eight of nine bladder cancer tissues (Figures 1b and c).

CDC20 is a potential cancer therapeutic target

CDC20 is known to regulate separation of chromosomes by inducing ubiquitination and degradation of securin, and subsequently promote transition from G_2/M phase to G_1 phase. Therefore, we expected that knockdown of CDC20 expression might result in the growth suppression of tumor cells. Small interference RNA (siRNA) expression vector that was designed to suppress CDC20 expression (psiU6BX-CDC20) was transfected to H1299 cells. Reverse transcription (RT)–PCR analysis demonstrated a significant decrease of endogenous CDC20 expression by introduction of psiU6BX-CDC20 (Figure 2a). Neither of control plasmids suppressed CDC20 expression, indicating

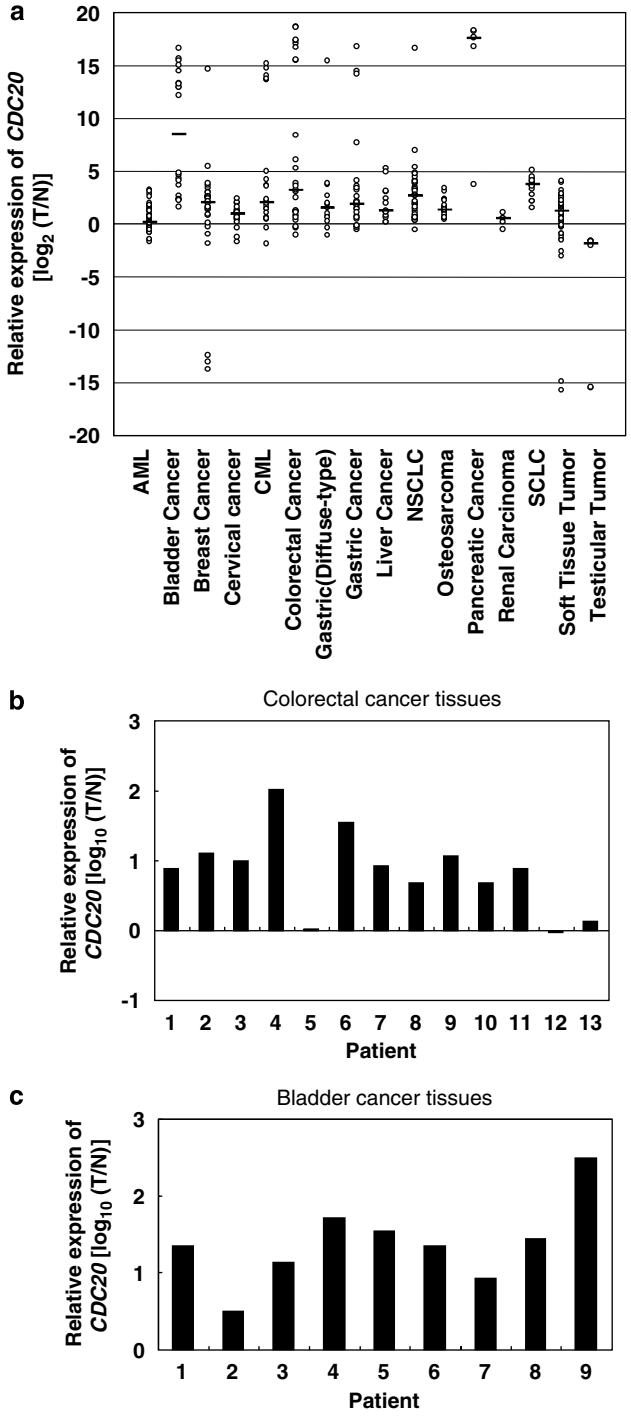

Figure 1 Upregulation of *CDC20* in various cancer tissues. (**a**) Relative expression of *CDC20* in various cancer tissues indicated by cDNA microarray analysis. Solid lines indicate the median of *CDC20* expression in each cancer sample. (**b** and **c**) Relative expression of *CDC20* in colorectal cancer (**b**) and bladder cancer tissues (**c**) determined by quantitative real-time PCR. Expression of *CDC20* was increased more than fivefolds in 10 of 13 (77%) colorectal cancer tissues and in 8 of 9 (89%) bladder cancer tissues.

sequence-specific inhibition of CDC20 by psiU6BX-CDC20. In concordance with the knockdown effect, (4,5 dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT) and colony formation assays revealed the

Table 1 Increased expression of *CDC20* in cancer tissues examined by cDNA mircroarray analyses

Cancer tissues	*Overexpression of CDC20* (*> threefold*), n (%)	*Total samples* (n)	*Reference*
AML	9(18)	50	Okutsu *et al.* (2002)
Bladder cancer	20(100)	20	Takata *et al.* (2005)
Breast cancer	18(60)	30	Nishidate *et al.* (2004)
Cervical cancer	3(16)	19	Kitahara *et al.* (2002)
Choleangiocellular carcinoma	7(100)	7	Obama *et al.* (2005)
CML	14(58)	24	Kaneta *et al.* (2002)
Colon and rectum carcinoma	16(59)	27	Kitahara *et al.* (2001)
Esophageal cancer	2(11)	19	Yamabuki *et al.* (2006)
Gastric cancer	8(32)	25	Hasegawa *et al.* (2002)
Gastric cancer (Diffuse type)	6(46)	13	Jinawath *et al.* (2004)
Liver cancer	6(46)	13	Okabe *et al.* (2001)
Lung cancer (NSCLC)	23(66)	35	Kikuchi *et al.* (2003)
Lung cancer (SCLC)	14(93)	15	Taniwaki *et al.* (2006)
Osteosarcoma	7(29)	24	Ochi *et al.* (2004)
Pancreatic cancer	6(100)	6	Nakamura *et al.* (2004)
Prostate cancer	8(27)	30	Ashida *et al.* (2004)
Renal carcinoma	0(0)	19	Hirota *et al.* (2006)
Soft tissue tumor	27(44)	62	Nagayama *et al.* (2002)
Testicular tumor	0(0)	7	Okada *et al.* (2003)
Total	194(44)	445	

Abbreviations: AML, acute myeloid leukemia; CML, chronic myeloid leukemia; NSCLC, non-small cell lung cancer; SCLC, small-cell lung cancer.

growth inhibitory effect by siRNA specific to CDC20 (Figure 2a).

Then H1299 cells were transfected with siRNA oligonucleotide for CDC20 (siCDC20) or control siRNA (siEGFP), and synchronized at G_1 phase by incubating the cells in the presence of $2\,\mu g\,ml^{-1}$ of aphidicolin for 24 h. After the release from the G_1 arrest, significant increase of G_2/M cell population and significant reduction of G_1 population were observed in the siCDC20-treated cells (Figure 2b). These data indicate that knockdown of CDC20 suppresses cancer cell growth by inducing G_2/M arrest.

Identification of CDC20 as p53-suppressive gene

Then we analysed the molecular mechanism how p53 regulates CDC20 expression. First, we examined the effect of p53 on the expression of CDC20 mRNA using other cancer cell lines. Negative regulation of CDC20 by ectopic expression of p53 was also confirmed in H1299 cells similarly to U373MG cells (Figure 3a). Secondly, we examined whether endogenous p53 could suppress CDC20 expression. HCT116 cells expressing wild-type p53 (HCT116 $p53^{+/+}$) and its isogenic mutant cells lacking p53 (HCT116 $p53^{-/-}$) were exposed to Adriamycin or ionizing radiation. As shown in Figure 3b, accumulation of p53 protein and suppression of CDC20 were observed in HCT116 $p53^{+/+}$ cells as early as 12 h after the treatment with $0.5\,\mu g\,ml^{-1}$ of Adriamycin. However, decrease of CDC20 expression was not observed in HCT116 $p53^{-/-}$ cells. The G_2/M arrest was induced in HCT116 $p53^{+/+}$ cells after Adriamycin treatment (St Clair *et al*., 2004) and our data indicated CDC20 expression was peaked at G_2/M phase (Figure 2b). Therefore, negative regulation of CDC20 by Adriamycin treatment was not considered as the indirect effects of cell cycle arrest. Suppression of CDC20 expression by the exposure to 14 Gy of ionizing radiation was also observed in the p53-dependent manner (Figure 3b). Similarly, when we treated various cancer cells with Adriamycin, we found the decrease of CDC20 expression in A549 cells with wild-type p53, but not in U373MG, SKBR3, T47D or H1299 cells that had no wild-type p53, (Figure 3c), further supporting that the expression of CDC20 was regulated in the p53-dependent manner. Then we treated normal human dermal fibroblast (NHDF) cells with siRNA designed to suppress p53 expression and found that CDC20 expression was remarkably upregulated (Figure 3d). Thus, our data suggested that increased expression of CDC20 in cancer tissues was caused by inactivation of p53.

Identification of p53 regulatory element in CDC20 gene

To clarify the transcriptional regulatory mechanism by p53, we fused the DNA fragment of CDC20 promoter from -1495 to $+26$ (relative to transcription start site) into a pGL3-basic vector (pCDC20-Luc) and also constructed a series of deletion mutant vectors (Figure 4a, Supplementary Figure 1b). Co-transfection with p53 expression vector remarkably suppressed luciferase activity of the cells that were transfected with pCDC20-Luc, in both U373MG and H1299 cells, whereas co-transfection with mutant p53 (R175H) did not suppress at all (Figure 4b). We found a putative p53-binding sequence (p53BS1, $-1469/-1448$) in the CDC20 promoter, however p53-dependent transcriptional suppression was still observed when we used another vector that lacked p53BS1 (Supplementary Figure 1b). According to the analyses of various deletion mutant vectors, the region from -79 to -19 was revealed to be essential for suppression by p53 (Figure 4b). In this region, we

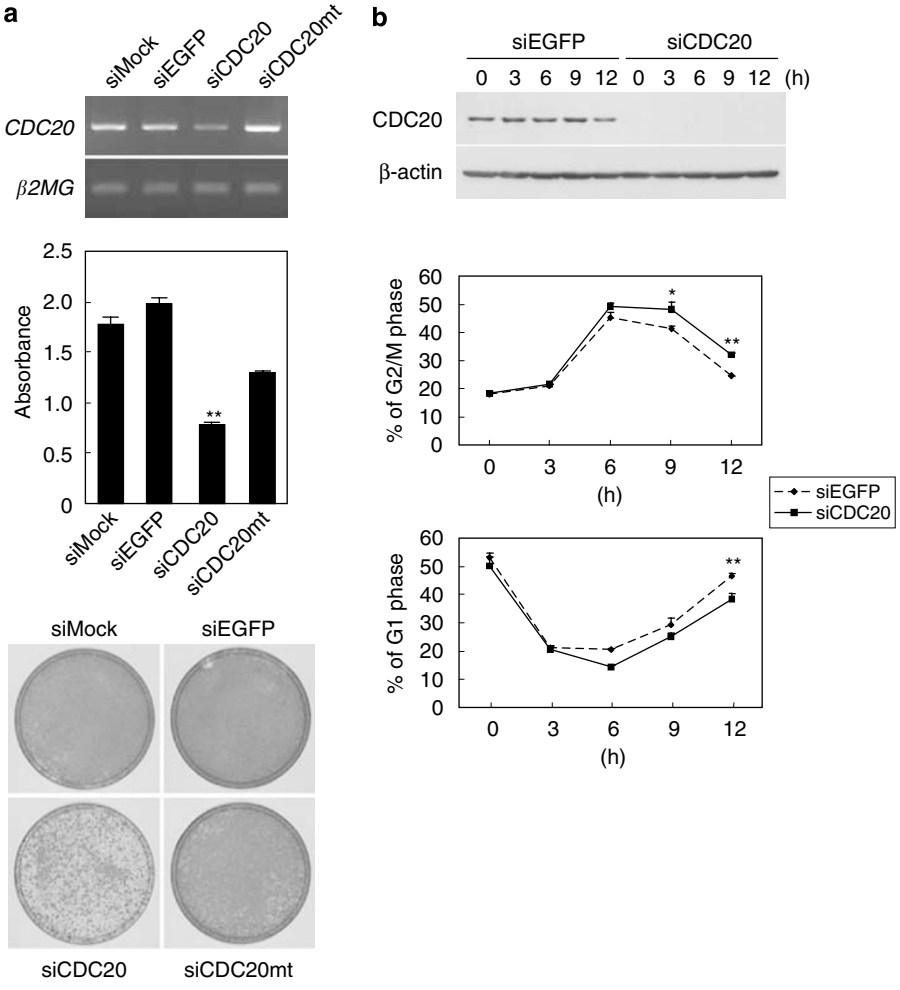

Figure 2 CDC20 is a potential therapeutic target. (**a**) Effect of *CDC20* knockdown on cell proliferation. H1299 cells were transfected with siRNA expression vectors for selective suppression of CDC20 (siCDC20). Mutated siRNA (siCDC20 mt), siMock and siEGFP vectors were used as controls. CDC20 mRNA was specifically suppressed by siCDC20, analysed by semiquantitative RT-PCR (upper panel). β2MG was used as an internal control. H1299 cells transfected with siCDC20 were significantly reduced in cell proliferation determined by MTT assay (middle panel) and colony formation assay (lower panel). The bars indicate standard deviation. $^{**}P<0.01$ (Student's *t*-test). (**b**) Effect of CDC20 siRNA on cell cycle. H1299 cells were treated with $2\,\mu g\,ml^{-1}$ of aphidicolin for 24 h to synchronize at G_1 phase and transfected with siRNA oligonucleotides for selective suppression of CDC20 (siCDC20), or control siRNA oligonucleotides (siEGFP). Cells that were harvested at each time point after releasing from the cell cycle arrest, analysed by western blotting (upper panel) and FACS analysis (middle and lower panel). The bars indicate standard deviation. $^{*}P<0.05$, $^{**}P<0.01$ (Student's *t*-test).

found the CCAAT box, and the CDE (cell cycle-dependent element)–CHR (cell cycle gene homology region) elements (Figure 4c). The CCAAT box is a common promoter element in many eukaryotic promoters (Maity *et al*., 1992). The CDE–CHR elements are found in the genes that were regulated in a cell cycle-dependent manner (Zwicker *et al*., 1995). Moreover, this genomic region was highly conserved between human and mouse genomes (Figure 4c). Therefore, we considered this region might be important for CDC20 gene transcription. Then, we constructed the mutant vector lacking the CCAAT box and the CDE–CHR elements (Figure 4a), and found that p53-dependent transcriptional suppression was not observed in this mutant construct (Figure 4d). Therefore we considered a genomic region from −50 to −13 of the CDC20 promoter to be a p53-regulatory region.

p21 suppressed CDC20 expression

It was previously reported that p21, a p53-inducible cell-cycle regulator, repressed CDC2 expression through the CDE–CHR elements in CDC2 promoter (Taylor *et al*., 2001). Therefore, we examined whether CDC20 could be suppressed by p21. When p21 was exogenously introduced in H1299 cells, the CDC20 expression was drastically suppressed as similar to the cells in which p53 protein was overexpressed (Figure 5a). To further clarify the role of p21 in the p53-dependent CDC20 suppression, HCT116 cells expressing wild-type p21

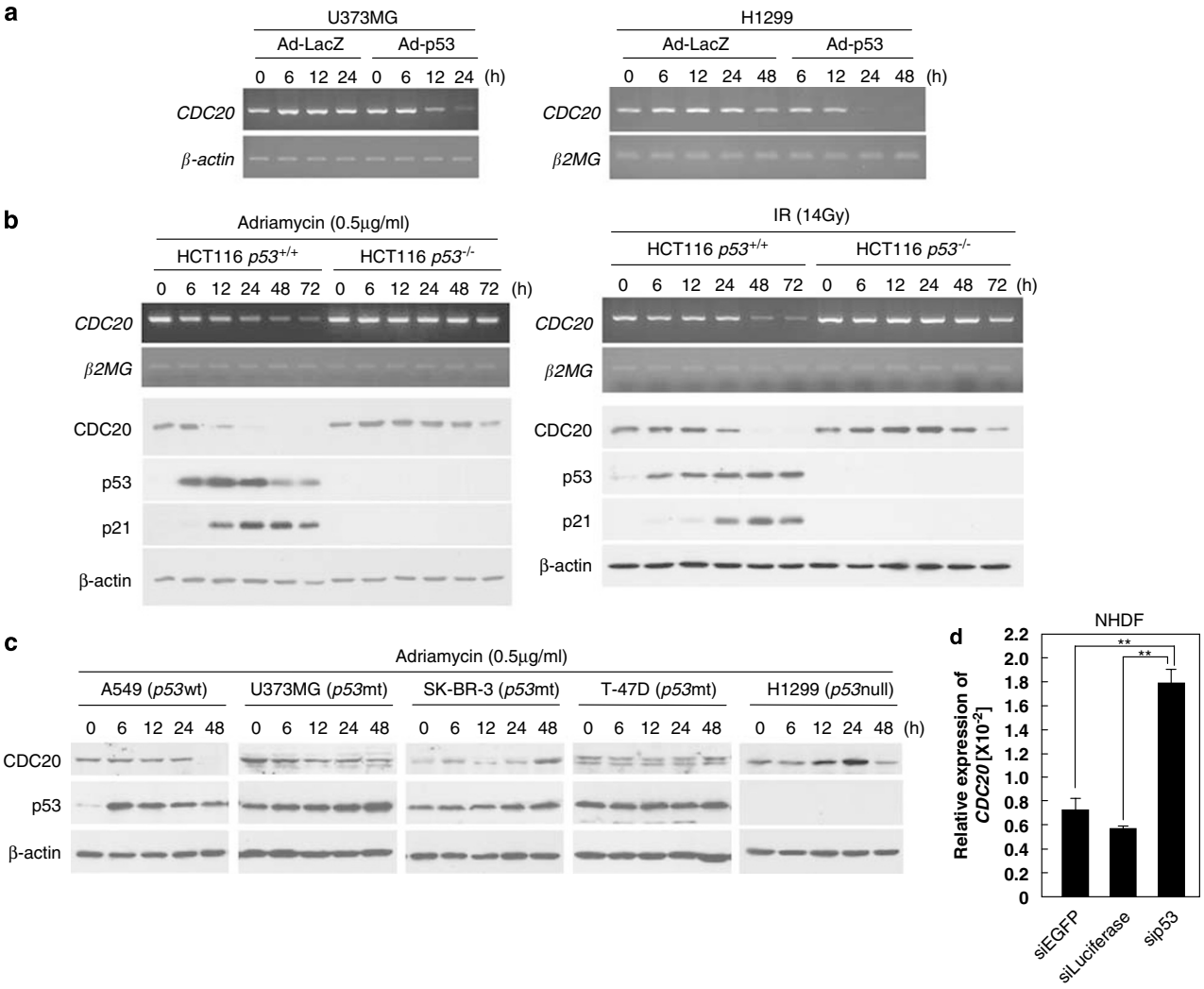

Figure 3 p53-dependent suppression of CDC20. (**a**) Suppression of CDC20 mRNA by overexpressing p53 using introduction of an adenovirus vector (Ad-p53) into U373MG (left panel) and H1299 (right panel) cells. Expression of β-actin and β2MG were used as internal controls. (**b**) Suppression of CDC20 mRNA (upper panel) and protein (lower panel) by endogenous p53. p53 wild type (+/+) and p53 null (−/−) HCT116 cells were treated with 0.5 μg ml^{-1} of Adriamycin (left panel) or 14 Gy of ionizing radiation (IR) (right panel). CDC20 was suppressed in a p53-dependent manner. Expression of p21 was served as a positive control, and β*2MG* (upper panel) and β-actin (lower panel) were used as internal controls. (**c**) p53-dependent suppression of CDC20. Adriamycin suppressed CDC20 expression in A549 (p53 wild type), but not in U373MG, SK-BR-3, T-47D (p53 mutant) and H1299 cells (p53 null). β-actin was used as an internal control. (**d**) Upregulation of CDC20 mRNA by knockdown of p53. NHDF, normal human dermal fibroblast cells, were transfected with siEGFP, siLuciferase as controls and sip53. The bars indicate standard deviation. $^{**}P<0.01$ (Student's *t*-test).

(HCT116 *p21*$^{+/+}$) and its isogenic mutant cells lacking p21 (HCT116 *p21*$^{-/-}$) were exposed to Adriamycin. We found that CDC20 was repressed by Adriamycin treatment in a dose- and time-dependent manner only in HCT116 *p21*$^{+/+}$ cells (Figure 5b). These data clearly indicated that p21 is an essential mediator for p53-mediate CDC20 suppression. Then we generated deletion mutant construct that lacked the CDE–CHR elements from pCDC20-Luc vector (Δ−50/−34, Figure 5c). Co-transfection of p21 significantly repressed the luciferase activity of pCDC20-Luc, but the deletion of the CDE–CHR elements almost diminished the suppressive effect of p21 in both H1299 and U373MG cells (Figure 5c). To investigate the transcription factors that mediated p53/p21-dependent CDC20 suppression, we performed electromobility shift assay (EMSA). Radioisotope-labeled oligonucleotides corresponding to the genomic sequence from −55 to −29 was incubated with nuclear extracts from H1299 cells and electrophoresed. We found a strong shift band and its disappearance by competition of unlabeled self-oligonucleotides, indicating a presence of some nuclear protein(s) bound to this DNA fragment (Figure 5d). Since p21 was shown to inhibit p130/E2F4 binding to the CDE–CHR elements in CDC2 promoter (Taylor *et al*., 2001), we considered E2F to be a candidate to

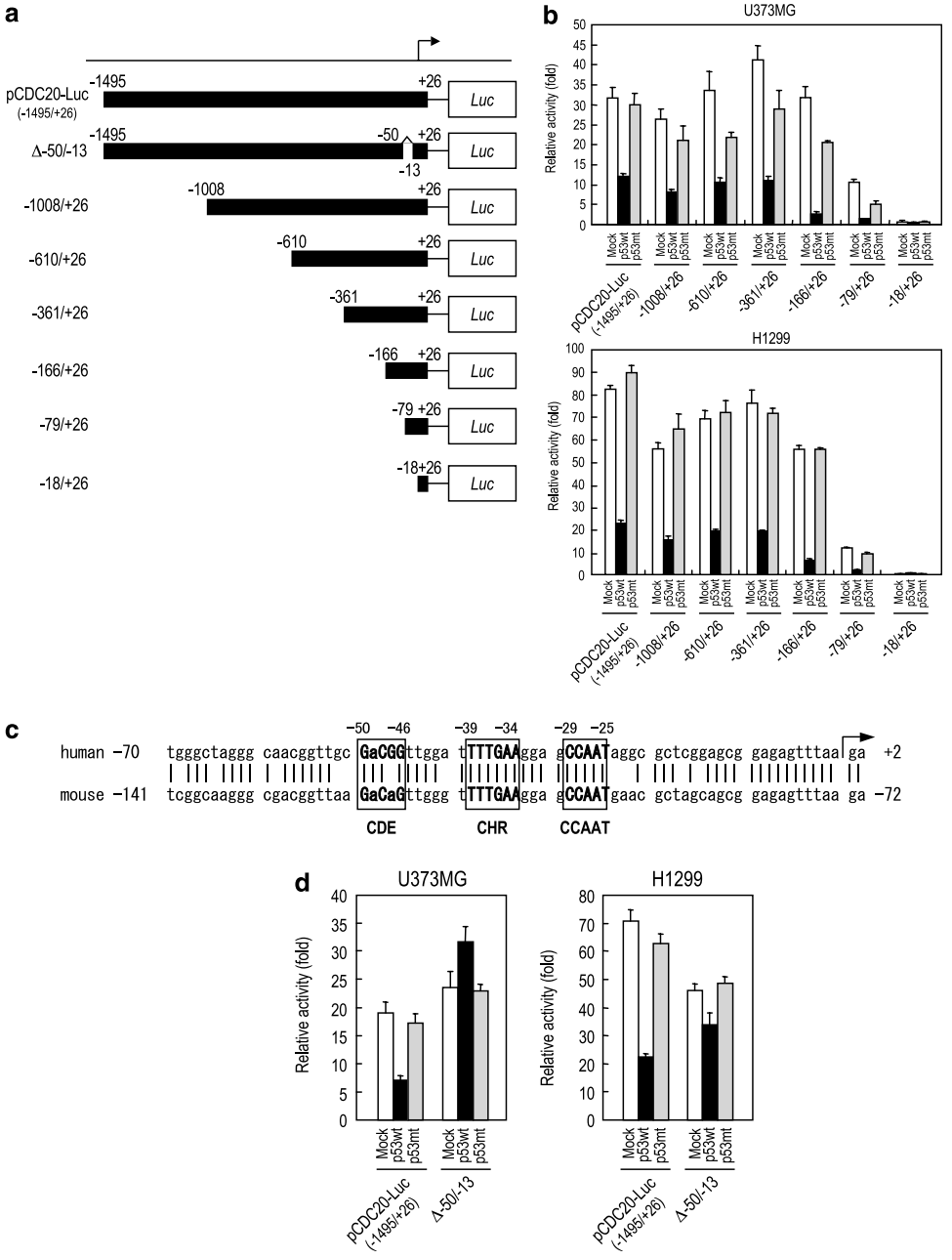

Figure 4 Identification of p53-regulatory element in the CDC20 promoter. (**a**) Schematic presentation of constructs of pCDC20-Luc vector and its deletion mutant vectors. Several DNA fragments (black thick line) were inserted into pGL3-basic vectors. The numbers indicate the positions relative to the transcription initiation site. (**b**) Identification of p53 regulatory element in the CDC20 promoter region by luciferase assay. U373MG (upper panel) and H1299 (lower panel) were transfected with pCDC20-Luc or with its deletion mutant vectors along with pRL-CMV and each of the expression vectors (Mock, p53wt or p53mt). The bars indicate standard deviation. (**c**) Sequence of human and mouse CDC20 promoter regions. The DNA sequences in this region is highly conserved between human and mouse genomes. The putative CDE (−50/−46), CHR (−39/−34) and CCAAT box (−29/−25) are indicated in the box. Nucleotides identical to the consensus sequence are shown in the capital letters. (**d**) A genomic region from −50 to −13 of the CDC20 promoter is essential for p53-dependent CDC20 suppression. Luciferase assay using pCDC20-Luc or its deletion mutant (Δ−50/−13) in U373MG (left panel) and H1299 (right panel) cells are shown. The bars indicate standard deviation.

bind to the CDE–CHR elements in the CDC20 promoter. As we expected, DNA–protein complex formation was completely inhibited with an excess of unlabeled E2F-binding consensus oligonucleotides (Figure 5d).

Discussion

Through the comparison of two data sets of microarray analyses, we identified CDC20 as a potential cancer therapeutic target. Recently genes that are activated in

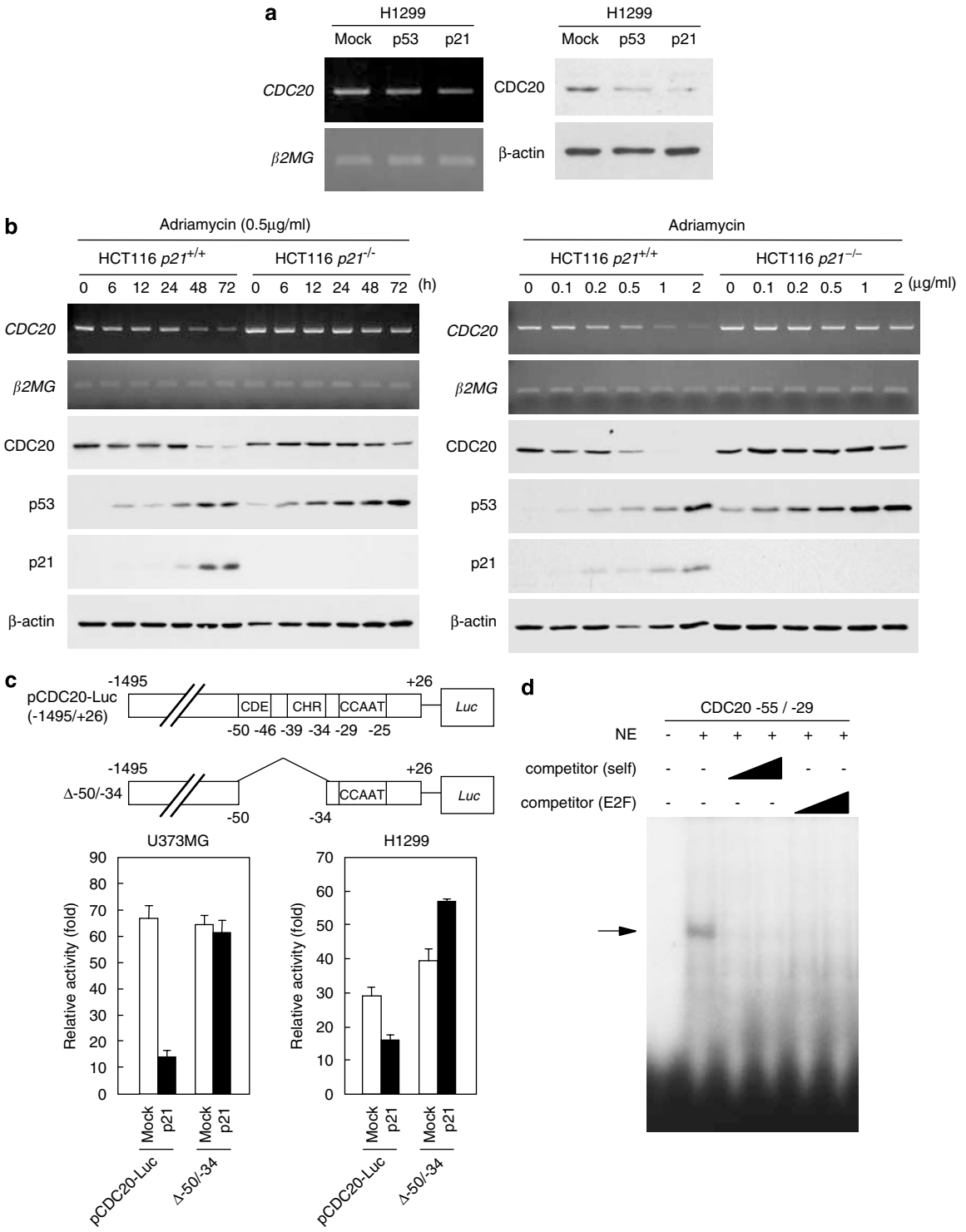

Figure 5 Regulation of CDC20 by p21. (**a**) Overexpression of p21 as well as p53-suppressed CDC20 mRNA (left panel) and protein (right panel) in H1299 cells transfected with mock, p53 or p21 expression vectors. *β2MG* (left panel) and β-actin (right panel) were used as internal controls. (**b**) Suppression of CDC20 mRNA (upper panel) and protein (lower panel) by endogenous p21. p21 wild type (+/+) or p21 null (−/−) HCT116 cells were treated with 0.5 μg ml^{-1} (left panel) or several dose (right panel) of Adriamycin. CDC20 was suppressed in a p21-dependent manner. β2MG (upper panel) and β-actin (lower panel) were used as internal controls. (**c**) Luciferase assay with pCDC20-Luc and its deletion mutant vector (upper panel) in U373MG and H1299 cells (lower panel). The CDE–CHR elements are essential for CDC20 suppression by p21. The bars indicate standard deviation. (**d**) Electromobility shift assay (EMSA). A shift band (arrow) appeared with ^{32}P-labeled double-strand DNA oligonucleotides of −55/−29 of the CDC20 promoter including CDE–CHR elements (lane 2). By addition of excess unlabeled DNA oligonucleotides corresponding to −55/−29 of CDC20 (self, lanes 3–4) or E2F consensus sequence (E2F, lane5–6), the shift band was disappeared.

cancer tissues and involved in carcinogenesis are intensively analysed as therapeutic targets. For instance, Trastuzumab, a recombinant humanized monoclonal antibody to the extracellular domain of HER2 provides substantial clinical benefits in patients with HER2-overexpressing breast cancer (Slamon *et al*., 2001). However, since HER2 is elevated in only 15–25% of human breast cancers (Slamon *et al*., 1989), therapeutic targets for a broad spectrum of cancer are urgently desired.

Notably, overall frequency of CDC20 upregulation observed in cancer tissues was as much as 44% (194 in 445 cancer tissues, Table 1). Quantitative real-time PCR experiments also indicated remarkable upregulation of CDC20 in colorectal and bladder cancer tissues. Enhanced expression of CDC20 was recently reported in oral squamous cell carcinoma as well as gastric cancer (Kim *et al*., 2005; Mondal *et al*., 2007). Therefore, upregulation of CDC20 is considered as a common event in various cancer tissues.

CDC20 is one of the regulators of spindle checkpoint. Mammalian CDC20 interacts with the anaphase-promoting complex/cyclosome (APC/C), and is involved in anaphase onset and late mitotic events (Fung and Poon, 2005). Treatment of cancer cells with siRNA designed to suppress CDC20 expression led to growth suppression by inducing G_2/M arrest, suggesting that CDC20 is a potential therapeutic target for various cancers.

Inactivation of p53 renders cancer cells more resistant to current therapies due to lack of p53-mediated apoptosis and/or cell-cycle arrest (Sun, 2006). Therefore, extensive studies have been conducted to employ the p53 pathway as cancer therapeutics. One approach is the introduction of p53 itself or its downstream genes that mediate p53-dependent apoptotic pathway into cancer cells (Matsuda *et al*., 2002; Roth, 2006). Another approach is the identification of compounds or peptides that manipulate the p53 function. Several small molecular compounds were developed to restore mutant p53 conformation to wild type or to disrupt MDM2-p53 binding and increase the stability of p53 (Bykov *et al*., 2002; Issaeva *et al*., 2004). As a result of p53 inactivation, we considered that some p53-suppressive genes playing significant roles in carcinogenesis might be upregulated in cancer tissues. Our data clearly indicated that CDC20 was remarkably upregulated by knockdown of p53 and also suppressed by DNA damage in a p53-dependent manner. Taken together, p53 protein acts as the transcriptional suppressor of CDC20 gene and inactivation of p53 in cancer tissues is likely to cause enhancement of CDC20 expression. We here demonstrated that an approach to employ genes negatively regulated by p53 could identify candidate therapeutic targets.

The molecular basis for p53-dependent gene silencing is not fully understood so far. The binding of the p53 tetramer to the p53 consensus sequence is required for the p53-dependent transcriptional activation, however, downregulation by p53 does not necessarily require the binding of p53 to consensus sequences (el-Deiry, 1998). One of the possible mechanisms is the ability of p53 to interact with other transcription factors such as TATA-binding protein (TBP) (Seto *et al*., 1992) and SP1 (Innocente and Lee, 2005). Here, we showed that p21 was essential for p53-mediated suppression of CDC20. We also indicated that the CDE–CHR elements in the CDC20 promoter region were required for negative regulation of CDC20 by p21. p21 is known to participate in the G_2/M checkpoint as well as at the G_1 arrest (Bunz *et al*., 1998). p21 inhibits the activity of CDKs and subsequently blocks the phosphorylation of RB tumor suppressor protein. Unphosphorylated RB sequesters E2F proteins, thereby inhibiting transcriptional activation of E2F (Wells *et al*., 2000). The CDE–CHR elements are concerned with the regulation of several cell cycle regulatory genes including E2F. Consequently p53–p21 pathway regulates CDC20 expression through the CDE–CHR elements.

In this study, we revealed the mechanism of CDC20 repression that mediates the tumor suppressive function of p53. Inactivation of p53 observed in various cancer tissues is likely to be the cause of the CDC20 upregulation. Our findings indicated specific inhibitor(s) for CDC20 might be a potential therapeutics for the patients with CDC20-positive cancers.

Materials and methods

cDNA microarrays

We obtained mRNAs from U373MG glioblastoma cells that were infected with Ad-p53 or Ad-LacZ (Tanikawa *et al*., 2003). These RNAs were amplified by T7-based RNA amplification methods. After amplified RNAs (aRNAs) were labeled with Cy3 or Cy5, respectively, we carried out hybridization between labeled aRNAs and cDNA microarray slides containing 36 864 spots and detected the signals. Expression of CDC20 in various cancer tissues and their corresponding normal tissues were examined by using data sets that were analysed previously (Ono *et al*., 2000; Kitahara *et al*., 2001; Okabe *et al*., 2001; Hasegawa *et al*., 2002; Kaneta *et al*., 2002; Kitahara *et al*., 2002; Nagayama *et al*., 2002; Okutsu *et al*., 2002; Kikuchi *et al*., 2003; Okada *et al*., 2003; Ashida *et al*., 2004; Jinawath *et al*., 2004; Nakamura *et al*., 2004; Nishidate *et al*., 2004; Ochi *et al*., 2004; Obama *et al*., 2005; Takata *et al*., 2005; Hirota *et al*., 2006; Taniwaki *et al*., 2006; Yamabuki *et al*., 2006). To select genes that were suppressed by p53 and upregulated in various cancer tissues, the following two criteria were used: (1) genes whose expression were more than threefold decreased by Ad-p53 and (2) genes whose expression were more than threefold increased in various cancer tissues compared with its corresponding normal tissues.

Cell lines

Human cancer cell lines H1299 (lung adenocarcinoma), U373MG (glioma), A549 (lung carcinoma), SK-BR-3 (breast adenocarcinoma), T-47D (breast ductal carcinoma) were purchased from American Type Culture Collection (Manassas, VA, USA). NHDF cells were obtained from Lonza Biologics, Inc. (Portsmouth, NH, USA). HCT116 ($p53^{+/+}$, $p53^{-/-}$, $p21^{+/+}$ and $p21^{-/-}$) cell lines were gifts from B Vogelstein (Johns Hopkins University, Baltimore, MD, USA). All cells were cultured under the conditions recommended by their respective depositors. For induction of endogenous p53, cells were treated with 0.5 μg ml^{-1} of Adriamycin or 14 Gray of ionizing radiation.

RT–PCR
Total RNA from cultured cells was extracted with RNeasy Mini kit (Qiagen, Valencia, CA, USA). Extracted RNAs were reverse-transcribed using oligo$(dT)_{12-18}$ primer and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). cDNAs of colorectal and bladder cancer tissues were prepared as described previously (Kitahara *et al*., 2001; Takata *et al*., 2005). *CDC20* was quantified by real-time RT–PCR that was performed in an Applied Biosystems 7700 sequence detector system with SYBR Premix Ex Taq (Takara, Tokyo, Japan). The primer sets used for RT–PCR are listed in Supplementary Table 1.

Western blotting
Total protein was purified using RIPA buffer (50 mM Tris–HCl, pH8.0, 150 mM sodium chloride, 1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate) added with 1 mM phenyl methylsulphonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT). Equal amount of protein was run to 10% SDS–polyacrylamide gels and transferred to nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, England). The membranes were incubated with primary antibodies overnight at 4 °C, and then incubated with horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences). The primary antibody against p53 (DO-1) and p21 (Ab-1) were purchased from Calbiochem (San Diego, CA, USA), CDC20 (H-175) from Santa Cruz (Santa Cruz, CA, USA) and β-actin (AC-15) from Sigma-Aldrich (St Luois, MO, USA). Signals were detected by an enhanced chemiluminescence kit (Amersham Biosciences).

Gene silencing effect by RNA interference
Plasmids expressing siRNA were constructed by cloning double-strand oligonucleotides into the psiU6BX vector containing the neomycin resistance gene. The oligonucleotides used for siRNA are listed in Supplementary Table 1.

Cell growth assay
Each siRNA expression vector was transfected with Lipofectamine 2000 (Invitrogen) into H1299 cells and these cells were cultured with 0.8 mg ml^{-1} of G418 (Invitrogen), a neomycin analogue for 2 weeks. Cell growth was analysed by MTT assay with Cell counting kit-8 (Dojindo, Kumamoto, Japan) and colony formation assay with Giemsa-staining method.

FACS analysis
siRNA oligonucleotides for *CDC20* (siCDC20) and *EGFP* (siEGFP) were transfected in H1299 cells with Lipofectamine 2000 (Invitrogen) and these cells were treated with 2 μg ml^{-1} of aphidicolin (Sigma-Aldrich) for 24 h. Cells were harvested 0, 3, 6, 9 and 12 h after removing aphidicolin from medium and fixed with 70% ethanol at 4 °C for overnight. After washing with cells were treated with 1 mg ml^{-1} of RNase A at 37 °C for 30 min and stained with 50 μg ml^{-1} of propidium iodide. Cells were analysed with Becton Dickinson FACScalibur using the CellQuest software.

Luciferase assay
Each DNA fragments of the CDC20 promoter were amplified by PCR, digested with *MLu*I and *Xho*I and subcloned into pGL3-basic vector (Promega, Madison, WI, USA). H1299 and U373MG cells that were seeded in 12-well plates (1×10^5 cells per well) 24 h before co-transfected with 125 ng of reporter vector, 25 ng of pRL-CMV (Promega) and 125 ng of wild-type or mutant p53, p21 or mock vector, using FuGENE6 reagent (Roche Diagnostic, Mannheim, Germany). After 36 h, luciferase activity was measured using the picagene dual kit (TOYO Ink, Tokyo, Japan).

Electromobility shift assay
The double-strand DNA oligonucleotides were prepared by annealing sense and antisense oligonucleotides (Supplementary Table 1) and labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Toyobo, Osaka, Japan). Nuclear extracts from H1299 cells were used in this assay. For competition assay, a 10- or 100-fold molar excess of unlabeled DNA oligonucleotides were added before incubation. After incubation, each sample was electrophoresed in a native 4% polyacrylamide gels using 0.5 × TBE buffer. The gels were dried and exposed for autoradiography at −80°C.

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References

Ashida S, Nakagawa H, Katagiri T, Furihata M, Iiizumi M, Anazawa Y *et al*. (2004). Molecular features of the transition from prostatic intraepithelial neoplasia (PIN) to prostate cancer: genome-wide gene-expression profiles of prostate cancers and PINs. *Cancer Res* **64**: 5963–5972.

Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP *et al*. (1998). Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* **282**: 1497–1501.

Bykov VJ, Issaeva N, Shilov A, Hultcrantz M, Pugacheva E, Chumakov P *et al*. (2002). Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. *Nat Med* **8**: 282–288.

el-Deiry WS. (1998). Regulation of p53 downstream genes. *Semin Cancer Biol* **8**: 345–357.

Fang G, Yu H, Kirschner MW. (1998). The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. *Genes Dev* **12**: 1871–1883.

Fung TK, Poon RY. (2005). A roller coaster ride with the mitotic cyclins. *Semin Cell Dev Biol* **16**: 335–342.

Hasegawa S, Furukawa Y, Li M, Satoh S, Kato T, Watanabe T *et al*. (2002). Genome-wide analysis of gene expression in intestinal-type gastric cancers using a complementary DNA microarray representing 23,040 genes. *Cancer Res* **62**: 7012–7017.

Hirota E, Yan L, Tsunoda T, Ashida S, Fujime M, Shuin T *et al*. (2006). Genome-wide gene expression profiles of clear cell renal cell carcinoma: identification of molecular targets for treatment of renal cell carcinoma. *Int J Oncol* **29**: 799–827.

Hoffman WH, Biade S, Zilfou JT, Chen J, Murphy M. (2002). Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. *J Biol Chem* **277**: 3247–3257.

Hollstein M, Rice K, Greenblatt MS, Soussi T, Fuchs R, Sorlie T *et al*. (1994). Database of p53 gene somatic mutations in human tumors and cell lines. *Nucleic Acids Res* **22**: 3551–3555.

Innocente SA, Lee JM. (2005). p53 is a NF-Y- and p21-independent, Sp1-dependent repressor of cyclin B1 transcription. *FEBS Lett* **579**: 1001–1007.

Issaeva N, Bozko P, Enge M, Protopopova M, Verhoef LG, Masucci M *et al*. (2004). Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. *Nat Med* **10**: 1321–1328.

Jinawath N, Furukawa Y, Hasegawa S, Li M, Tsunoda T, Satoh S *et al*. (2004). Comparison of gene-expression profiles between diffuse- and intestinal-type gastric cancers using a genome-wide cDNA microarray. *Oncogene* **23**: 6830–6844.

Kaneta Y, Kagami Y, Katagiri T, Tsunoda T, Jin-nai I, Taguchi H *et al*. (2002). Prediction of sensitivity to STI571 among chronic myeloid leukemia patients by genome-wide cDNA microarray analysis. *Jpn J Cancer Res* **93**: 849–856.

Kikuchi T, Daigo Y, Katagiri T, Tsunoda T, Okada K, Kakiuchi S *et al*. (2003). Expression profiles of non-small cell lung cancers on cDNA microarrays: identification of genes for prediction of lymph-node metastasis and sensitivity to anti-cancer drugs. *Oncogene* **22**: 2192–2205.

Kim JM, Sohn HY, Yoon SY, Oh JH, Yang JO, Kim JH *et al*. (2005). Identification of gastric cancer-related genes using a cDNA microarray containing novel expressed sequence tags expressed in gastric cancer cells. *Clin Cancer Res* **11**: 473–482.

Kitahara O, Furukawa Y, Tanaka T, Kihara C, Ono K, Yanagawa R *et al*. (2001). Alterations of gene expression during colorectal carcinogenesis revealed by cDNA microarrays after laser-capture microdissection of tumor tissues and normal epithelia. *Cancer Res* **61**: 3544–3549.

Kitahara O, Katagiri T, Tsunoda T, Harima Y, Nakamura Y. (2002). Classification of sensitivity or resistance of cervical cancers to ionizing radiation according to expression profiles of 62 genes selected by cDNA microarray analysis. *Neoplasia* **4**: 295–303.

Maity SN, Sinha S, Ruteshouser EC, de Crombrugghe B. (1992). Three different polypeptides are necessary for DNA binding of the mammalian heteromeric CCAAT binding factor. *J Biol Chem* **267**: 16574–16580.

Matsuda K, Yoshida K, Taya Y, Nakamura K, Nakamura Y, Arakawa H. (2002). p53AIP1 regulates the mitochondrial apoptotic pathway. *Cancer Res* **62**: 2883–2889.

Mondal G, Sengupta S, Panda CK, Gollin SM, Saunders WS, Roychoudhury S. (2007). Overexpression of Cdc20 leads to impairment of the spindle assembly checkpoint and aneuploidization in oral cancer. *Carcinogenesis* **28**: 81–92.

Nagayama S, Katagiri T, Tsunoda T, Hosaka T, Nakashima Y, Araki N *et al*. (2002). Genome-wide analysis of gene expression in synovial sarcomas using a cDNA microarray. *Cancer Res* **62**: 5859–5866.

Nakamura T, Furukawa Y, Nakagawa H, Tsunoda T, Ohigashi H, Murata K *et al*. (2004). Genome-wide cDNA microarray analysis of gene expression profiles in pancreatic cancers using populations of tumor cells and normal ductal epithelial cells selected for purity by laser microdissection. *Oncogene* **23**: 2385–2400.

Nakamura Y. (2004). Isolation of p53-target genes and their functional analysis. *Cancer Sci* **95**: 7–11.

Nishidate T, Katagiri T, Lin ML, Mano Y, Miki Y, Kasumi F *et al*. (2004). Genome-wide gene-expression profiles of breast-cancer cells purified with laser microbeam microdissection: identification of genes associated with progression and metastasis. *Int J Oncol* **25**: 797–819.

Obama K, Ura K, Li M, Katagiri T, Tsunoda T, Nomura A *et al*. (2005). Genome-wide analysis of gene expression in human intrahepatic cholangiocarcinoma. *Hepatology* **41**: 1339–1348.

Ochi K, Daigo Y, Katagiri T, Nagayama S, Tsunoda T, Myoui A *et al*. (2004). Prediction of response to neoadjuvant chemotherapy for osteosarcoma by gene-expression profiles. *Int J Oncol* **24**: 647–655.

Oda K, Arakawa H, Tanaka T, Matsuda K, Tanikawa C, Mori T *et al*. (2000). p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell* **102**: 849–862.

Okabe H, Satoh S, Kato T, Kitahara O, Yanagawa R, Yamaoka Y *et al*. (2001). Genome-wide analysis of gene expression in human hepatocellular carcinomas using cDNA microarray: identification of genes involved in viral carcinogenesis and tumor progression. *Cancer Res* **61**: 2129–2137.

Okada K, Katagiri T, Tsunoda T, Mizutani Y, Suzuki Y, Kamada M *et al*. (2003). Analysis of gene-expression profiles in testicular seminomas using a genome-wide cDNA microarray. *Int J Oncol* **23**: 1615–1635.

Okutsu J, Tsunoda T, Kaneta Y, Katagiri T, Kitahara O, Zembutsu H *et al*. (2002). Prediction of chemosensitivity for patients with acute myeloid leukemia, according to expression levels of 28 genes selected by genome-wide complementary DNA microarray analysis. *Mol Cancer Ther* **1**: 1035–1042.

Ono K, Tanaka T, Tsunoda T, Kitahara O, Kihara C, Okamoto A *et al*. (2000). Identification by cDNA microarray of genes involved in ovarian carcinogenesis. *Cancer Res* **60**: 5007–5011.

Roth JA. (2006). Adenovirus p53 gene therapy. *Expert Opin Biol Ther* **6**: 55–61.

Seto E, Usheva A, Zambetti GP, Momand J, Horikoshi N, Weinmann R *et al*. (1992). Wild-type p53 binds to the TATA-binding protein and represses transcription. *Proc Natl Acad Sci USA* **89**: 12028–12032.

Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE *et al*. (1989). Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **244**: 707–712.

Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A *et al*. (2001). Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* **344**: 783–792.

Soussi T, Asselain B, Hamroun D, Kato S, Ishioka C, Claustres M *et al*. (2006). Meta-analysis of the p53 mutation database for mutant p53 biological activity reveals a methodologic bias in mutation detection. *Clin Cancer Res* **12**: 62–69.

St Clair S, Giono L, Varmeh-Ziaie S, Resnick-Silverman L, Liu WJ, Padi A *et al*. (2004). DNA damage-induced downregulation of Cdc25C is mediated by p53 via two independent mechanisms: one involves direct binding to the cdc25C promoter. *Mol Cell* **16**: 725–736.

Sun Y. (2006). p53 and its downstream proteins as molecular targets of cancer. *Mol Carcinog* **45**: 409–415.

Takata R, Katagiri T, Kanehira M, Tsunoda T, Shuin T, Miki T *et al*. (2005). Predicting response to methotrexate, vinblastine, doxorubicin, and cisplatin neoadjuvant chemotherapy for bladder cancers through genome-wide gene expression profiling. *Clin Cancer Res* **11**: 2625–2636.

Tanaka H, Arakawa H, Yamaguchi T, Shiraishi K, Fukuda S, Matsui K *et al*. (2000). A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. *Nature* **404**: 42–49.

Tanikawa C, Matsuda K, Fukuda S, Nakamura Y, Arakawa H. (2003). p53RDL1 regulates p53-dependent apoptosis. *Nat Cell Biol* **5**: 216–223.

Taniwaki M, Daigo Y, Ishikawa N, Takano A, Tsunoda T, Yasui W *et al*. (2006). Gene expression profiles of small-cell lung cancers: molecular signatures of lung cancer. *Int J Oncol* **29**: 567–575.

Taylor WR, Schonthal AH, Galante J, Stark GR. (2001). p130/E2F4 binds to and represses the cdc2 promoter in response to p53. *J Biol Chem* **276**: 1998–2006.

Vogelstein B, Lane D, Levine AJ. (2000). Surfing the p53 network. *Nature* **408**: 307–310.

Wells J, Boyd KE, Fry CJ, Bartley SM, Farnham PJ. (2000). Target gene specificity of E2F and pocket protein family members in living cells. *Mol Cell Biol* **20**: 5797–5807.

Yamabuki T, Daigo Y, Kato T, Hayama S, Tsunoda T, Miyamoto M *et al*. (2006). Genome-wide gene expression profile analysis of esophageal squamous cell carcinomas. *Int J Oncol* **28**: 1375–1384.

Zaffaroni N, Pennati M, Daidone MG. (2005). Survivin as a target for new anticancer interventions. *J Cell Mol Med* **9**: 360–372.

Zhou M, Yeager AM, Smith SD, Findley HW. (1995). Overexpression of the MDM2 gene by childhood acute lymphoblastic leukemia cells expressing the wild-type p53 gene. *Blood* **85**: 1608–1614.

Zwicker J, Lucibello FC, Wolfraim LA, Gross C, Truss M, Engeland K *et al*. (1995). Cell cycle regulation of the cyclin A, cdc25C and cdc2 genes is based on a common mechanism of transcriptional repression. *EMBO J* **14**: 4514–4522.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).