Oncogene (2007) 26, 945–957
© 2007 Nature Publishing Group All rights reserved 0950-9232/07 $30.00
www.nature.com/onc

ORIGINAL ARTICLE

SIRT2, a tubulin deacetylase, acts to block the entry to chromosome condensation in response to mitotic stress

T Inoue[1], M Hiratsuka[1], M Osaki[2,3], H Yamada[1,2], I Kishimoto[1,2], S Yamaguchi[1,2], S Nakano[1,2], M Katoh[1], H Ito[3] and M Oshimura[1,2]

[1]Department of Human Genome Science, Graduate School of Medical Science, Tottori University, Yonago, Tottori, Japan; [2]Department of Biomedical Science, Institute of Regenerative Medicine and Biofunction, Graduate School of Medical Science, Tottori University, Yonago, Tottori, Japan; and [3]Division of Organ Pathology, Department of Microbiology and Pathology, Faculty of Medicine, Tottori University, Yonago, Tottori, Japan

We previously identified SIRT2, an nicotinamide adenine dinucleotide (NAD)-dependent tubulin deacetylase, as a protein downregulated in gliomas and glioma cell lines, which are characterized by aneuploidy. Other studies reported SIRT2 to be involved in mitotic progression in the normal cell cycle. We herein investigated whether SIRT2 functions in the mitotic checkpoint in response to mitotic stress caused by microtubule poisons. By monitoring chromosome condensation, the exogenously expressed SIRT2 was found to block the entry to chromosome condensation and subsequent hyperploid cell formation in glioma cell lines with a persistence of the cyclin B/cdc2 activity in response to mitotic stress. SIRT2 is thus a novel mitotic checkpoint protein that functions in the early metaphase to prevent chromosomal instability (CIN), characteristics previously reported for the CHFR protein. We further found that histone deacetylation, but not the aberrant DNA methylation of SIRT2 5′untranslated region is involved in the downregulation of SIRT2. Although SIRT2 is normally exclusively located in the cytoplasm, the rapid accumulation of SIRT2 in the nucleus was observed after treatment with a nuclear export inhibitor, leptomycin B and ionizing radiation in normal human fibroblasts, suggesting that nucleo-cytoplasmic shuttling regulates the SIRT2 function. Collectively, our results suggest that the further study of SIRT2 may thus provide new insights into the relationships among CIN, epigenetic regulation and tumorigenesis.
Oncogene (2007) **26,** 945–957. doi:10.1038/sj.onc.1209857; published online 14 August 2006

Keywords: SIRT2; mitotic checkpoint; endoreduplication; epigenetic modification; subcellular localization

Correspondence: Professor M Oshimura, Department of Biomedical Science, Institute of Regenerative Medicine and Biofunction, Graduate School of Medical Science, Tottori University, 86 Nishi-cho, Yonago, Tottori 683-8503, Japan.
E-mail: oshimura@grape.med.tottori-u.ac.jp
Received 1 September 2005; revised 8 May 2006; accepted 30 June 2006; published online 14 August 2006

Introduction

A checkpoint is a regulatory mechanism that ensures genomic integrity and prevents the propagation of transformed cells (Hartwell and Kastan, 1994). An impairment of the molecules involved in mitotic checkpoints may play an important role in tumorigenesis by inducing genomic instability and the alteration of ploidy (Paulovich *et al*., 1997). The best known mitotic checkpoint is the spindle-assembly checkpoint that delays chromosome separation and entry to anaphase until the chromosomes are attached correctly to the chromosome segregation machinery (Cortez and Elledge, 2000). MAD2 and BUB1 are involved in this checkpoint (Musacchio and Hardwick, 2002). In a second mitotic checkpoint found in yeast, EB1 delays the final production of two daughter cells in response to an incorrectly oriented spindle (Muhua *et al*., 1998). In a third mitotic checkpoint, which was recently defined, chromosome condensation in response to microtubule poisons is delayed at the G2/early M phase. To date, only CHFR, an ubiquitin ligase (Kang *et al*., 2002), has been shown to be involved in the third mitotic checkpoint (Scolnick and Halazonetis, 2000). CHFR is frequently inactivated in human cancers, mostly owing to the hypermethylation of its promoter region (Mizuno *et al*., 2002; Honda *et al*., 2004; Takahashi *et al*., 2004; Brandes *et al*., 2005).

Gliomas are characterized by marked aneuploidy (Bigner and Vogelstein, 1990), suggesting that a defective mitotic spindle checkpoint may be one of the causes of chromosomal instability (CIN) in this type of cancer (Cleveland *et al*., 2003). We previously identified the SIRT2 protein as a protein downregulated in gliomas and gastric carcinomas (Hiratsuka *et al*., 2003; unpublished observation). The downregulation of SIRT2 protein in gliomas and glioma cell lines such as U251MG and HTB14, is at least partially caused by a decrease in the SIRT2 mRNA levels (Hiratsuka *et al*., 2003). SIRT2 has been reported to be an nicotinamide adenine dinucleotide (NAD)-dependent tubulin deacetylase (North *et al*., 2003). During the cell cycle, the levels of SIRT2 protein increase during mitosis. Furthermore, the overexpression of SIRT2 causes a

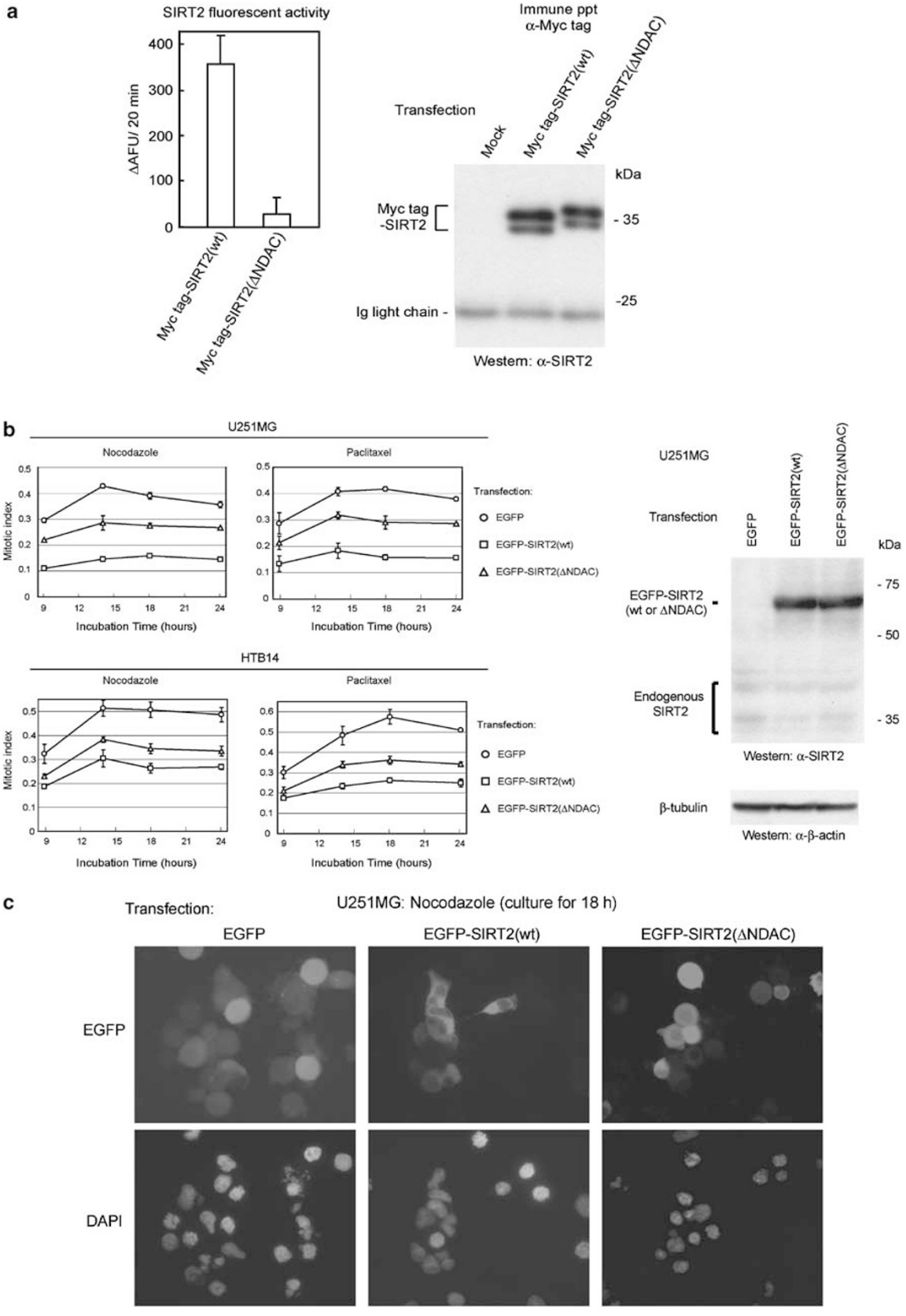

Figure 1 *Continued*

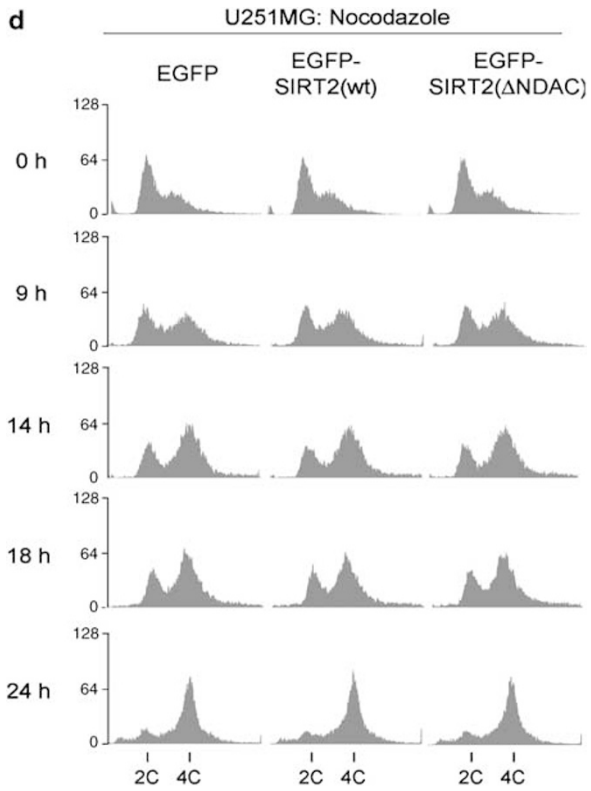

Figure 1 Transiently expressed SIRT2 blocks chromosome condensation in response to arrest caused by microtubule poisons. (**a**) SIRT2 fluorescent activity assay of SIRT2(wt) and SIRT2(ΔNDAC). Myc-tagged proteins were produced in 293 cells and the immunoprecipitated proteins were subjected to SIRT2 fluorescent activity. The assay was performed in triplicate and error bars denote the s.d. The equal input of SIRT2 proteins was confirmed by immunoblotting. (**b**) Mitotic index of U251MG and HTB14 cells expressing EGFP, EGFP-SIRT2(wt) or EGFP-SIRT2(ΔNDAC) after exposure to nocodazole or paclitaxel. The plasmids were transiently transfected into cells and EGFP-positive cells were analysed. EGFP-SIRT2(wt) and EGFP-SIRT2(ΔNDAC) were confirmed to be expressed at equal levels in the total population by immunoblotting. (**c**) Representative images of the cells expressing the indicated constructs after 18 h of nocodazole treatment. (**d**) A cell cycle analysis of the cells expressing EGFP, EGFP-SIRT2 (wt) or EGFP-SIRT2 (ΔNDAC) after exposure to nocodazole. EGFP-positive cells were analysed.

marked extension of the mitotic phase in the normal cell cycle (Dryden *et al*., 2003), thus suggesting the involvement of SIRT2 in mitosis in the normal cell cycle. These previous insights led us to further examine the possibility that SIRT2 may thus function at a mitotic checkpoint in response to mitotic stress to ensure chromosomal stability, such that the downregulation of SIRT2 may thus lead to CIN and consequently tumorigenesis.

In this study, the involvement of SIRT2 in the mitotic checkpoints elicited by mitotic stress was investigated. The results provide clear evidence that exogenously expressed SIRT2 blocks entry to mitotic arrest with a persistence of the cyclin B/cdc2 activity and subsequent hyperploid cell formation in U251MG glioma cells in response to mitotic stress caused by nocodazole, a microtubule-disrupting agent, in its tubulin deacetylase-dependent manner. SIRT2 may thus act at a checkpoint that functions in early metaphase to prevent CIN, similar to CHFR. We further provide a molecular basis of SIRT2 inactivation in gliomas and report a nucleo-cytoplasmic shuttling of SIRT2 as a possible regulatory mechanism of the SIRT2 function, which is a useful finding for the planning of future studies to elucidate the involvement of SIRT2 in tumorigenesis.

Results

SIRT2 elicits a deacetylase activity-dependent change in response to microtubule poisons

To examine whether SIRT2 is involved in a mitotic checkpoint, cells were transiently transfected with enhanced green fluorescent protein (EGFP)-fused SIRT2 and then treated with nocodazole and paclitaxel, which elicit mitotic stress by inducing microtubule depolymerization and polymerization, respectively. We used the glioma cell lines U251MG and HTB14, which showed a decreased expression of SIRT2 in comparison to normal brain tissues in our previous study (Hiratsuka *et al*., 2003, see also Figure 2a). To examine whether any function of SIRT2 requires its NAD-dependent tubulin deacetylase activity, we generated an NAD-dependent tubulin deacetylase activity deficient mutant (referred to as EGFP-SIRT2(ΔNDAC)) by changing the codons for three amino acids (Q130A, N131A and H150Y) (Figure 1a) (Finnin *et al*., 2001; Dryden *et al*., 2003; North *et al*., 2003). The cells were transfected with these constructs and then were cultured in the presence of nocodazole or paclitaxel. We scored the fraction of cells that have condensed chromosomes with nuclear membrane destruction (mitotic index) among EGFP-positive cells expressing EGFP-fused wild-type SIRT2 (EGFP-SIRT2(wt)) proteins at 9, 14, 18 and 24 h after the treatment.

As shown in Figure 1b, the cells that express EGFP had a high mitotic index after treatment with nocodazole and paclitaxel; however, the cells that express EGFP-SIRT2(wt) had a comparatively lower mitotic index. In the latter samples, the cells without an EGFP signal had a comparable mitotic index (data not shown), thus suggesting that the lower mitotic index observed in EGFP-positive cells is attributable to SIRT2 expression. Importantly, cells expressing EGFP-SIRT2(ΔNDAC) exhibited a higher mitotic index than EGFP-SIRT2(wt) and also showed a moderate restoration of the mitotic index. Representative images are shown in Figure 1c. The kinetics of the mitotic index was similar in all the samples (Figure 1b). It is thus likely that exogenously expressed SIRT2 blocks the entry to chromosome condensation in about half of the cell population in response to microtubule poisons, whereas the other cells were all able to escape from the SIRT2-mediated arrest and thus behaved similarly to control cells. In the absence of nocodazole, EGFP-SIRT2 did not have a significant effect on the cell cycle distribution for at least 24 h (data not shown). A flow cytometric analysis of the EGFP-positive population revealed that the patterns of the cell cycle progression and accumulation in

G2/M phase in the presence of nocodazole are comparable among the cells transfected with EGFP, EGFP-SIRT2(wt) or EGFP-SIRT2(ΔNDAC) (Figure 1d). These results implicate ectopically expressed SIRT2 protein blocks entry to chromosome condensation in U251MG and HTB14 cells in response to mitotic stress. Furthermore, the block appears to occur at G2 or the early M phase, and the SIRT2-induced block depends on its NAD-dependent tubulin deacetylase activity. However, we cannot rule out the possibility that some unknown functions of SIRT2 other than an NAD-dependent tubulin deacetylase may also play a role to block the entry to chromosome condensation in response to microtubule poisons, because the cells expressing EGFP-SIRT2(ΔNDAC) also exhibited a partial blockade of entry to chromosome condensation (Figure 1b).

SIRT2 prevents microtubule poison-induced hyperploid cell formation via a block of entry to chromosome condensation

After the prolonged exposure to nocodazole, U251MG cells, which carry a p53 mutation (Alonso *et al*., 2003), undergo a transient arrest at mitosis and subsequently escape from mitotic arrest and then undergo DNA replication, but they do not complete chromosome segregation and cell division. This phenomenon is termed 'mitotic slippage' and represents a failure to maintain a mitotic arrested state (Di Leonardo *et al*., 1997; Nitta *et al*., 2002). As a result of mitotic slippage, U251MG cells undergo DNA re-replication and form hyperploid cells, which are considered to be a frequent precursor of aneuploidy during tumorigenesis (Shackney *et al*., 1989). In the last transient transfection experiment, it was difficult to monitor the mitotic index and cell cycle progression of transfected cells over a longer culture period, owing to the disappearance of EGFP signals. As a result, in the experiment shown in Figure 1, we cannot strictly rule out the possibility that SIRT2(wt)-expressing cells arrest at a subsequent G1 phase after undergoing mitotic slippage, and not at the phase before entry to chromosome condensation. To clearly distinguish between these possibilities, we investigated whether mitotic slippage and DNA rereplication occur after a prolonged exposure to microtubule poisons in the SIRT2-expressing cells. To do this, we isolated U251MG clones that stably express EGFP, EGFP-SIRT2(wt) or EGFP-SIRT2(ΔNDAC). The population doubling time, cell cycle distribution and mitotic index in the absence of mitotic stress were similar among all the clones used in this study (data not shown). The levels of EGFP-SIRT2(wt) or EGFP-SIRT2(ΔNDAC) were also comparable among clones 1 and 2 for each transformant; and these levels are comparable to the level of endogenous SIRT2 observed in non-tumor brain tissue (Figure 2a). The intensity of EGFP signal was homogenous in each clone even after many rounds of passages, thus suggesting the homogenous and stable expression of transfected genes (data not shown).

The mitotic index of the clones (clones 1 and 2 for each transfectant) was examined after a prolonged microtubule poison exposure. Consistent with the results obtained in transiently transfected U251MG cells, 14 and 18 h after exposure to nocodazole, only two pools of EGFP-SIRT2(wt)-expressing clones had a much lower fraction of condensed chromosomes as compared to each of two pools of EGFP- or EGFP-SIRT2(ΔNDAC)-expressing clones (Figure 2b). At all data points between 9 and 30 h after the treatment, the mitotic index was significantly lower in the clones expressing EGFP-SIRT2(wt) than in the clones expressing EGFP or EGFP-SIRT2(ΔNDAC). In all clones, the mitotic index reaches a peak at 18 h of treatment and then decreases as the cells approach 36 h of treatment, thus suggesting that SIRT2 functions to block the entry to chromosome condensation which is consistent with the results obtained in Figure 1. Importantly, all the clones show the same kinetics of the mitotic index. This suggests that there are two populations in the cells expressing EGFP-SIRT2(wt), one which does not condense their chromosomes during the prolonged exposure to nocodazole and is kept at a stage before chromosome condensation until 72 h of the start of treatment, and another which undergoes chromosome condensation and mitotic slippage similar to U251MG cells expressing EGFP or EGFP-SIRT2(ΔNDAC) with the same kinetics.

To directly determine whether mitotic slippage and DNA re-replication occur in these clones, we performed a flow cytometric analysis and chromosome analysis of U251MG stable clones during prolonged exposure to nocodazole. The flow cytometric analysis revealed that clones expressing EGFP or EGFP-SIRT2(ΔNDAC) have a hyperploid DNA content after 72 h (Figure 2c), consistent with the previous report on parental U251MG cells (Tsuiki *et al*., 2001). A chromosome analysis revealed that the chromosome number increases to nearly twice the normal levels after prolonged exposure to nocodazole in all the clones (Figure 2d and e). As a result, in the clones expressing EGFP or EGFP-SIRT2(ΔNDAC), almost all of the cells undergo mitotic slippage and form hyperploid cells. On the other hand, in cells expressing EGFP-SIRT2(wt), about half the cells had a 4C DNA content after 72 h with nocodazole treatment, whereas the remaining fraction had a hyperploid DNA content (Figure 2d). After 72 h of nocodazole treatment, almost all cells in metaphase had nearly twice the chromosome number. Cells with a normal of chromosome number range were rarely observed (Figure 2e). This suggests that cells in metaphase were derived from the population that underwent mitotic slippage by escaping the mitotic checkpoint evoked by SIRT2, and additionally, that the other population was held in the first G2/early M phase by SIRT2 function and did not undergo mitotic slippage. This observation is consistent with the result obtained in the flow cytometric analysis in which there were two populations in the aspect of response after the treatment with nocodazole in EGFP-SIRT2(wt)-expressing cells (Figure 2c). Even so, our data revealed that SIRT2 functions to block entry to chromosome condensation and subsequent hyperploidy at least

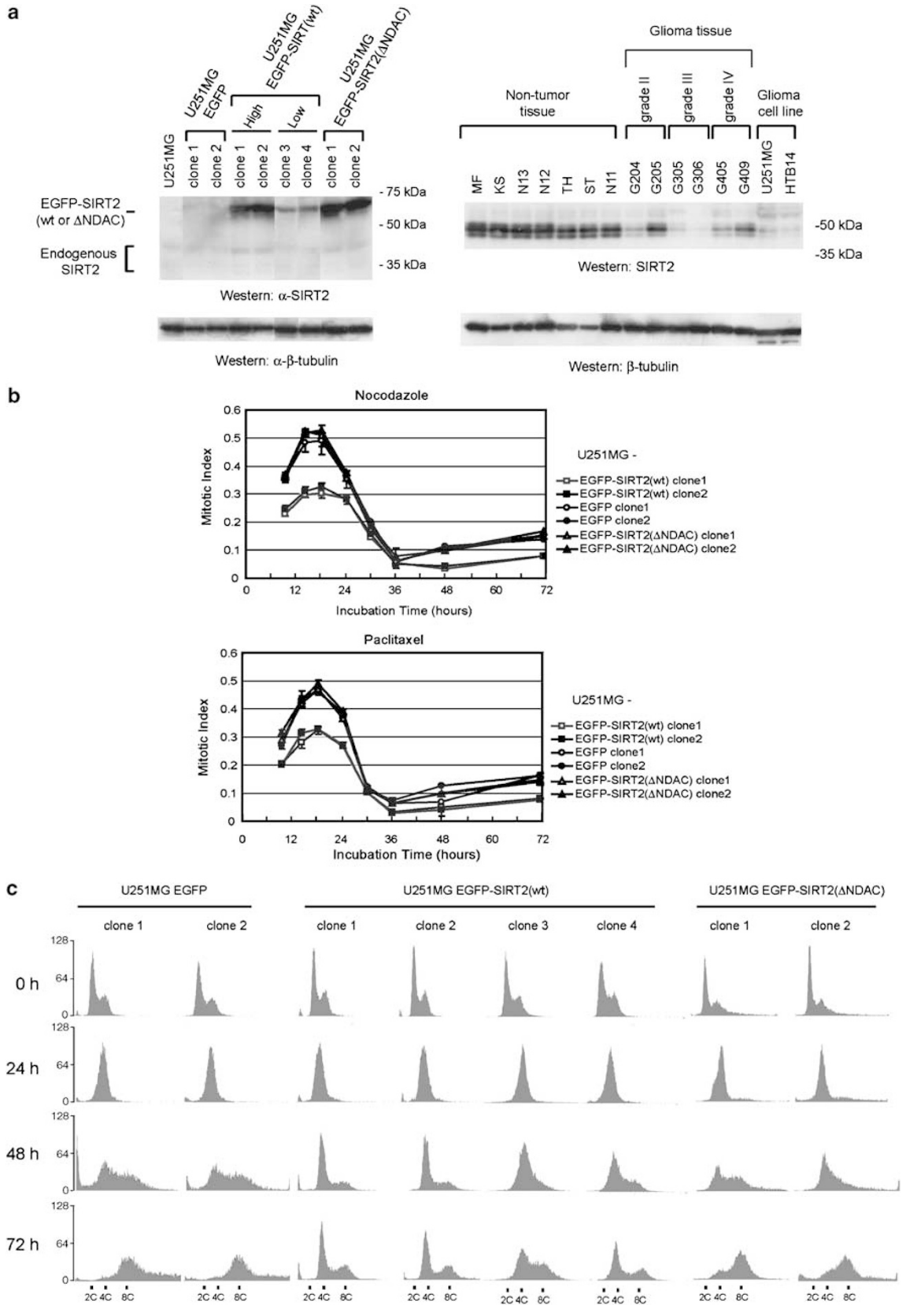

Figure 2 *Continued*

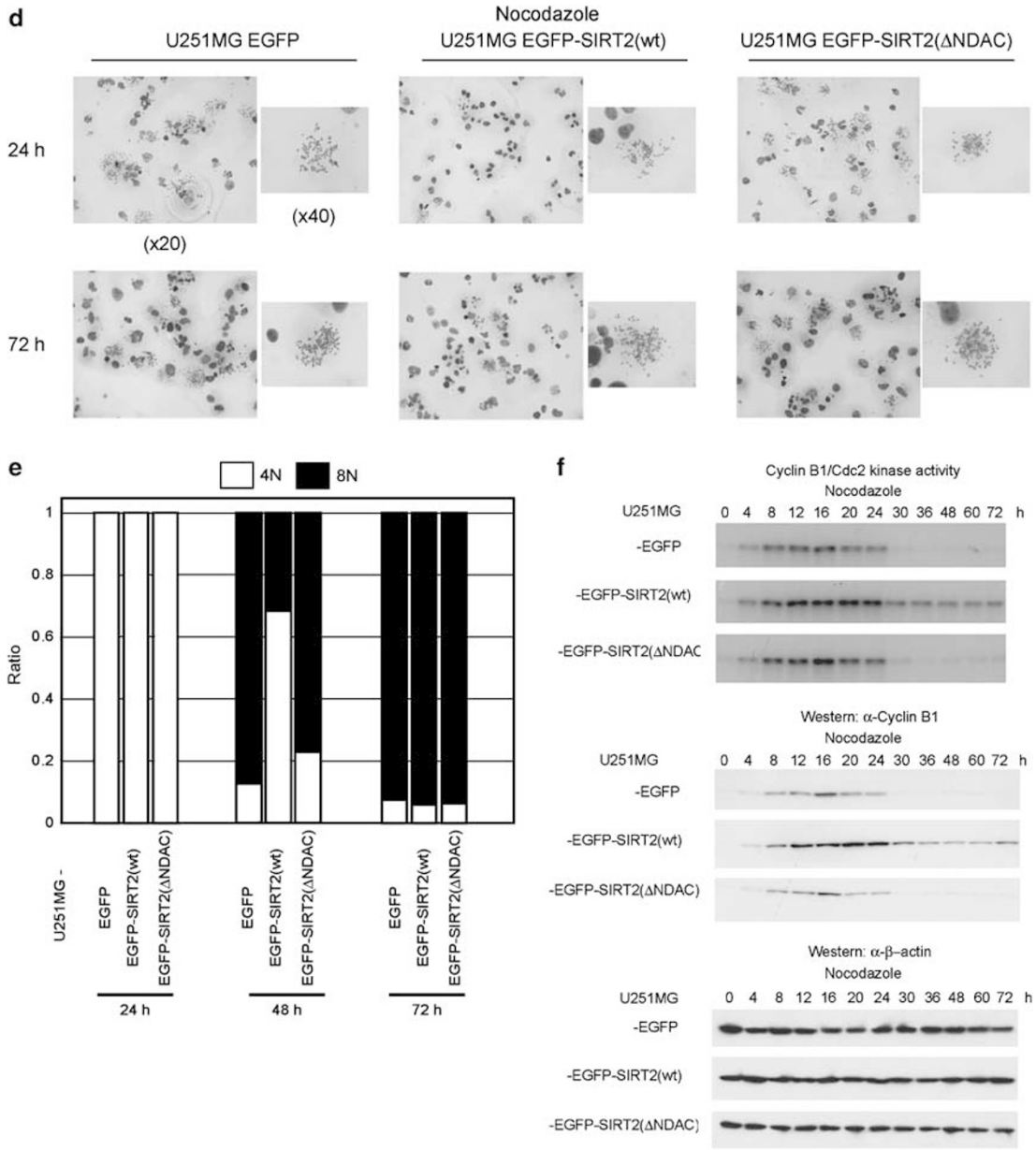

Figure 2 Characterization of stably transfected U251MG clones expressing EGFP, EGFP-SIRT2(wt) or EGFP-SIRT2(ΔNDAC). (**a**) Expression of EGFP-SIRT2(wt), or EGFP-SIRT2(ΔNDAC) proteins in stably transfected clones. The levels of endogenous SIRT2 protein, which is known to have two isoforms (GenBank accession numbers: NM_012237.2, and NM_030593.1), are shown, along with phosphorylated forms (Dryden *et al*., 2003; see also Figure 5d). The levels of endogenous SIRT2 protein in gliomas and non-tumor brain tissues are also shown. The equal loading of protein was confirmed by immunoblotting for β-tubulin. (**b**) Mitotic index after exposure to nocodazole or paclitaxel. (**c**) Cell cycle analysis of each stable transformant after prolonged exposure to nocodazole. (**d**) Chromosome analysis of stable clones after exposure to nocodazole. Cells were fixed, stained with Giemsa to determine chromosome number. Note that most of cells which exhibited metaphase have nearly twice the number of chromosomes (8N) at 72 h, thus indicating that these cells underwent DNA endoreduplication. (**e**) Ratio of cells with a normal number of chromosomes (4N; 85–95) to those with nearly twice number of chromosomes (8N: 175–190). At least 50 metaphase cells were scored. (**f**) Cyclin B/cdc2 kinase activity and the level of cyclin B of stable clones after exposure to nocodazole.

partially in U251MG. In order to further confirm that moderate levels of SIRT2 expression can prevent hyperploid cell formation, we isolated U251MG stable clones expressing EGFP-SIRT2(wt) whose levels were modestly higher than the level of endogenous SIRT2 (U251MG EGFP-SIRT2(wt) clones 3 and 4) (Figure 2a). A flow cytometric analysis during the prolonged exposure to nocodazole was performed in these clones (Figure 2c). These clones showed the same trend as U251MG EGFP-SIRT2(wt) clones 1 and 2. These data underscores the role of SIRT2 to block the entry to chromosome condensation in response to mitotic stress.

To further define the specific phases of the cell cycle at which SIRT2(wt)-expressing cells arrest, we used the cyclin B/cdc2 levels as a marker, which are elevated in the prophase and metaphase and low in G2 and anaphase (Amon, 1999). The cyclin B/cdc2 kinase

activity and the cyclin B expression increased after exposure to nocodazole and reached maximum levels 12–16 h after treatment in all clones (Figure 2f). Both were barely detectable after 30 h of treatment in EGFP- or EGFP-SIRT2(ΔNDAC)-expressing clones. This was in contrast to the EGFP-SIRT2(wt)-expressing cells wherein moderate cyclin B/cdc2 expression levels persisted for 30–72 h after treatment.

Considering the data as a whole, we conclude that EGFP-SIRT2(wt)-expressing cells with a 4C DNA content after prolonged exposure to nocodazole are thus able to arrest the cell cycle progression at the phase that precedes the entry to chromosome condensation but not at metaphase, anaphase or the subsequent G1 phase. In other words, SIRT2 functions in the prophase to block the entry to chromosome condensation in response to mitotic stress in U251MG cells.

Analysis of the DNA methylation status of the SIRT2 gene

We next addressed the molecular basis of the downregulation of SIRT2 protein observed in gliomas and glioma cell lines. In our previous study, we showed that the downregulation of SIRT2 is caused at least in part by a decrease in the SIRT2 mRNA levels (Hiratsuka *et al*., 2003). It is widely accepted that the aberrant methylation at the cytosine guanine dinucleotide (CpG) sites in the region near the transcription start site is associated with gene silencing. Because the 5′ upstream region of the SIRT2 gene is highly enriched in CpG (Figure 3), we analysed the methylation status in this region of glioma tissues that were confirmed to exhibit a significantly decreased expression of SIRT2 mRNA in our previous study (Hiratsuka *et al*., 2003), as well as that of non-tumor brain tissues, using bisulfite genomic DNA sequencing. As shown in Figure 3, however, none of the 21 CpGs were methylated in five non-tumor tissues and five glioma tissues. Although we cannot rule out the possibility that the methylation status of other regions of the SIRT2 gene that is important for the transcriptional regulation is different between the non-tumor tissues and glioma tissues, our results thus suggest that aberrant DNA methylation near the transcription start sites is not involved in the decreased expression of SIRT2.

Trichostatin A activates SIRT2 expression

We next investigated the possibility that the histone modification of the SIRT2 gene is involved in downregulation of the SIRT2 in gliomas. To test this possibility directly, we treated U251MG cells with the histone deacetylase inhibitor trichostatin A (TSA). After 24 h of treatment with TSA, SIRT2 protein was upregulated in U251MG cells, whereas SIRT2 protein levels are not altered by treatment with the demethylating agent 5-aza-2′-deoxycytidine (5-Aza-dC) (Figure 4a). In addition, similar effects on the expression of SIRT2 protein were observed in HTB14 cells treated with the same agents (data not shown), and our observation is consistent with a previous report by another group which showed that

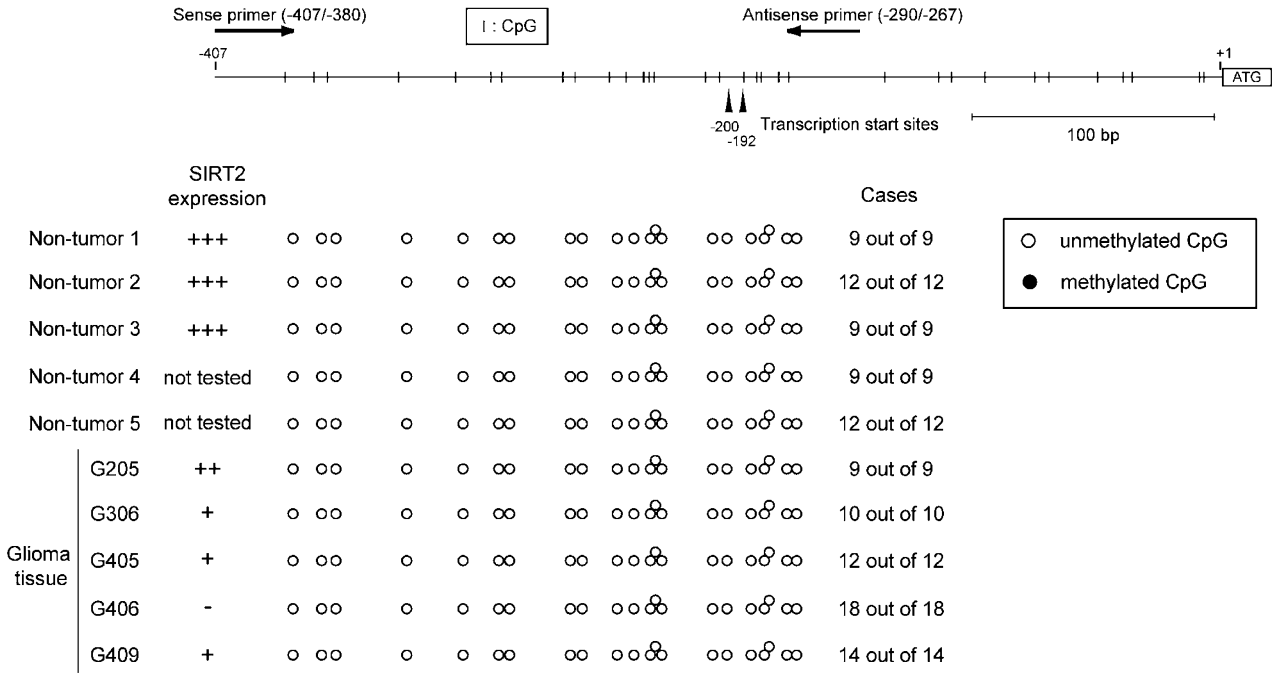

Figure 3 Methylation status analysis of SIRT2. Schematic representation of the CpG-rich region of the SIRT2 genes. This CpG map is based on the published genomic DNA sequence (Ensembl Gene ID ENSG00000068903). CpG sites are represented by vertical lines. Nucleotide positions are numbered relative to the translation start site (+1). The genomic position of the putative transcription start sites and the positions for primers are represented by arrow heads and arrows, respectively. Note that none of the 21 CpGs were methylated in all the tissue samples. The numbers of analysed clones from different bacterial colonies are indicated on each tissue sample.

TSA or *n*-butyrate treatment can induce an upregulation of SIRT2 mRNA in Neuro-2a, a mouse neuroblastoma cell line (Kyrylenko *et al*., 2003). Chromatin immunoprecipitation (ChIP) assays showed that treatment with TSA causes the hyperacetylation of histone H3 and H4 in 5′ upstream region of the SIRT2 gene (Figure 4b). These data suggest that histone acetylation is a potent determinant of the SIRT2 expression. Additional evidence in favor of this model comes from the observation that SIRT2 is downregulated in gastric carcinomas, and that in a gastric carcinoma cell line, TSA but not 5-Aza-dC induces the upregulation of SIRT2 (manuscript in preparation). Taken together, these results suggest that SIRT2 is inactivated by histone deacetylation in several types of tumors.

Nucleo-cytoplasmic shuttling of SIRT2

Several lines of studies including our previous study suggest that SIRT2 is detected only in the cytoplasm. However, during this study, it was reported that SIRT2 interacts with the homeobox transcription factor HOXA10 (Bae *et al*., 2004), which is detected in the nucleus (Qian *et al*., 2005). We thus suspected that SIRT2 has the ability to shuttle between the cytoplasm and the nucleus, a possible novel regulatory mechanism of the SIRT2 function. The size of this protein is about 64 kDa, and we assumed that it does not undergo passive diffusion as the molecular masses of protein that can freely pass through the nuclear membrane are less than 40 kDa (Mattaj and Englmeier, 1998). To test whether the cytoplasmic localization of SIRT2 is due to active nuclear export, which is mediated by the nuclear export receptor *cis*-regulatory modules (CRM)1 in many cases, the cells expressing EGFP-SIRT2 were treated with leptomycin B (LMB), a CRM-specific nuclear export inhibitor (Kudo *et al*., 1999). Within 1 h with treatment, we observed a partial accumulation of EGFP-SIRT2(wt) in the nucleus, and at 2 h of treatment, most of the EGFP-SIRT2(wt) in the cell had accumulated in the nucleus (Figure 5a). Moreover, the accumulation of endogenous SIRT2 in the nucleus was observed in normal human diploid fibroblasts, TIG-1, after 2 h treatment of LMB by an immunohistochemical analysis (Figure 5b). These results suggest that the cytoplasmic localization of SIRT2 protein is due to CRM1-dependent active nuclear export, thus raising the possibility that nucleo-cytoplasmic shuttling is a regulatory mechanism of SIRT2.

To examine whether the alteration in the subcellular localization of SIRT2 is induced in response to stresses that can evoke G2/M checkpoint caused by DNA damage, we exposed the TIG-1 cells to ionizing radiation (IR), UV and nocodazole and performed immunostaining with anti-SIRT2 antibody. In parallel, we also performed immunoblotting with anti-SIRT2 antibody to examine whether the change in the expression level of SIRT2, and/or phosphorylation of SIRT2 are involved in the stress responses. Intriguingly, we observed that SIRT2 is localized in the nucleus in addition to cytoplasm at 8–12 h after IR (Figure 5c). The accumulation of SIRT2 in the nucleus diminished by 24 h after IR. No significant change in the protein level or phosphorylation of SIRT2 was observed after IR (Figure 5d). Neither the alteration in the subcellular localization of SIRT2 nor the change in the expression level of SIRT2 and phosphorylation of SIRT2 was observed by UV irradiation and nocodazole treatment (data not shown). This result raises the possibility that SIRT2 may be involved in the DNA damage response caused by IR through the alteration in the subcellular localization of SIRT2 rather than the change in the expression level and phosphorylation of SIRT2. Although the functional role of the accumulation of SIRT2 in the nucleus in response to IR remains to be clarified, these data support the notion that nucleo-cytoplasmic shuttling regulates the SIRT2 function.

We have not determined the region(s) responsible for the cytoplasmic localization of SIRT2 experimentally but we suspect the presence of a nuclear export signal (NES) in SIRT2. The widely accepted NES consensus is L-x(2,3)-[LIVFM]-x(2,3)-L-x-[LI] although certain variations are allowed (la Cour *et al*., 2003, 2004). Although SIRT2 does not have the canonical NES consensus sequence, the NES prediction program

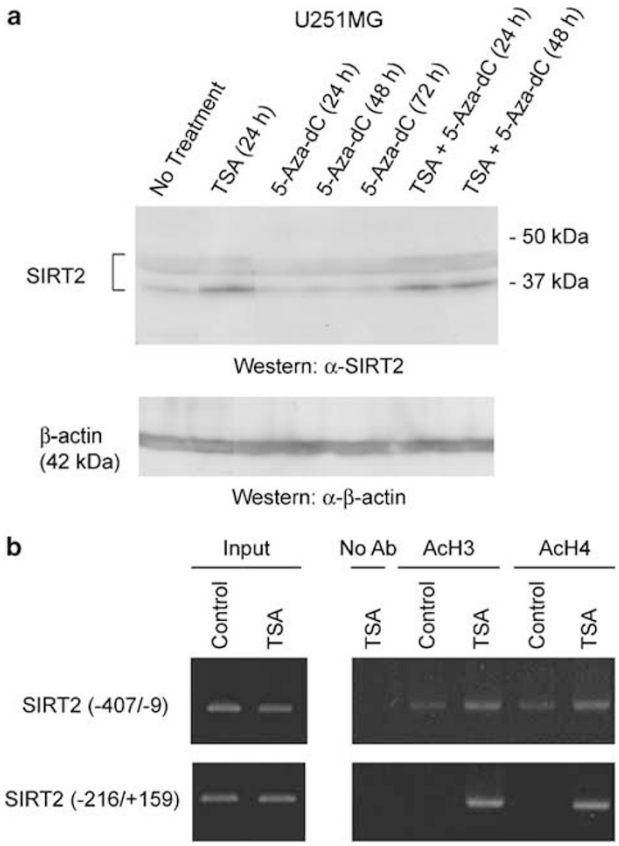

Figure 4 Effects of TSA and 5-Aza-dC on the expression of SIRT2 protein. (**a**) U251MG cells were treated with ethanol (control), TSA (0.2 μM) and/or 5-Aza-dC (2 μM), and subjected to immunoblot analysis with anti-SIRT2 antibody. (**b**) ChIP analysis was performed using U251MG cells treated with ethanol (control) or TSA. Immunoprecipitation was performed by incubation with anti-acetyl-histone H3 or anti-acetyl-histone H4 antibody, and PCR was performed using primers specific for the SIRT2 promoter region.

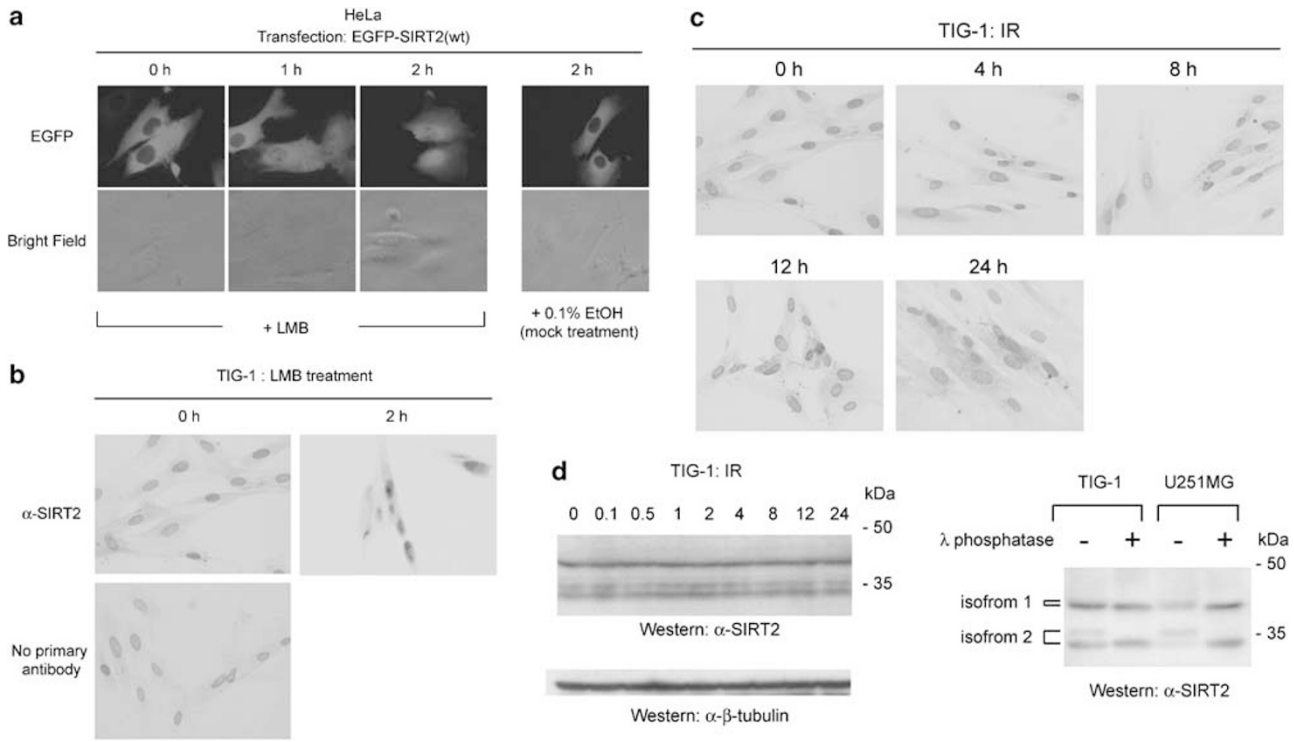

Figure 5 Nucleo-cytoplasmic shuttling of SIRT2. (**a**) Effects of LMB treatment on the subcellular localization of EGFP-SIRT2. HeLa cells were transiently transfected with EGFP-SIRT2(wt) and cultured for 1 day. Next, the cells were incubated in the presence of LMB or 0.1% ethanol (control). (**b**) Effects of LMB treatment on subcellular localization of endogenous SIRT2. TIG-1 cells with treatment of LMB were immunostained with anti-SIRT2 antibody. Diaminobenzidine was used as the chromogen for the immunoperoxidase reaction. The slides were counterstained with methylgreen. Endogenous SIRT2 was not detectable in an immnocytochemical analysis in HeLa, U251MG and HTB14 cells (data not shown). (**c**) The effects of IR on subcellular localization of endogenous SIRT2. TIG-1 cells treated with IR were immunostained with anti-SIRT2 antibody. (**d**) An immunoblotting analysis of SIRT2 in TIG-1 cells treated with IR. Cell lysates treated with or without λ phosphatase, subjected to immunoblotting with anti-SIRT2 antibody and used as markers for the phosphorylation status.

NetNES (http://www.cbs.dtu.dk/, (la Cour *et al*., 2003)) does predict a possible NES between residues 17 and 27 (QKERLLDELTL).

Discussion

In a previous study, Dryden *et al*. (2003) report that cells stably overexpressing SIRT2 showed a marked increase in the cell population whose DNA content was 4C and they suggest that SIRT2 regulates mitotic progression in the normal cell cycle in the osteoblastic cell line Saos2. Bae *et al*. (2004) also observed a similar phenomena in the myelomonocytic cell line U937. In the present study, however, exogenous expression of SIRT2 did not affect the cell cycle distribution in U251MG glioma cells. Instead, we observed that SIRT2 functions in early metaphase to prevent entry to chromosome condensation in response to mitotic stress in a manner dependent on deacetylase activity of SIRT2. To date, only CHFR had been reported as a protein that delays chromosome condensation in response to mitotic stress, although several proteins, such as MAD, BUB and EB1, are known to function as mitotic checkpoint proteins later in mitosis (Cortez and Elledge, 2000). Therefore, SIRT2 is the second protein to be identified as functioning in a mitotic checkpoint that blocks chromosome condensation in response to mitotic stress.

The next question to be answered is how SIRT2 functions in this checkpoint. Our results suggest that SIRT2 NAD-dependent tubulin deacetylase activity is required for its function. Acetylated tubulin is abundant in stable microtubules but it is absent from dynamic cellular structure such as the leading edges of fibroblast (Piperno *et al*., 1987). Moreover, HDAC6, another tubulin deacetylase (Hubbert *et al*., 2002), increases cell motility (Saji *et al*., 2005) and inhibits the clustering of CD3 in the central region of the immune synapse and of LFA-1 in the periphery by decreasing the acetylation of microtubules at contact regions (Serrador *et al*., 2004). However, how the acetylation status of tubulin is involved in the mitotic checkpoint or spindle formation at mitosis is not well known. One possible model of the SIRT2 function is that SIRT2 regulates microtubules in specific intracellular spaces. Although this point remains unclear, our study underscores a possible link between the acetylation status of tubulin and entry to chromosome condensation. In this context, it is notable that our previous proteomic studies found that the expression of a group of tubulin-related proteins is altered in gliomas or gastric carcinomas in comparison to normal tissues (Hiratsuka *et al*., 2003; Nishigaki *et al*., 2005). Although

how CHFR contributes to mitotic checkpoints is not fully understood, several mechanisms of CHFR function have been proposed. Kang *et al*. (2002) reported that CHFR targets polo-like kinase 1 for ubiquitination, which is important as polo-like kinase 1 is required at multiple stages of mitosis and is upregulated in many human malignancies (Takai *et al*., 2005; Xie *et al*., 2005). Recently, Yu *et al*. (2005) suggested that CHFR controls the expression levels of key mitotic proteins, including Aurora A, which is a component for mitotic commitment. Therefore, the involvement of SIRT2 in CHFR-regulated molecular events should be investigated. In order to clarify the interdependency between SIRT2 and CHFR, it would be useful to compare the expression levels of SIRT2 and CHFR in tumors, including gliomas.

It is widely accepted that tumorigenesis and cancer progression result from a progressive accumulation of genetic alterations. In solid cancers, two major types of genetic instability have been described, based on the findings initially observed in colorectal cancer (Lengauer *et al*., 1998). The first is microsatellite instability, which is tightly associated with a mismatch repair deficiency. The second is instability at the chromosome level, thus leading to aneuploidy, abnormal rates of chromosome instability, and/or a loss of heterozygosity (LOH). This second type of defect is referred to as CIN and it is assumed that a disruption of the checkpoints is necessary for CIN. Alterations in the expression or mutation of checkpoint proteins, including MAD2 and BUB1 (Imai *et al*., 1999), have been reported in only a few cases, with the exception of CHFR, which is frequently inactivated by aberrant DNA methylation in cancer cases. However, CHFR inactivation is not associated with CIN in colon cancers (Bertholon *et al*., 2003). SIRT2 is located on 19q13, a region known to be frequently deleted in human gliomas. In addition, the present study suggests that epigenetic alteration can also inactivate SIRT2 gene expression. Collectively, SIRT2 may thus be a new candidate for a mitotic checkpoint protein whose inactivation gives rise to CIN.

To examine whether the downregulation of SIRT2 affects mitotic checkpoints, we used a plasmid-based short hairpin RNA system to knockdown the SIRT2 gene (unpublished). Although the expression of SIRT2 protein was moderately suppressed by this vector, any significant change was not observed by the knockdown of SIRT2 in the mitotic index at 18 and 24 h after the treatment with nocodazole in TIG-1 cells. Considering the possibility that either the remaining SIRT2 protein may be sufficient to elicit checkpoints or other factors may compensate for the SIRT2 function, the knockout of SIRT2 gene in normal cells and/or a long-term culture in the condition in which SIRT2 is inactivated is required to clarify the involvement of SIRT2 in CIN or early stage of tumorigenesis.

In addition, the systematic analysis of SIRT2 expression in primary human tumors from the aspect of LOH, mutation, epigenetic alteration and subcellular localization of SIRT2 protein is merited in order to explore whether SIRT2 can be referred to as a tumor suppressor gene.

When considering the therapeutic context, it should be noted that in the U251MG cells, SIRT2 blocks entry to chromosome condensation and subsequent mitotic slippage, and in turn, the DNA re-replication that promotes CIN, including both numerical and structural chromosomal changes (Nitta *et al*., 2002). Particularly, the formation of hyperploid cells was at least partially prevented by exogenous expression of SIRT2 in U251MG cells, which harbor a p53 mutation. Although the overexpression of CHFR delays the entry to metaphase in DLD cells by about 6 h (Scolnick and Halazonetis, 2000), the overexpression of SIRT2 blocks at least half of U251MG cells for more than 48 h (Figure 2c). This implies that SIRT2 more potently arrests cell cycle progression than CHFR. Furthermore, the finding that SIRT2 expression can be upregulated in glioma and gastric carcinoma by treatment with TSA provides additional evidence that targeting SIRT2 has the potential to provide significant therapeutic benefits. However, it should be noted that even in EGFP-SIRT2(wt)-expressing cells, a population of cells underwent mitotic slippage and formed hyperploid cells after long exposure to nocodazole (Figure 2c), although the level of protein expression was homogenous among the cells by measure of EGFP intensity (data not shown). One plausible explanation is that the expression levels of other factors that regulate the mitotic processes vary from cell to cell.

The results obtained in a subcellular localization study suggest that SIRT2 is actively exported from the nucleus via a CRM1-dependent mechanism. Intriguingly, an accumulation of SIRT2 in the nucleus was observed after IR. Although much remains to be clarified regarding how the regulation of SIRT2 by subcellular localization relates to DNA damage response and G2/M checkpoint, these results suggest that nucleo-cytoplasmic shuttling regulates the SIRT2 function. The present study also supports the idea that SIRT2 and HOXA10 are physically interacted, which was observed in a previous report (Bae *et al*., 2004).

In conclusion, this study provides the first evidence that SIRT2 can act in a mitotic checkpoint which blocks chromosome condensation in response to mitotic stress, similar to the activity previously reported for CHFR. Further studies of SIRT2 could provide insights into a novel avenue of mitotic checkpoints in the early stages, as well as insight into a possible link between tubulin regulation, mitotic checkpoints and CIN.

Materials and methods

Cell culture and DNA transfection

DNA transfection to glioma cell lines was performed using Lipofectamine 2000 (Invitrogen, Paisley, UK), according to the manufacturer's instructions.

U251MG clones stably expressing fusion protein of wild or mutant form of SIRT2 with EGFP were selected for 12–14 days in the presence of 800 μg/ml G418 (Invitrogen). For the microscopic analysis of mitotic index, the cells were re-plated on gelatin-coated coverslips (Fisher, Ottawa, ON, USA) at 30–40% confluence in order to keep cells exponentially growing and to facilitate the microscopic analysis.

Nocodazole (Sigma, St Louis, MO, USA) and Paclitaxel (Sigma) were kept as 8.3 and 3.9 mM stocks in dimethylsulfoxide, respectively, and added to the medium with a final concentration of 200 and 16 nM, respectively. TSA (Sigma) and 5-Aza-dC (ICN Biochemical, Aurora, OH, USA) were kept as a 3.3 mM stock in ethanol and a 1 mM stock in water, respectively, and added to the medium with a final concentration of 200 nM and 2 μM, respectively. LMB (Sigma) was kept as a 9.2 μM stock in ethanol, and added to the medium with a final concentration of 2.3 nM.

For ionizing irradiation (IR) (10 Gy) and UV irradiation (20 J/m^2), an X-ray irradiation machine (MBR-1505R2, Hitachi Medico) and a FUNA-UV-LINKER (Funakoshi, Tokyo, Japan) were used, respectively.

DNA constructs

pEGFP-SIRT2(wt), a construct encoding a fusion of wild-type SIRT2 with EGFP, was described in our previous study (Hiratsuka *et al*., 2003). pEGFP-SIRT2(ΔNDAC), a mutant version of pEGFP-SIRT2(wt), lacks an NAD-dependent tubulin deacetylase activity and was generated by mutation of codons for three amino (Q130A, N131A and H150Y) as reported by other groups (Finnin *et al*., 2001; Dryden *et al*., 2003; North *et al*., 2003), in two sequential steps using the QuickChange II XL site-directed mutagenesis kit (Stratagene). pcDNA3-Myc-SIRT2(wt) and pcDNA3-Myc-SIRT2(ΔNDAC) constructs encoding Myc-tagged SIRT2(wt) and SIRT2(ΔNDAC), respectively, were generated by standard recombination techniques using pcDNA3 (Invitrogen) as a parental vector.

Antibodies, immunoblotting and immunocytochemistry

Antibody to SIRT2 was raised in New Zealand White rabbits with a peptide antigen corresponding to the C-terminal 12 amino-acid residues of human SIRT2 protein (DEARTTEREKPQ) and affinity purified.

For immunoblotting, equal amounts of protein samples or protein samples derived from an equal number of cells were separated on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Pharmacia, Québeck, Canada). Immunoblotting was carried out using 1:1000 anti-SIRT2 antibody or 1:300 anti-cyclin B monoclonal antibody (Santa Cruz, Heidelberg, Germany) as a primary antibody. Alternatively, 1:2000 anti-β-actin monoclonal antibody (Sigma) or 1:2000 anti-β-tubulin (Amersham Pharmacia) was used as the primary antibody to confirm equal protein loading in each lane. A 1:3000 dilution of peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin (Amersham Pharmacia) was used as the secondary antibody. Signals were visualized using the ECL detection system (Amersham Pharmacia).

For immunostaining, the cells were fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS) for 20 min at 4°C and permeabilized with 0.25% Triton X-100/PBS for 20 min at 4°C. Endogenous peroxidase activity was blocked by immersing the slides in 0.6% hydrogen peroxide in methanol for 30 min at room temperature. Immunocytochemistry was performed using the streptavidin–biotin-peroxidase complex method (Histofine, Nichirei), according to the manufacturer's instruction. Diaminobenzidine was used as the chromogen for the immunoperoxidase reaction. The slides were counterstained with methylgreen.

Microscopic analysis of chromosomes

Cells plated on 18 mm-coverslips (Fisher) were fixed with 4% paraformaldehyde/PBS for 20 min at room temperature, stained with 4, 6-diamidino-2-phenylindole (Sigma) and mounted on glass microscope slides (Matsunami, Osaka, Japan). The mitotic index was determined by inspecting more than 400 cells per data point with a fluorescent microscope (ELPIPSE TE300; Nikon, Tochigi, Japan).

For metaphase preparation, cells were treated with a hypotonic solution of 75 mM KCl for 15 min at room temperature. Next, the cells were fixed with methanol/acetic acid (3:1) and dropped onto glass microscope slides. The slides were dried and stained with 5% Giemsa for 30 min.

Flow cytometry analysis

For DNA content evaluation of cells expressing EGFP-fusion proteins, cells were collected by trypsinization, fixed in 4% paraformaldehyde/PBS at room temperature and then in cold 70% EtOH/30% PBS, and stored at −20°C until flow cytometry analysis (Chu *et al*., 1999). A flow cytometric analysis for EGFP and propidium iodide fluorescence was performed using an EPICS ELITE 4.0 (Coulter, Fullerton, CA, USA). For each analysis, 10 000 gated events were collected to permit cell cycle analysis of EGFP-positive cell populations. The data analysis and figure generation were performed using the WinMDI 2.8 software program (freeware).

ChIP assays

ChIP assays were performed using the ChIP Assay Kit (Upstate Biotechnology, Milano, Italy) according to the manufacturers instructions with anti-acetylated histone H3 antibody (Upstate Biotechnology) and anti-acetylated histone H4 antibody (Upstate Biotechnology). Two pairs of primers were used for polymerase chain reaction (PCR): TGATTCA CCCAATGATAATATGCTGAAT and TGCTGAAGCCCT TGAGGCTGTCACC that spanned −409 to −9 of the SIRT2 gene, TAACTGGGGCGCTCTGGGTGTTGTACGAAA and AAGCAACAGCCCTCAGAATCCCACTC that spanned −216 to +156 of the SIRT2 gene.

Cyclin B/Cdc2 kinase assays

Cyclin B/Cdc2 kinase assays were performed as reported (Scolnick and Halazonetis, 2000). Briefly, whole-cell extracts were prepared in 50 mM Tris (pH 7.9), 120 mM NaCl, 0.5% NP-40, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM ethyleneglycoltetraacetate, 1.5 mM $MgCl_2$, 1 mM dithiothreitol and a cocktail of protease inhibitors (Roche). Whole-cell extracts were incubated with 1.5 μg histone H1 (Calbiochem) as a substrate in 21 μl of 50 mM Tris (pH 7.9), 150 mM NaCl, 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, 25 μM ATP and 5 Ci ^{32}P-γ-ATP (Amersham Pharmacia) for 30 min at 30°C. The reactions were subjected to polyacrylamide gel electrophoresis using 10% gels followed by autoradiography.

SIRT2 fluorescent activity assay

SIRT2 fluorimetric drug discovery kit (BIOMOL) was used to measure the lysyl deacetylase activity of SIRT2. Transfection to 293 cells using the calcium phosphate DNA precipitation method, preparation of whole-cell extract and immunoprecipitation were performed as we previously reported (Roberts *et al*., 2001). Immunoprecipitated materials were washed with the assay buffer two times and used for the SIRT2 activity assay. Fluorescence was excited with 360 nm light and the emitted light (460 nm) was detected on a CytoFluor II plate reader (PersPective Biosystems, gain = 85).

Abbreviations

5-Aza-dC, 5-aza-2′-deoxycytidine; ChIP, chromatin immunoprecipitation; CIN, chromosomal instability; EGFP, enhanced green fluorescent protein; IR, ionizing irradiation; TSA, trichostatin A; LMB, leptomycin B; NES, nuclear export signal.

Acknowledgements

We thank S Abe (Tottori University) and Dr A Kurimasa (Tottori University) for technical assistance with the metaphase preparations and for valuable suggestions, respectively. We thank Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku University) for a kind gift of TIG-1 cells. This work was supported in part by grants from the Public Trust Haraguchi Memorial Cancer Research Fund (T. I.), the Ministry of Education, Culture, Sports, Science and Technology of Japan (T. I., M. H., M.O. and M. O.), and The 21st Century COE Program: The Research Core for Chromosome Engineering Technology (T. I. and M. O.).

References

Alonso M, Tamasdan C, Miller DC, Newcomb EW. (2003). Flavopiridol induces apoptosis in glioma cell lines independent of retinoblastoma and p53 tumor suppressor pathway alterations by a caspase-independent pathway. *Mol Cancer Ther* **2**: 139–150.

Amon A. (1999). The spindle checkpoint. *Curr Opin Genet Dev* **9**: 69–75.

Bae NS, Swanson MJ, Vassilev A, Howard BH. (2004). Human histone deacetylase SIRT2 interacts with the homeobox transcription factor HOXA10. *J Biochem* **135**: 695–700.

Bertholon J, Wang Q, Falette N, Verny C, Auclair J, Chassot C *et al*. (2003). Chfr inactivation is not associated to chromosomal instability in colon cancers. *Oncogene* **22**: 8956–8960.

Bigner SH, Vogelstein B. (1990). Cytogenetics and molecular genetics of malignant gliomas and medulloblastoma. *Brain Pathol* **1**: 12–18.

Brandes JC, van Engeland M, Wouters KA, Weijenberg MP, Herman JG. (2005). CHFR promoter hypermethylation in colon cancer correlates with the microsatellite instability phenotype. *Carcinogenesis* **26**: 1152–1156.

Chu YW, Wang R, Schmid I, Sakamoto KM. (1999). Analysis with flow cytometry of green fluorescent protein expression in leukemic cells. *Cytometry* **36**: 333–339.

Cleveland DW, Mao Y, Sullivan KF. (2003). Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling. *Cell* **112**: 407–421.

Cortez D, Elledge SJ. (2000). Conducting the mitotic symphony. *Nature* **406**: 354–356.

Di Leonardo A, Khan SH, Linke SP, Greco V, Seidita G, Wahl GM. (1997). DNA rereplication in the presence of mitotic spindle inhibitors in human and mouse fibroblasts lacking either p53 or pRb function. *Cancer Res* **57**: 1013–1019.

Dryden SC, Nahhas FA, Nowak JE, Goustin AS, Tainsky MA. (2003). Role for human SIRT2 NAD-dependent deacetylase activity in control of mitotic exit in the cell cycle. *Mol Cell Biol* **23**: 3173–3185.

Finnin MS, Donigian JR, Pavletich NP. (2001). Structure of the histone deacetylase SIRT2. *Nat Struct Biol* **8**: 621–625.

Hartwell LH, Kastan MB. (1994). Cell cycle control and cancer. *Science* **266**: 1821–1828.

Hiratsuka M, Inoue T, Toda T, Kimura N, Shirayoshi Y, Kamitani H *et al*. (2003). Proteomics-based identification of differentially expressed genes in human gliomas: downregulation of SIRT2 gene. *Biochem Biophys Res Commun* **309**: 558–566.

Honda T, Tamura G, Waki T, Kawata S, Nishizuka S, Motoyama T. (2004). Promoter hypermethylation of the Chfr gene in neoplastic and non-neoplastic gastric epithelia. *Br J Cancer* **90**: 2013–2016.

Hubbert C, Guardiola A, Shao R, Kawaguchi Y, Ito A, Nixon A *et al*. (2002). HDAC6 is a microtubule-associated deacetylase. *Nature* **417**: 455–458.

Imai Y, Shiratori Y, Kato N, Inoue T, Omata M. (1999). Mutational inactivation of mitotic checkpoint genes, hsMAD2 and hBUB1, is rare in sporadic digestive tract cancers. *Jpn J Cancer Res* **90**: 837–840.

Kang D, Chen J, Wong J, Fang G. (2002). The checkpoint protein Chfr is a ligase that ubiquitinates Plk1 and inhibits Cdc2 at the G2 to M transition. *J Cell Biol* **156**: 249–259.

Kudo N, Matsumori N, Taoka H, Fujiwara D, Schreiner EP, Wolff B *et al*. (1999). Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region. *Proc Natl Acad Sci USA* **96**: 9112–9117.

Kyrylenko S, Kyrylenko O, Suuronen T, Salminen A. (2003). Differential regulation of the Sir2 histone deacetylase gene family by inhibitors of class I and II histone deacetylases. *Cell Mol Life Sci* **60**: 1990–1997.

la Cour T, Gupta R, Rapacki K, Skriver K, Poulsen FM, Brunak S. (2003). NESbase version 1.0: a database of nuclear export signals. *Nucleic Acids Res* **31**: 393–396.

la Cour T, Kiemer L, Molgaard A, Gupta R, Skriver K, Brunak S. (2004). Analysis and prediction of leucine-rich nuclear export signals. *Protein Eng Des Sel* **17**: 527–536.

Lengauer C, Kinzler KW, Vogelstein B. (1998). Genetic instabilities in human cancers. *Nature* **396**: 643–649.

Mattaj IW, Englmeier L. (1998). Nucleocytoplasmic transport: the soluble phase. *Annu Rev Biochem* **67**: 265–306.

Mizuno K, Osada H, Konishi H, Tatematsu Y, Yatabe Y, Mitsudomi T *et al*. (2002). Aberrant hypermethylation of the CHFR prophase checkpoint gene in human lung cancers. *Oncogene* **21**: 2328–2333.

Muhua L, Adames NR, Murphy MD, Shields CR, Cooper JA. (1998). A cytokinesis checkpoint requiring the yeast homologue of an APC-binding protein. *Nature* **393**: 487–491.

Musacchio A, Hardwick KG. (2002). The spindle checkpoint: structural insights into dynamic signaling. *Nat Rev Mol Cell Biol* **3**: 731–741.

Nitta M, Tsuiki H, Arima Y, Harada K, Nishizaki T, Sasaki K *et al*. (2002). Hyperploidy induced by drugs that inhibit formation of microtubule promotes chromosome instability. *Genes Cells* **7**: 151–162.

Nishigaki R, Osaki M, Hiratsuka M, Toda T, Murakami K, Jeang KT *et al*. (2005). Proteomic identification of differentially-expressed genes in human gastric carcinomas. *Proteomics* **5**: 3205–3213.

North BJ, Marshall BL, Borra MT, Denu JM, Verdin E. (2003). The human Sir2 ortholog, SIRT2, is an NAD+-dependent tubulin deacetylase. *Mol Cell* **11**: 437–444.

Paulovich AG, Toczyski DP, Hartwell LH. (1997). When checkpoints fail. *Cell* **88**: 315–321.

Piperno G, LeDizet M, Chang XJ. (1987). Microtubules containing acetylated alpha-tubulin in mammalian cells in culture. *J Cell Biol* **104**: 289–302.

Qian K, Chen H, Wei Y, Hu J, Zhu G. (2005). Differentiation of endometrial stromal cells *in vitro*: down-regulation of suppression of the cell cycle inhibitor p57 by HOXA10? *Mol Hum Reprod* **11**: 245–251.

Roberts EC, Deed RW, Inoue T, Norton JD, Sharrocks AD. (2001). Id helix-loop-helix proteins antagonize pax transcription factor activity by inhibiting DNA binding. *Mol Cell Biol* **21**: 524–533.

Saji S, Kawakami M, Hayashi S, Yoshida N, Hirose M, Horiguchi S *et al*. (2005). Significance of HDAC6 regulation via estrogen signaling for cell motility and prognosis in estrogen receptor-positive breast cancer. *Oncogene* **24**: 4531–4539.

Scolnick DM, Halazonetis TD. (2000). Chfr defines a mitotic stress checkpoint that delays entry into metaphase. *Nature* **406**: 430–435.

Serrador JM, Cabrero JR, Sancho D, Mittelbrunn M, Urzainqui A. (2004). HDAC6 deacetylase activity links the tubulin cytoskeleton with immune synapse organization. *Immunity* **20**: 417–428.

Shackney SE, Smith CA, Miller BW, Burholt DR, Murtha K, Giles HR *et al*. (1989). Model for the genetic evolution of human solid tumors. *Cancer Res* **49**: 3344–3354.

Takahashi T, Shivapurkar N, Riquelme E, Shigematsu H, Reddy J, Suzuki M *et al*. (2004). Aberrant promoter hypermethylation of multiple genes in gallbladder carcinoma and chronic cholecystitis. *Clin Cancer Res* **10**: 6126–6133.

Takai N, Hamanaka R, Yoshimatsu J, Miyakawa I. (2005). Polo-like kinases (Plks) and cancer. *Oncogene* **24**: 287–291.

Tsuiki H, Nitta M, Tada M, Inagaki M, Ushio Y, Saya H. (2001). Mechanism of hyperploid cell formation induced by microtubule inhibiting drug in glioma cell lines. *Oncogene* **20**: 420–429.

Xie S, Xie B, Lee MY, Dai W. (2005). Regulation of cell cycle checkpoints by polo-like kinases. *Oncogene* **24**: 277–286.

Yu X, Minter-Dykhouse K, Malureanu L, Zhao WM, Zhang D, Merkle CJ *et al*. (2005). Chfr is required for tumor suppression and Aurora A regulation. *Nat Genet* **37**: 401–406.