Oncogene (2007) 26, 1110–1121

www.nature.com/onc

ORIGINAL ARTICLE

RGC32, a novel p53-inducible gene, is located on centrosomes during mitosis and results in G2/M arrest

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To identify target genes for the hemizygous deletions of chromosome 13 that are recurrently observed in malignant gliomas, we performed genome-wide DNA copy-number analysis using array-based comparative genomic hybridization and gene expression analysis using an oligonucleotide-array. The *response gene to complement 32* (*RGC32*) at 13q14.11 was identified as a deletion target, and its expression was frequently silenced in glioma cell lines compared with normal brain. Levels of *RGC32* mRNA tended to decrease toward higher grades of primary astrocytomas, especially in tumors with mutations of *p53*. Expression of *RGC32* mRNA was dramatically increased by exogenous p53 in a p53-mutant glioma cell line, and also by endogenous p53 in response to DNA damage in $p53^{+/+}$ colon-cancer cells, but not in isogenic $p53^{-/-}$ cells. Chromatin immunoprecipitation and reporter assays demonstrated binding of endogenous p53 protein to the promoter region of the *RGC32* gene, implying p53-dependent transcriptional activity. Transiently and stably overexpressed *RGC32* suppressed the growth of glioma cells, probably owing to induction of G2/M arrest. Immunocytochemical analysis revealed a concentration of RGC32 protein at the centrosome during mitosis. RGC32 formed a protein complex with polo-like kinase 1 and was phosphorylated *in vitro*. These observations implied a novel mechanism by which p53 might negatively regulate cell-cycle progression by way of this newly identified transcriptional target. Our results provide the first evidence that *RGC32* might be a possible tumor-suppressor for glioma, that it is directly induced by p53, and that it mediates the arrest of mitotic progression.
Oncogene (2007) **26,** 1110–1121. doi:10.1038/sj.onc.1210148; published online 4 December 2006

Keywords: *RGC32*; p53; Plk1; glioma; array-CGH

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Received 22 May 2006; revised 12 September 2006; accepted 17 October 2006; published online 4 December 2006

Introduction

Astrocytomas, composed predominantly of astrocyte-like cells, are the most common neoplasm of the central nervous system (Kleihues *et al*., 2002). According to the World Health Organization (WHO) grading system, grades II–IV astrocytomas are biologically malignant and diffusely infiltrate the brain. Grade IV astrocytoma, or glioblastoma multiforme (GBM), is among the most aggressive of human cancers, with a median survival of less than 1 year. Unfortunately, this prognosis has not changed significantly over the past two decades despite advances in neurosurgery, radiation and chemotherapy (Holland, 2001; Maher *et al*., 2001; Zhu and Parada, 2002). As accumulation of genetic alterations within a cell lineage leads to development of malignant phenotypes of solid tumors, increasing knowledge about the genetic control of cellular proliferation can provide a basis for rational design of specific therapeutic strategies aimed at the regulation of proliferative disorders.

Abnormalities of the *p53* tumor-suppressor gene are found in a significant proportion of astrocytomas (Ohgaki *et al*., 2004). Low-grade diffuse astrocytomas (grade II), anaplastic astrocytomas (grade III) and secondary GBMs (grade IV) show a high incidence of *p53* mutations. In *de novo* GBMs, however, *p53* mutations are significantly less frequent, in spite of high malignancy (Ohgaki *et al*., 2004; Okamoto *et al*., 2004). Endogenous expression of wild-type p53, where present, is activated transcriptionally and post-translationally by a variety of cellular stresses, which in turn can suppress tumor formation through transcriptional regulation of genes that possess p53-binding sites (Ko and Prives, 1996). Growth arrest, DNA repair and apoptosis are thought to constitute the core mechanisms of p53-dependent tumor suppression. Although many p53-target genes have been reported, many more seem to remain unidentified based on the number of potential binding sites for p53 that are present in the human genome (Tokino *et al*., 1994). Transcriptional targets for p53, which may play important roles in the suppression of glioma formation, may be inactivated through *p53* mutation or other mechanisms during gliomagenesis.

In the course of a program to screen a panel of glioma cell lines for copy-number aberrations in a genome-wide manner using an in-house bacterial artificial chromosome (BAC)/P1-derived artificial chromosome (PAC) array (Inazawa *et al*., 2004), we identified frequent loss of 13q (Saigusa *et al*., 2005). Using that method in combination with analysis of genome-wide expression using an oligonucleotide-array, we analysed the same cell lines of glioma and identified *RGC32* at 13q14.11 as a gene frequently inactivated in gliomas, although it is present in normal brain. As tumors carrying *p53* mutations showed lower levels of *RGC32* expression than gliomas with wild-type p53, we investigated whether *RGC32* could be a direct transcriptional target of p53 and might function as a tumor suppressor for glioma. The results of those experiments suggested that *RGC32* is a novel p53-inducible gene and that its product might inhibit G2/M progression by possibly interacting polo-like kinase 1 (Plk1).

Given its frequent loss in aggressive gliomas, we propose that *RGC32* may be involved in gliomagenesis when it is inactivated by genetic aberrations and/or transcriptional dysregulation owing to *p53* mutation in glial cells.

Results

Possible target genes for 13q deletion in malignant gliomas

We previously observed frequent copy-number losses at 13q among 22 glioma cell lines by array-based comparative genomic hybridization (array-CGH) analysis (Saigusa *et al*., 2005). To identify possible target gene(s) for this deletion, we performed expression-array analysis of one of the cell lines (Marcus) showing 13q deletion, in comparison with normal brain. We compiled a list of four genes (*MRP63*, *ALOX5AP*, *RGC32*, *F10*) on 13q that showed $\log_2$ ratios < -0.76 (-2 s.d.) on the expression-array (Figure 1a, Table 1). We examined the status of mRNA expression for each of those four genes in our panel of 22 glioma cell lines by reverse transcriptase–polymerase chain reaction (RT–PCR) (Figure 1b), and confirmed reduced expression of *RGC32* in 21 of the 22 cell lines (95.5%) and of *F10* in 16 of them (72.7%). However, we detected no mutations in the *RGC32* gene in any of the glioma cell lines examined (data not shown).

Analysis of RGC32 *expression in primary astrocytomas*

We determined the level of *RGC32* mRNA expression in 35 primary astrocytomas using real-time quantitative RT–PCR, and compared the results with pathological grades. These experiments revealed a significant difference between low-grade diffuse astrocytoma (grade II) and GBM (grade IV) ($P = 0.003$, Student's *t*-test), and showed that median values of mRNA expression tended to decrease with higher grade of the tumor (Figure 1c).

Among the 35 primary astrocytomas we examined, nine (25.7%) carried mutations in exons 5–8 of the *p53* gene. Although the number of cases in each grade (grades II–IV) was too small for statistical analysis, *RGC32* mRNA expression appeared to be lower in *p53*-mutated tumors compared with tumors containing wild-type *p53*, within each grade (Figure 1d). These, along with earlier observations of frequent hemizygous deletions and silencing of the *RGC32* gene in multiple glioma cell lines, prompted us to examine whether *RGC32* is a transcriptional target for p53 and a possible tumor suppressor in glial tissue.

Induction of RGC32 *by p53 or DNA damage*

To examine whether ectopic expression of p53 could increase expression of endogenous *RGC32* mRNA in human cells, U-373 MG cells, whose p53 does not function, were infected with the *p53* gene by means of adenovirus-mediated transfer. Infection with a replication-deficient adenoviral vector harboring human *p53* (Ad-p53) induced expression of *RGC32* mRNA in U-373 MG cells, whereas a control adenoviral vector harboring human *β-galactosidase* (Ad-lacZ) did not (Figure 2a). To confirm regulation of *RGC32* expression by wild-type p53, we examined whether endogenous *RGC32* could be induced by DNA damage in a p53-dependent manner using isogenic $p53^{+/+}$ and $p53^{-/-}$ HCT116 colon-cancer cell lines (Figure 2b). After treatment with adriamycin (ADR), *RGC32* mRNA was significantly induced in HCT116 ($p53^{+/+}$) but not in HCT116 ($p53^{-/-}$) when endogenous p53 and p21 were induced in HCT116 ($p53^{+/+}$).

p53-responsive elements in the RGC32 *gene*

We found 10 potential p53-binding sites of 20 nucleotides around the *RGC32* gene, designated RGC32-RE1 to RGC32-RE10 (Figure 2c). Each of them matched the consensus p53-binding sequence by at least 80% (el-Deiry *et al*., 1992). To determine whether the RGC32-RE1 to RGC32-RE10 sequences were involved in p53-dependent transcriptional activity, we first performed a heterologous promoter-reporter assay using luciferase vectors prepared by cloning genomic sequences containing each potential binding site (RGC32-RE1 to RE10) upstream of the simian virus 40 (SV40) minimal promoter in pGL3. Among the 10 reporter plasmids, when co-transfected with a p53-expression (pCMV-Tag3-p53) or control plasmid (pCMV-Tag3-p53Mut and pCMV-Tag3-mock) into SaOS2 cells, the genomic fragment containing RGC32-RE2 (present in intron 2) showed increased luciferase activity in the presence of the wild-type p53 expression vector (data not shown). However, luciferase activity did not increase when the vector contained mutant p53 (data not shown). We constructed two reporter vectors, in which one or two copies ($\times 2$) of a synthesized oligonucleotide corresponding to the RGC32-RE2 sequence was cloned upstream of the SV40 minimal promoter in pGL3. As shown in Figure 2d, wild-type p53, but not mutant p53, induced more than threefold and 60-fold higher luciferase activities in cells transfected with pGL3-RGC32-RE2 and pGL3-RGC32-RE2 $\times 2$, respectively.

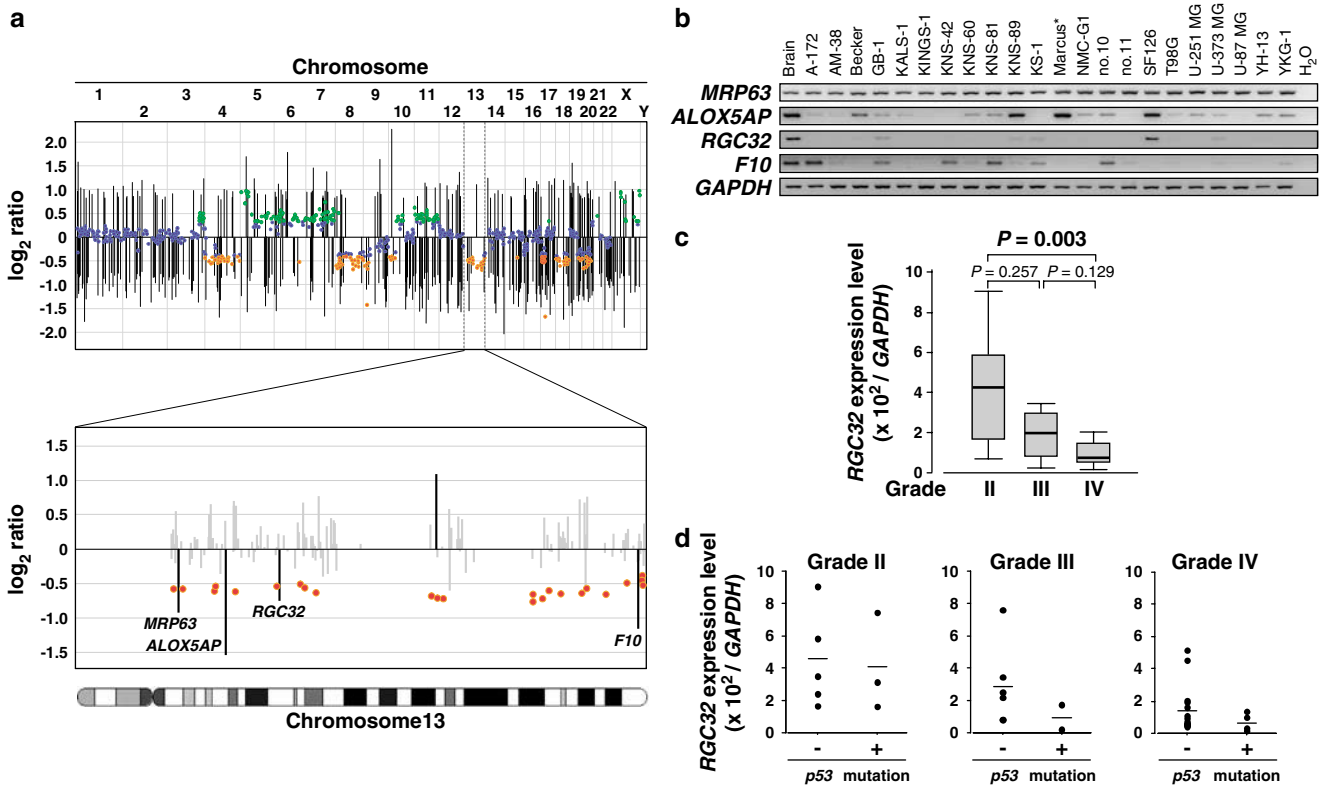

Figure 1 (**a**) Genome-wide (upper panel) and chromosome 13 (lower panel) profiles of copy-number and mRNA expression in the glioma cell line, Marcus. Clones are ordered from chromosomes 1–22, X and Y, and within each chromosome on the basis of the UCSC mapping position (http://genome.ucsc.edu/). Colored spots indicate BACs with normal genomic copy-number ratio (blue), with increased ratio (green), or with decreased ratio (red) on the MCG Cancer Array-800. Vertical black bars indicate genes with expression copy-number ratios larger than ±2 s.d., and gray bars denote genes with ratios within ±2 s.d., according to results of experiments using the AceGene Human oligo chip 30K. Gray bars are depicted only for chromosome 13 because the upper panel is too crowded to demonstrate differences in this way. (**b**) Expression of *MRP63*, *ALOX5AP*, *RGC32* and *F10* as determined by RT–PCR in 22 glioma cell lines and normal brain. An asterisk indicates the cell line (Marcus) with 13q deletion that was used for the array analyses depicted in Figure 1a. Note that 21 of the 22 cell lines (95.5%) showed very low levels of *RGC32* expression compared with normal brain. (**c**) Expression of *RGC32* mRNA in primary astrocytomas as determined by real-time quantitative RT–PCR experiments, compared with histopathological grade. Mean values are indicated by the horizontal bars within the boxes; vertical bars indicate s.e. Grades II, III and IV indicate, respectively, low-grade diffuse astrocytoma, anaplastic astrocytoma and GBM. These experiments revealed a significant difference between grade II and IV in terms of *RGC32* expression ($P = 0.003$, Student's *t*-test). *RGC32* mRNA expression in grades III and IV tumors tended to be lower than in grades II and III, but the differences did not reach statistical significance ($P = 0.257$ and $P = 0.129$, respectively, Student's *t*-test). (**d**) Expression of *RGC32* mRNA among primary astrocytomas of each grade, compared with *p53* mutation status. Horizontal bars represent means of the expression levels.

Table 1 Downregulated genes in glioma cells with 13q deletion

Acc. number	*LocusLink ID*	*Location*	*Gene*		log_2 *ratio*[a]
			Symbol	*Gene name*	
NM_024026_1	78988	13p11.1–q11	*MRP63*	Mitochondrial ribosomal protein 63	−0.99
NM_001629_1	241	13q12	*ALOX5AP*	Arachidonate 5-lipoxygenase-activating	−1.61
NM_014059_1	28984	13q14.11	*RGC32*	RGC32 protein	−0.83
NM_000504_1	2159	13q34	*F10*	Coagulation factor X	−1.22

[a]Cy3-labeled Marcus cell line sample/Cy5-labeled normal brain sample (see Figure 1a).

To verify whether p53 could bind to this candidate site, we used chromatin immunoprecipitation (ChIP) assays to analyse cell lysates extracted from HCT116 ($p53^{+/+}$) cells treated with ADR, which would imply interaction with endogenous p53 in response to genotoxic stress. As expected, endogenous p53 protein resided at the *p21* promoter containing a p53-responsive element *in vivo*. An anti-p53 antibody precipitated the genomic fragment including RGC-RE2 as a p53 protein–DNA complex (Figure 2e). In contrast, DNA fragments lacking RGC32-RE2 were amplified in the input control for PCR but not in the p53 protein–DNA

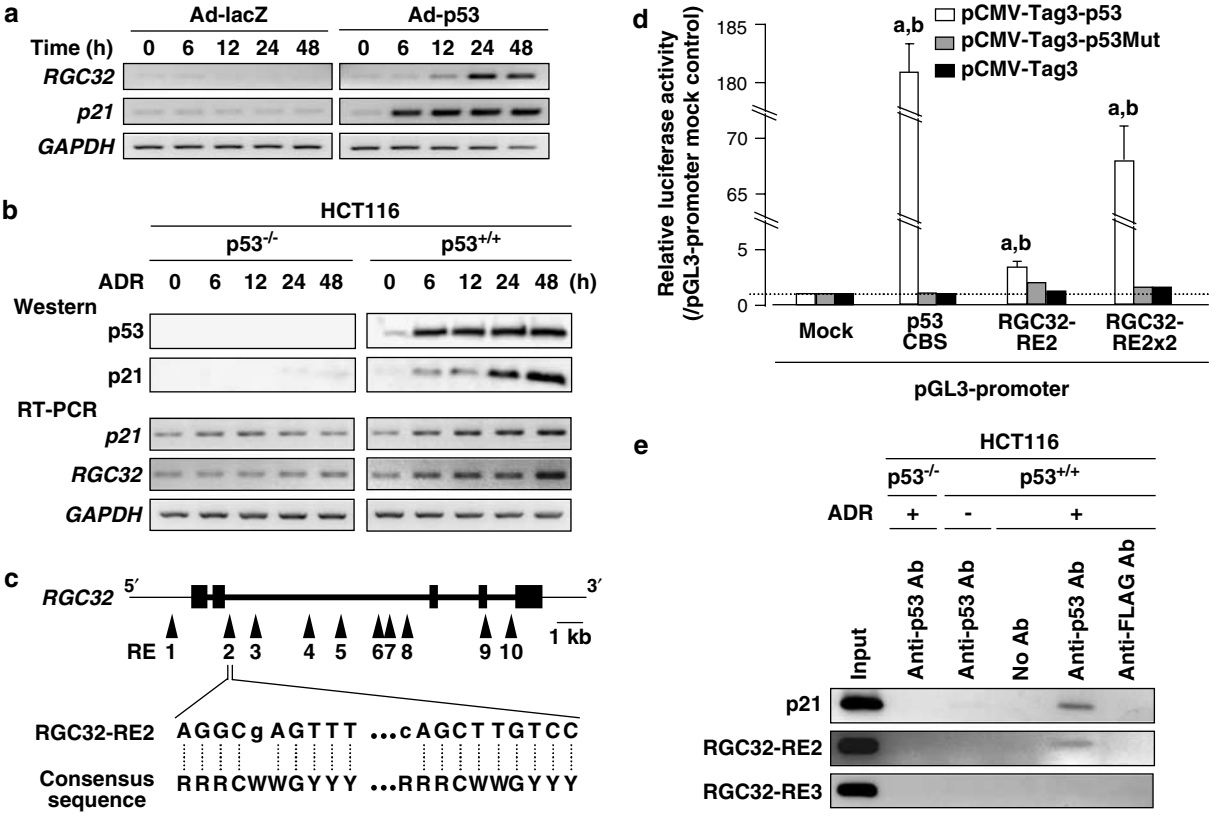

Figure 2 (**a**) RT–PCR analysis of *RGC32* mRNA expression in U-373 MG cells at the indicated times after infection with Ad-p53 or Ad-lacZ. *p21* and *GAPDH* were used as positive and internal controls, respectively. (**b**) Induction of *RGC32* transcription by endogenous p53 in $p53^{+/+}$ HCT116 cells after DNA damage from 1 μg/ml ADR. Expression of *p21* and *GAPDH* served, respectively, as positive and loading controls. (**c**) Genomic organization of *RGC32*. Black boxes indicate the locations and relative sizes of five exons. Arrowheads indicate potential p53-responsive elements (RGC32-RE1-10). The sequence of RGC32-RE2 is shown in comparison to the consensus p53-binding sequence (p53CBS). Capital letters represent nucleotides identical to the p53CBS; lowercase letters identify disparities. The RGC32-RE1-10 sequences contain only one- to four-mismatched nucleotides compared with p53CBS. R, purine; Y, pyrimidine; W, A or T. (**d**) Luciferase assay for RGC32-RE2 and RGC32-RE2 $\times$ 2 (two copies of RGC32-RE2) sequences. SaOS2 cells were co-transfected with a reporter plasmid containing either the p53CBS in the promoter region of *p21* (pGL3-p53CBS), one copy of RGC32-RE2 (pGL3-RGC32-RE2) or two copies of RGC32-RE2 (pGL3-RGC32-RE2 $\times$ 2) with minimal SV40 promoter, together with phRL-TK vector, and either a wild-type p53 (pCMV-Tag3-p53) or mutant p53 expression construct (pCMV-Tag3-p53Mut) or a control expression vector (pCMV-Tag3). Relative luciferase activity was defined as the measured activity divided by that of the pGL3-promoter mock control. The histograms represent the average of three independent experiments, and error bars show the s.e. Statistical analysis used the Mann–Whitney *U*-test: (a) pCMV-Tag3-p53 co-transfected cells versus pCMV-Tag3-p53Mut co-transfected cells; (b) pCMV-Tag3-p53 co-transfected cells versus pCMV-Tag3 co-transfected cells. All $P<0.05$. (**e**) ChIP assay showing that p53 protein binds to RGC32-RE2 sites *in vivo*. Immunoprecipitation of DNA–protein complexes using an antibody against p53 (Ab-6) was performed on cross-linked extracts from untreated (lane 3) and ADR-treated (lane 5) HCT116 ($p53^{+/+}$) cells; immunoprecipitated samples containing *p21* promoter, RGC32-RE2 or RGC32-RE3 were amplified by PCR. A portion of the sonicated chromatin before immunoprecipitation (input, lane 1), served as a positive control. Immunoprecipitates from anti-p53 antibody in HCT116 ($p53^{-/-}$) lysates (lane 2) or anti-FLAG antibody in HCT116 ($p53^{+/+}$) lysates (lane 6) or in the absence of antibody (lane 4) served as negative controls. The DNA fragment containing the RGC32-RE2 was detected only in the sample immunoprecipitated with the anti-p53 antibody in ADR-treated HCT116 ($p53^{+/+}$) cells (lane 5); one of the other candidate sequences, RGC32-RE3, was amplified only in the input control.

complex precipitated with an anti-p53 antibody (Figure 2e and data not shown), suggesting that endogenous p53 protein associates with RGC32-RE2 *in vivo*. Taken together, these results clearly indicate that *RGC32* is a genuine transcriptional target for p53.

Suppression of cell growth after restoration of RGC32

To gain further insight into the potential role of RGC32 loss in the genesis of glioma, we investigated whether restoration of RGC32 would suppress growth of glioma cells that lack expression in this gene. We performed colony-formation assays using the full coding sequence of *RGC32* cloned into a mammalian expression vector (pCMV-Tag3-RGC32). Three weeks after transfection and subsequent selection of drug-resistant colonies, the number of large colonies (>2 mm) produced by RGC32-transfected U-87 MG cells decreased compared to cells containing empty vector ($P=0.02$, Student's *t*-test; Figure 3a). Furthermore, stable RGC32 transfectants established from glioma cell line U-87 MG, which does not express this gene, showed a lower growth rate than cells transfected with the control vector (Figure 3b).

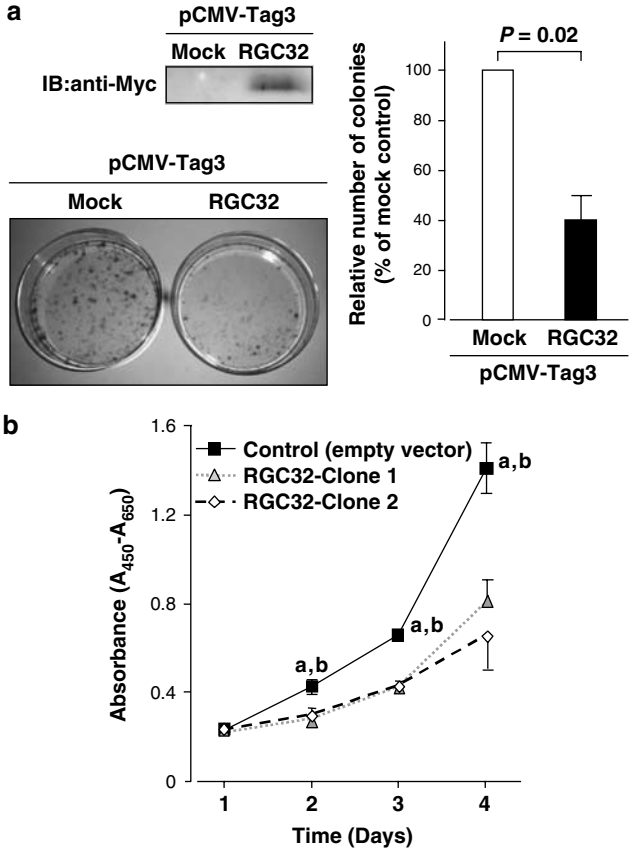

Figure 3 (**a**) Effect of restored RGC32 on growth of glioma cells. A Myc-tagged expression construct containing RGC32 (pCMV-Tag3-RGC32), or empty vector (pCMV-Tag3-Mock) as a control, was transfected into U-87 MG cells lacking expression of the *RGC32* gene. Western blotting using 10 μg of protein extract and anti-Myc antibody demonstrated that cells transiently transfected with pCMV-Tag3-RGC32 expressed Myc-tagged RGC32 protein (left, top). Three weeks after transfection and subsequent selection of G418-resistant colonies in 10-cm plates, the colonies formed by RGC32-transfected cells were less numerous than those formed by mock-transfected cells (left, bottom). The right panel shows a quantitative analysis of colony formation; the relative number of >2 mm colonies of RGC32-expressing cells, defined as the number divided by that of the mock control, was decreased to 39.7% ($P=0.02$, Student's *t*-test). The histograms represent the average of three independent experiments, each performed in triplicate, and an error bar shows the s.e. (**b**) The growth rate of U-87 MG cells stably expressing RGC32 was lower than that of cells transfected with mock plasmid. U-87 MG cells were transfected with pCMV-Tag3-RGC32 or pCMV-Tag3-Mock (empty vector), and selected with G418 to establish clones stably expressing RGC32. Cell viability was determined by WST assay at the indicated times. Each point indicates the means of three separate experiments; error bars show s.e. Statistical analysis used the Mann–Whitney *U*-test: (a) control versus clone 1; (b) control versus clone 2. All $P<0.05$.

Subcellular localization of RGC32 protein throughout the cell cycle

We transiently transfected a Myc-tagged RGC32 expression plasmid into U-87 MG cells, and stained the cells using an anti-Myc antibody along with anti-β-tubulin (Figure 4a) or anti-γ-tubulin antibody (Figure 4b). Ectopically expressed RGC32 protein was located in the cytoplasm during interphase, with the strongest signal around the nuclear membrane. Progression into prophase was marked by an increase in the centrosomal signal of the RGC32 protein. Centrosome staining with RGC32 increased further and seemed to be maximal at prometaphase and metaphase. During telophase and cytokinesis, low levels of RGC32 protein remained in the centrosome. Similar staining patterns were observed in HeLa Tet-On cells expressing green fluorescence protein (GFP)-tagged RGC32, after treatment with doxycycline (Dox) and subsequent visualization of the ectopically expressed protein with anti-GFP antibody (data not shown), and in SF126 cells expressing endogenous RGC32 visualized using antibody specific to RGC32 (Figure 4c).

G2/M arrest after overexpression of RGC32

As RGC32 seemed to be a centrosome-associated protein, we used flow cytometry to determine the effect of ectopic RGC32 expression on progression of the cell cycle. When expression of GFP protein alone or GFP-tagged RGC32 protein (GFP-RGC32) was induced by Dox in HeLa Tet-On cells, each protein was detected as early as 12 h after induction and the expression levels increased until 24 h after induction (data not shown). An increase in the proportion of cells in G2/M was observed in asynchronous cultures of HeLa Tet-On cells that overexpressed GFP-RGC32 (24.87%) compared with control cells (16.53%, Figure 5a). When cell-cycle analysis was performed using synchronized cells (Figure 5b), HeLa Tet-On cells that were overexpressing GFP-RGC32 protein showed simultaneous transition into G2/M phase from G1 (0–8 h after release), but showed delayed transition into G1 phase from G2/M phase (10–14 h after release) compared with control cells. Cells overexpressing RGC32 did not show an apoptotic phenotype, such as a sub-G1 population, in flow cytometry.

Binding of RGC32 to Plk1 and phosphorylation of RGC32 by Plk1 in vitro

As ectopically expressed RGC32 protein induced delayed transition into G1 phase from G2/M and localized at centrosomes during mitosis, we examined whether RGC32 would interact with mitotic kinases in mammalian cells. First, we compared levels of Plk1, cyclin B1, Aurora-A and Aurora-B proteins throughout progression of the cell cycle, between RGC32-overexpressing cells and control cells, by immunoblotting synchronized lysates. HeLa Tet-On cells overexpressing GFP-RGC32 protein showed similar expression levels to those expressing GFP, through mitosis and the timing of destruction of cyclin B1, Aurora-A and Aurora-B proteins. On the other hand, Plk1 protein was observed longer in cells expressing GFP-RGC32 than in cells expressing GFP during cell-cycle progression (Figure 5c), suggesting that the degradation of Plk1 might be suppressed in RGC32-overexpressing cells. Interestingly, slow-migrating form (arrow in Figure 5c) of GFP-RGC32 protein was detected following release from double thymidine block, especially 6–12 h after

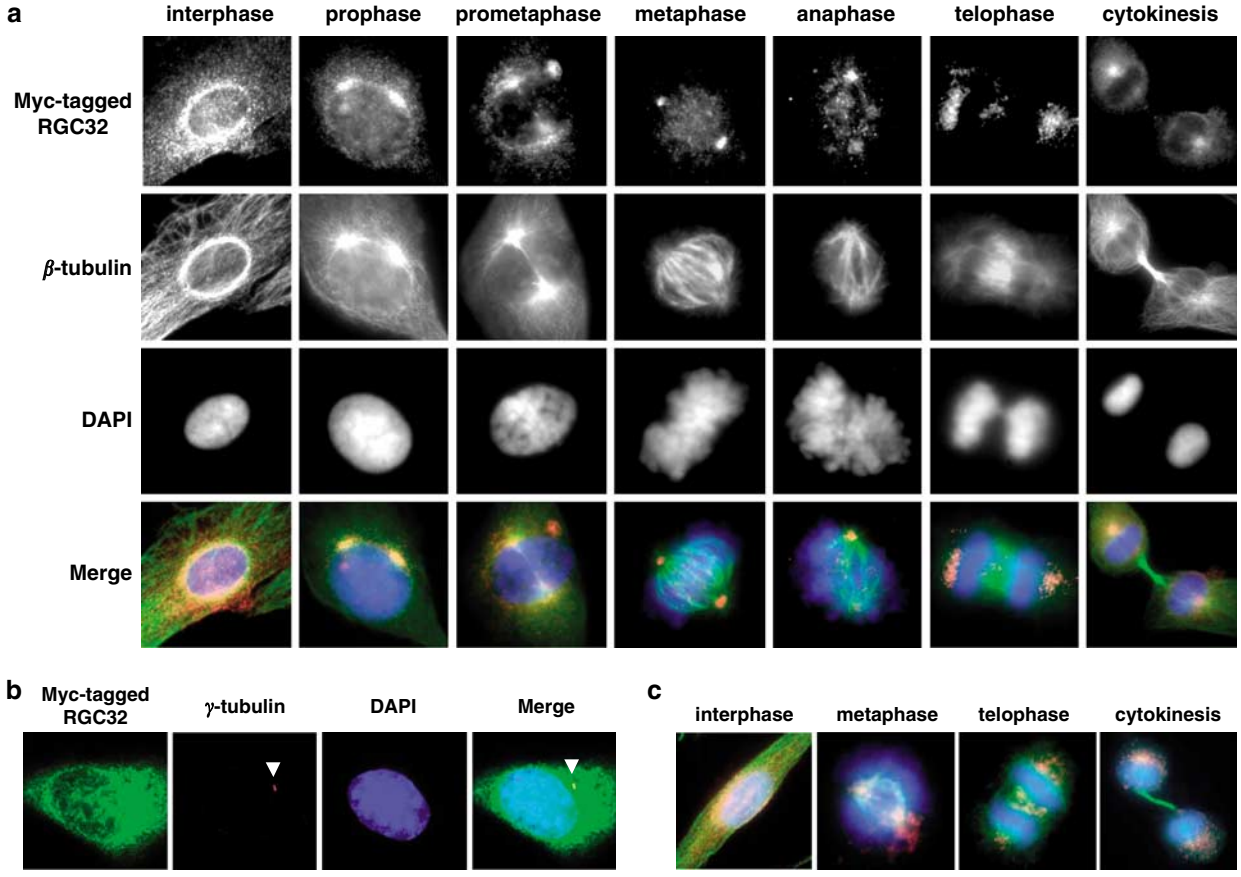

Figure 4 Subcellular localization of RGC32 protein. (**a**) U-87 MG cells were transiently transfected with RGC32 expression vector (pCMV-Tag3-RGC32) containing Myc epitope-tagged RGC32. Cells were fixed, stained with antibodies to the Myc epitope (red) and to endogenous β-tubulin (green), and treated with appropriate secondary antibodies. Nuclei were detected by staining cells with DAPI (blue). Ectopic RGC32 protein was located in the cytoplasm during interphase, with the strongest signal around the nuclear membrane. RGC32 protein appeared mainly on centrosomes during mitosis, especially at metaphase. (**b**) U-87 MG cells transiently transfected with Myc-tagged RGC32 were stained with antibodies to the Myc epitope (green) and to endogenous γ-tubulin (red), and treated with appropriate secondary antibodies. Nuclei were detected by staining cells with DAPI (blue). During interphase, ectopic RGC32 protein was located in the cytoplasm but not on centrosomes. (**c**) SF126 cells expressing endogenous RGC32 was stained with anti-RGC32 antibody (red) and anti-β-tubulin (green) using appropriate secondary antibodies. Nuclei were detected by staining cells with DAPI (blue). Although expression level of endogenous RGC32 is too low to specifically and clearly detect by antibody, similar localization pattern was obtained in interphase, metaphase, telophase and cytokinesis.

release, suggesting that RCG32 protein might be modified during cell-cycle progression. The slow-migrating form of GFP-RGC32 protein in whole-cell lysates from mitotic phase GFP-RGC32-expressing HeLa Tet-On cells shifted down to the same position as its fast-migrating form after treatment with λ-protein phosphatase (λ-PPase) and this effect was inhibited by the λ-PPase inhibitor vanadate (Figure 5d), suggesting that phosphorylation might be one of modifications observed in RGC32.

We next determined whether mitotic kinases and RGC32 physically interact. FLAG-tagged RGC32 and Myc-tagged PlK1 or FLAG-tagged Plk1 and Myc-tagged RGC32 coimmunoprecipitated when transiently expressed in human embryonic kidney (HEK) 293-T cells (Figure 6a), whereas no physical interaction between RGC32 and cyclin B1, Aurora-A or Aurora-B was not detected (Figure 6a). To determine the involvement of Plk1 polo-box and the kinase domains in Plk1 interaction with RGC32, anti-FLAG immunoprecipitates from lysates of HEK 293-T cells transiently coexpressing FLAG- or Myc-tagged Plk1, Plk1(1-330), or Plk1(330-CT) and Myc- or FLAG-tagged RGC32, respectively, were immunoblotted with anti-Myc antibody (Figure 6b). Interaction of RGC32 with Plk1 or Plk1(330-CT) was clearly detected, whereas only a trace of interaction was observed between RGC32 and Plk1(1-330), suggesting that RGC32 may predominantly interact with Plk1(330-CT) containing Plk1 polo-box domain.

In addition, when a lysate of cells expressing GFP-RGC32 was treated with antibody to GFP, immunoblotting revealed the presence of endogenous Plk1 in the resulting precipitate (Figure 6c).

To examine the phosphorylation of RGC32 by Plk1 *in vitro*, we mixed a recombinant His_6-RGC32 protein with recombinant Plk1 protein, and assayed whether Plk1 phosphorylates RGC32 as a substrate. The result is

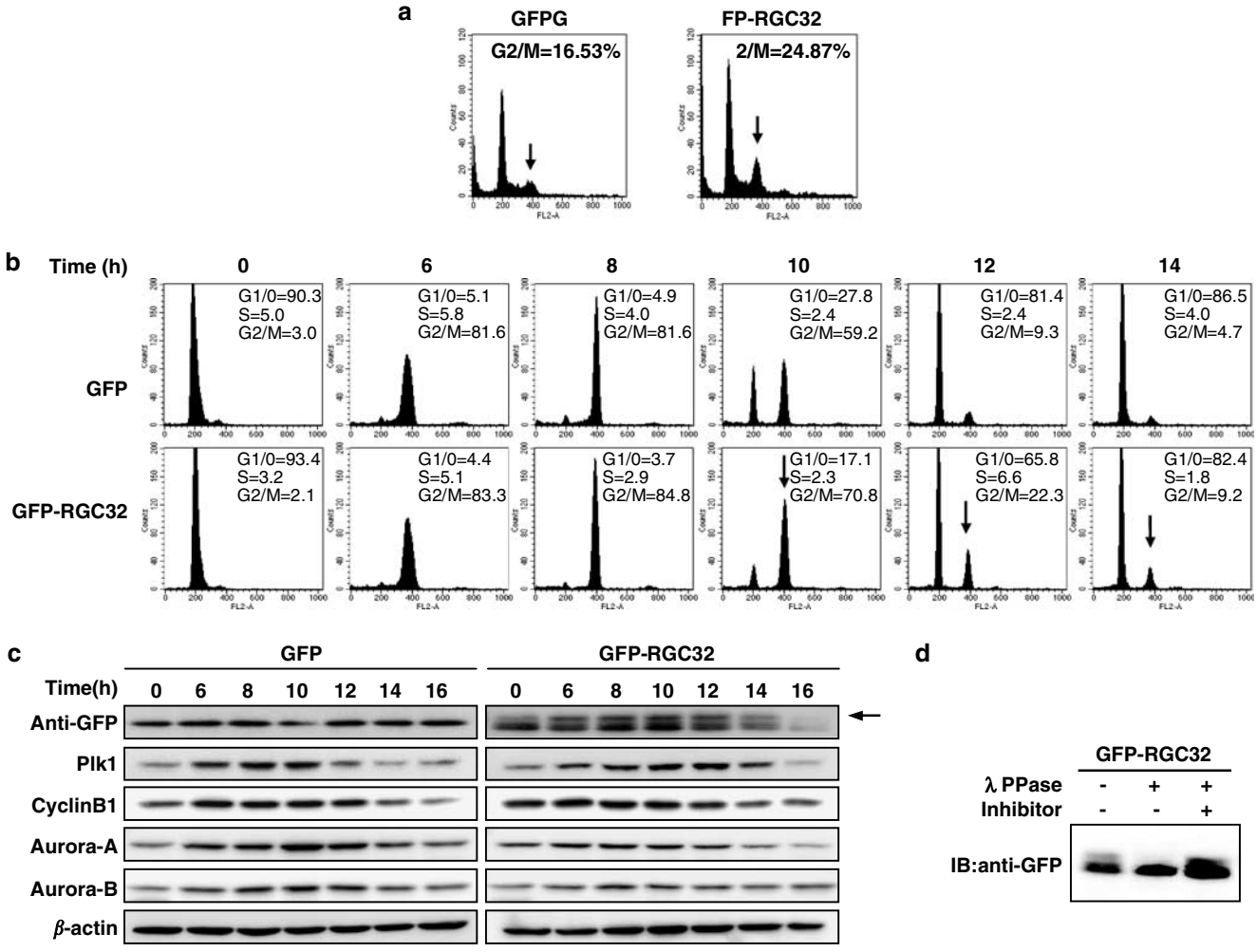

Figure 5 (**a**) FACS analysis of GFP- or GFP-RGC32-expressing HeLa Tet-On cells cultured in the presence of Dox. The 4N peak (G2/M phase) is indicated by arrows. (**b**) FACS analysis of GFP- or GFP-RGC32-expressing HeLa Tet-On cells cultured in the presence of Dox, synchronized at the G1/S boundary by double thymidine block and then released into fresh medium for the indicated times. GFP-RGC32-expressing HeLa Tet-On cells showed delayed mitotic exit (arrows) compared with control (GFP-expressing) cells. (**c**) Cell cycle-dependent expression of mitotic kinases in GFP- or GFP-RGC32-expressing HeLa Tet-On cells synchronized by double thymidine block and collected at indicated times after release from arrest at the G1/S boundary. Levels of GFP or GFP-RGC32, Plk1, cyclin B1, Aurora-A and Aurora-B were determined by immunoblotting. Cell-cycle synchronization was confirmed by flow cytometry. Slow-migrating form (arrow) of GFP-RGC32 protein was detected following release from double thymidine block, especially 6–12 h after release, suggesting that RCG32 protein might be modified during cell-cycle progression. (**d**) Whole-cell lysates from mitotic phase GFP-RGC32-expressing HeLa Tet-On cells were mock treated or treated with λ-PPase in the presence or absence of the PPase inhibitor vanadate. Western blotting was performed as in panel **c**. Note that, after treatment with λ-PPase, the slow-migrating form of GFP-RGC32 protein shifted down to the same position as its fast-migrating form, and this effect was inhibited by vanadate.

that His_6-RGC32 was phosphorylated by Plk1 (Figure 5e), suggesting that RGC32 may be a normal substrate of this enzyme; in fact, Ser9, Thr82 and Thr113 in the amino-acid sequence of RGC32 fit an emerging consensus sequence for Plk1 phosphorylation sites.

Discussion

In the study presented here, we identified *RGC32* as a novel target for 13q deletion in gliomas, through a genome-wide exploration using a combination of copy-number analysis by array-CGH and expression analysis by oligonucleotide-array. A clear inverse correlation emerged between histopathological grade and the expression status of *RGC32* in primary astrocytomas. Together with a demonstrated growth-suppressive effect of exogenous RGC32, the data suggested that *RGC32* is likely to be a tumor suppressor gene associated with clinical and/or biological aggressiveness of glial tumors. Our results further underscored the promise of a combination of array-CGH and oligonucleotide expression-array as a high-throughput screening method for revealing tumor-suppressor genes, as well as oncogenes, in cancer genomes.

RGC32 locates at 13q14.11, a chromosomal region that is often involved in loss of heterozygosity (LOH) or loss of copy number in glioma cells (Nishizaki *et al*.,

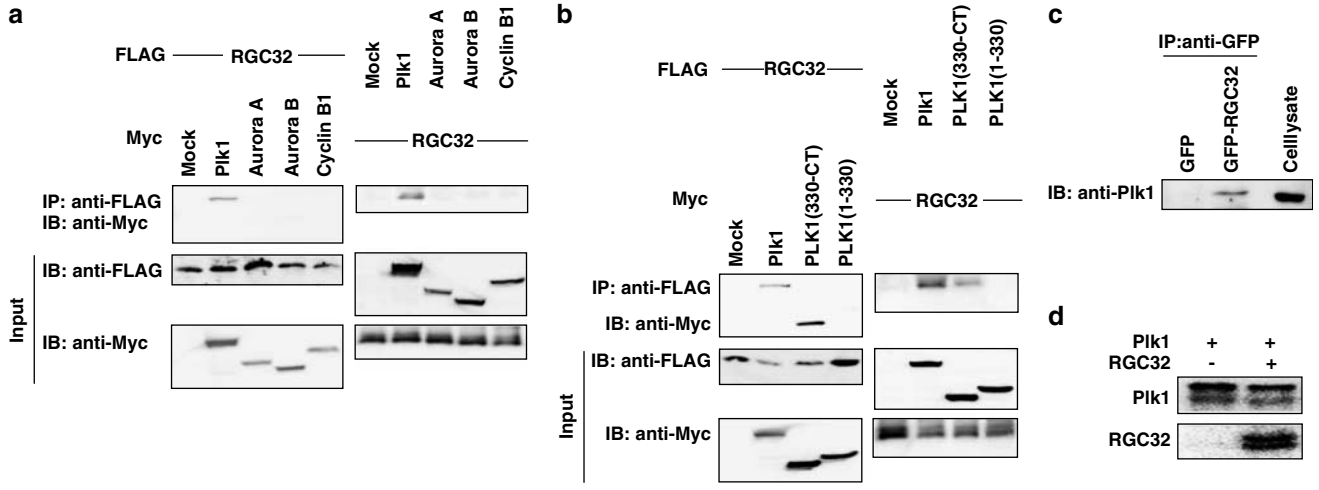

Figure 6 (**a**) Lysates from HEK 293-T cells transiently coexpressing FLAG- or Myc-tagged RGC32 and Myc- or FLAG-tagged kinases (PlK1, Aurora-A, Aurora-B or cyclin B1), respectively, were immunoprecipitated with mouse anti-FLAG antibody and immunoblotted with rabbit polyclonal anti-Myc antibody. Whole-cell lysates (input) were also immunoblotted with anti-FLAG or anti-Myc antibody. (**b**) Lysates from HEK 293-T cells transiently coexpressing FLAG- or Myc-tagged Plk1, Plk1(1-330), or Plk1(330-CT) and Myc- or FLAG-tagged RGC32, respectively, were immunoprecipitated with mouse anti-FLAG antibody and immunoblotted with rabbit polyclonal anti-Myc antibody. Whole-cell lysates (input) were also immunoblotted with anti-FLAG or anti-Myc antibody. (**c**) Formation of a complex between RGC32 and endogenous Plk1 in HeLa Tet-On cells expressing GFP or GFP-RGC32 in the presence of Dox. Whole-cell lysates were immunoprecipitated with anti-GFP antibody, and immunoblotted with anti-Plk1 antibody. Whole-cell lysates were also immunoblotted with anti-Plk1 antibody to show expression of endogenous Plk1. (**d**) Phosphorylation of RGC32 by Plk1 *in vitro*. Recombinant Plk1 was mixed with recombinant His_6-RGC32 in Raf buffer containing [γ-^{32}P]ATP. Using SDS–PAGE and autoradiography, we determined phosphorylation of His_6-RGC32 by Plk1 and autophosphorylation of Plk1.

1998; Kunwar *et al*., 2001). Indeed, by array-CGH analysis 14 of the 22 cell lines we examined for this study (63.6%) had shown losses in DNA copy number at 13q (Saigusa *et al*., 2005). Although *RB1*, one of the best known of all tumor-suppressor genes, locates at 13q14.2 and is often involved in LOH or copy-number losses in glioma cells, loss of RB1 function is not always associated with genetic loss (Burns *et al*., 1998). Moreover, cell lines (Saigusa *et al*., 2005) as well as clinical specimens (Inda *et al*., 2003) of glioma usually show large losses of genomic material from chromosome 13, suggesting that tumor-suppressor genes other than *RB1* are likely to lie in that region. Despite previous studies that have focused on the incidence and prognostic implication of 13q deletion in tumors of many types (Sato *et al*., 1991; Shaughnessy *et al*., 2000; Jongsma *et al*., 2002; Pan *et al*., 2005; Lu *et al*., 2006), except for *RB1* any tumor-suppressor genes within this region that might be relevant to specific diseases remain largely unknown. Further examination may clarify whether loss of RGC32 function might be involved in the pathogenesis of tumors arising in tissues other than neuroglia.

We found the *RGC32* gene to be a direct transcriptional target of p53 in human cells of various tissue origins, and we demonstrated that overexpression of *RGC32* in several cancer-cell lines triggered suppression of cell growth, especially mitotic progression. Cell-cycle regulation and apoptosis are the most important features of p53-dependent tumor suppression, each of those mechanisms being mediated by several p53-target genes. For example, p21 is a pivotal mediator for arrest of the cell cycle at G1 phase, whereas Bax and p53AIP1 mediate p53-dependent apoptosis (Miyashita and Reed, 1995; Oda *et al*., 2000). However, the effect of RGC32 is unlikely to be due to either cell-cycle arrest at G0/G1 phase or induction of apoptosis, because we observed no remarkable changes with respect to those phenomena in our transient- or inducible-transfection assays. The results suggested instead that another p53-dependent pathway to suppress cell growth was operating. Although several approaches have been previously employed to identify genes whose expression is regulated by p53, the *RGC32* gene has never before been reported as inducible by p53. One explanation might be that the expression level of *RGC32*, or its response to p53, may vary considerably among different cell types.

Given a lack of significant motifs in the RGC32 amino-acid sequence, we analysed the subcellular localization of RGC32 using an ectopically expressed, epitope-tagged protein. A detailed analysis indicated that RGC32 protein is concentrated on centrosomes and spindle poles during mitosis, and this observation prompted us to explore potential interactions between RGC32 protein and mitotic kinases that localize on centrosomes with the same timing as RGC32. Immunoprecipitation experiments revealed that RGC32 interacts with endogenous Plk1, but not with cdc2/cyclin B1, Aurora-A or Aurora-B. By *in vitro* kinase assays, we demonstrated that this enzyme phosphorylates RGC32, although functional differences between phosphorylated and dephosphorylated forms of RGC32 remain unknown.

As (a) fluorescence-activated cell sorting (FACS) analysis after cell-cycle synchronization demonstrated that overexpression of RGC32 delays mitotic progression and (b) immunocytochemical analysis showed that

RGC32 stays on centrosome/spindle poles during mitosis, whereas Plk1 is known to localize to the central spindle in anaphase–telophase (Barr *et al*., 2004), RGC32 may interact with and be phosphorylated by Plk1 during mitotic progression, especially between prophase and anaphase, and inhibit mitotic exit.

Plk1 is a key regulator of mitosis (Barr *et al*., 2004), but is also associated with oncogenesis; expression and kinase activity of Plk1 is elevated in many kinds of cancers including gliomas (Dietzmann *et al*., 2001; Takai *et al*., 2005). In some experiments, approximately 50% of cells transiently treated with small interfering RNA (siRNA) for Plk1 were arrested at G2/M, with 4N DNA content in FACS profiles, and showed a dumbbell-like DNA organization that suggested incomplete separation of sister chromatids (Liu and Erikson, 2002, 2003). Inactivation of Plk1 by a variety of methods has caused cell-cycle arrest at mitosis and/or apoptosis in cancer cells of several types (Lane and Nigg, 1996; Mundt *et al*., 1997; Elez *et al*., 2000; Liu and Erikson, 2002, 2003; Spankuch-Schmitt *et al*., 2002), suggesting that inhibition of Plk1 function might be a promising approach to cancer therapy (Gumireddy *et al*., 2005). As RGC32 binds to and is phosphorylated by Plk1 *in vitro*, it is possible that RGC32 may negatively regulate cell cycle through interaction with Plk1.

RGC32 was formerly identified as a gene induced in oligodendrocytes by activation of complement (Badea *et al*., 1998). Overexpression of RGC32 induces quiescent aortic smooth-muscle cells to enter into the S-phase (Badea *et al*., 2002). Others have reported that RGC32 physically associated with cyclin-dependent kinase $p34^{CDC2}$, and that its kinase activity increased through phosphorylation by $p34^{CDC2}$-cyclin B1 (Badea *et al*., 2002). In our experiments, however, overexpressed RGC32 inhibited cell growth in glioma cell lines as well as in HeLa cells. We found that RGC32 does physically associate with Plk1, but not with $p34^{CDC2}$-cyclin B1 *in vitro*. Moreover, in our preliminary experiments, RGC32 was not phosphorylated by $p34^{CDC2}$-cyclin B1. Although we have no evidence to explain the discrepancies between previous reported findings and our results, it is possible that RGC32 may function differently among different types of cells. Further examination will be needed to clarify the significance of RGC32 in normal physiology and in tumorigenesis, especially in progression of gliomas.

Materials and methods

Cell lines and primary tumors

Of the 22 glioma cell lines (Saigusa *et al*., 2005) employed in this study, all except U-87 MG and U-373 MG were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). U-87 MG and U-373 MG, as well as the SaOS2 cell line, were obtained from the American Type Culture Collection (Rockville, MD, USA). HeLa Tet-On cells (cervical-cancer derivative) were obtained from Clontech (Mountain View, CA, USA); HCT116 ($p53^{+/+}$) and HCT116 ($p53^{-/-}$) cell lines (colon-cancer derivatives) were generous gifts from Dr Bert Vogelstein. All lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/100 μg/ml streptomycin.

Primary tumor samples were obtained during surgery from 35 astrocytoma patients who underwent surgical resection at the Department of Neurosurgery, Tokyo Medical and Dental University Hospital, with prior written consent from each patient in the formal style and after approval by the local ethics committee. Hematoxylin–eosin staining was routinely performed in order to classify each tumor according to criteria of the WHO. Of the 35 cases, eight were low-grade diffuse astrocytomas (grade II), eight were anaplastic astrocytomas (grade III) and 19 were GBMs (grade IV). The tumor samples were frozen immediately in liquid nitrogen and stored at -80°C until required.

Array-CGH analysis and oligonucleotide expression-array analysis

Array-CGH analysis using our in-house BAC/PAC-array ('MCG Cancer Array-800') was carried out as described elsewhere (Sonoda *et al*., 2004; Saigusa *et al*., 2005; Takada *et al*., 2005). mRNA-expression profiling was performed as described elsewhere (Inoue *et al*., 2004), using the AceGene Human oligo chip 30K (DNA Chip Research, Yokohama, Japan). Test and reference cDNA probes labeled with aminoallyl-dUTP were synthesized using oligo(dT)12–18 primer, and coupled, respectively, with Cy3- or Cy5-mono-reactive dye. The hybridized slides were scanned with a GenePix 4000B (Axon Instruments, Foster City, CA, USA), and analysed with GenePix Pro 6.0 imaging software (Axon Instruments). Fluorescence ratios were normalized as described elsewhere (Inoue *et al*. 2004; Saigusa *et al*., 2005).

RT–PCR and real-time quantitative RT–PCR

Single-stranded cDNAs generated from total RNAs were amplified with primers specific for each gene (Sonoda *et al*., 2004). Primer sequences are available on request. Levels of *RGC32* mRNA in primary tumors were measured by means of a quantitative real-time fluorescence detection method (PRISM 7900HT, Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol (Takada *et al*., 2005). The glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) served as an endogenous control; the expression level of *RGC32* mRNA in each sample was normalized on the basis of the respective *GAPDH* content and recorded as a relative expression level. PCR amplification was performed in duplicate for each sample.

Detection of p53 *and* RGC32 *mutations*

Genomic DNA isolated from cell lines or frozen tissues was amplified by PCR with primer sets specific for exons 5–8 of the *p53* gene (Nakamura *et al*., 2001) and for all five coding exons of the *RGC32* gene, followed by direct sequencing.

Construction of recombinant adenovirus and infection of glioma cells

Replication-deficient recombinant viruses, Ad-p53 and Ad-lacZ, encoding p53 and lacZ, respectively, were generated and purified as described previously (Oda *et al*., 2000). U-373 MG cells were infected with viral solutions at indicated multiplicities of infection and incubated at 37°C until the time of harvest.

DNA-damaging treatments

HCT116 ($p53^{+/+}$) and HCT116 ($p53^{-/-}$) cells were seeded 24 h before treatment and were 70% confluent at the time of

treatment. To examine expression of *RGC32* in response to genotoxic stress, these cells were treated with ADR (1 μg/ml) for 0–48 h.

Gene-reporter assay
Ten genomic DNA fragments in and around *RGC32* that contained possible p53-binding sites (RGC32-RE1 to 10) were cloned into the pGL3-promoter vector (Promega, Madison, WI, USA). One or two copies of oligonucleotide 5′-AGGC gAGTTT-aag-cAGCTTGTCC-3′ (RGC32-RE2) and its antisense sequence were annealed, ligated into the pGL3-promoter vector and designated pGL3-RGC32-RE2 and pGL3-RGC32-RE2 $\times$ 2, respectively. Cultures of SaOS2 cells in 12-well dishes were transfected with each reporter plasmid, together with phRL-TK control vector (Promega) and pCMV-Tag3 mock vector (Stratagene, La Jolla, CA, USA) or a vector that either expressed p53 (pCMV-Tag3-p53) or contained p53 mutation R273H (pCMV-Tag3-p53Mut). Cells were harvested 36 h after transfection for measurement of luciferase activity, using the Dual-Luciferase Reporter Assay System (Promega). The ability to stimulate transcription was defined as the ratio of luciferase activity in the cells transfected with each reporter construct relative to the activity in the cells transfected with the non-responsive reporter-plasmid pGL3-promoter. A reporter construct containing the p53-consensus binding sequence within the *p21* promoter (pGL3-p53CBS-p21) served as a positive control.

ChIP assay
ChIP assay was performed using the ChIP Assay Kit (Upstate Biotechnology, Waltham, MA, USA). A total of 2×10^6 HCT116 ($p53^{+/+}$) and HCT116 ($p53^{-/-}$) cells were plated onto 10-cm dishes and treated with 1 μg/ml ADR for 24 h. Sonicated pre-cleared DNA–protein complexes from cell lysates were immunoprecipitated at 4°C for 16 h using anti-p53 antibody (Ab-6; Oncogene Research Products, San Diego, CA, USA) or anti-FLAG antibody (M2; Sigma, St Louis, MO, USA) as a control. The precipitated immune complexes were reverse crosslinked, and extracted DNA was re-suspended in 50 μl of Tris-ethylenediaminetetraacetic acid (EDTA). One microliter of each sample was used as a template for PCR amplification, using primer sets flanking the suspected p53-binding sites. Primers for the *p21* promoter were used as a control.

Transient transfection and colony-formation assays
A plasmid expressing a Myc-tagged RGC32 protein (pCMV-Tag3-RGC32) was obtained by cloning the full coding sequence of *RGC32* into the pCMV-Tag3 vector (Stratagene) in-frame, along with the Myc epitope. For colony-formation assays, pCMV-Tag3-RGC32, or the empty vector (pCMV-Tag3-Mock), was transiently transfected into cells as described elsewhere (Imoto *et al*., 2003; Misawa *et al*., 2005). Expression of RGC32 protein in transfected cells was confirmed 48 h after transfection by immunoblotting with anti-Myc antibody (Cell Signaling Technology, Beverly, MA, USA) as described below. After treatment for 3 weeks with appropriate concentrations of G418, cells were fixed and stained with crystal violet.

Establishment of stable RGC32 U-87 MG and HeLa Tet-On cell lines
Stable RGC32 transfectants and their controls were obtained by transfecting pCMV-Tag3-RGC32 or pCMV-Tag3-Mock, respectively, into U-87 MG cells following selection with G418 for 3 weeks.

We generated HeLa Tet-On cells by inserting the *RGC32* coding region into pTRE2hyg along with GFP in-frame (pTRE2-GFP-RGC32) and a Tet-On gene expression system (Clontech), according to the manufacturer's protocol. Control cell lines were generated by transfection with pTRE2-GFP expressing GFP alone. Stable cells were obtained under selection with 200 μg/ml hygromycin B and 100 μg/ml G418. Stable clones were induced to express GFP-RGC32 or GFP using 10 μg/ml Dox, and expression was verified by Western blotting of whole-cell extracts, using a rabbit anti-GFP antibody (MBL, Nagoya, Japan).

Growth assays
For measurements of cell growth, 1×10^3 stable RGC32 transfectants and their controls were seeded in 96-well plates. The numbers of viable cells were assessed by a colorimetric water-soluble tetrazolium salt (WST) assay (cell counting kit-8; Dojindo Laboratories, Kumamoto, Japan) as described elsewhere (Imoto *et al*., 2003).

Immunofluorescent staining
After pCMV-Tag3-RGC32 was transiently transfected into U-87 MG cells, 1×10^4 of the transfectants were seeded into an eight-well slide chamber, cultured for 24 h and fixed in cold acetone–methanol (1:1). The cells were covered with blocking solution (3% bovine serum albumin in phosphate-buffered saline (PBS)), and incubated for 2 h with anti-Myc antibody (1:200 dilution) together with anti-β-tubulin (1:200 dilution, Sigma) or anti-γ-tubulin antibody (1:1000 dilution, Sigma) in blocking solution. To stain endogenous RGC32 protein, RGC32-specific rabbit polyclonal antibody generated by injection with synthetic peptide KELEAFIADLDKTLASM (Operon Biotechnology, Tokyo, Japan) was used (1:200 dilution). There followed 1 h of incubation with fluorescein isothiocyanate-conjugated goat anti-mouse or anti-rabbit IgG (1:400 dilution; MBL) and Alexa 594-conjugated goat anti-rabbit or anti-mouse IgG (1:1000 dilution; Molecular Probes, Eugene, OR, USA). The cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI), and viewed with an ECLIPSE E800 fluorescence microscope (Nikon, Tokyo, Japan).

Flow cytometry
HeLa Tet-On cells (1×10^6) were plated in 10-cm dishes, and 24 h later each culture was treated with 10 μg/ml Dox for 24 h. For analysis by FACS, harvested cells were fixed in 70% cold ethanol, and treated with RNase A and propidium iodide. Samples were analysed on a FACSCalibur HG (Becton-Dickinson, San Jose, CA, USA). Data were analysed using BD CellQuest Pro (Becton-Dickinson).

Cell-cycle synchronization
Exponentially growing HeLa Tet-On cells were seeded at a density of 1×10^6 onto 10-cm dishes. A day after seeding, thymidine (Sigma) was added to the medium with 5% FBS to a final concentration of 10 mM, and the cells were cultured for 15 h at 37°C. After three washes with PBS, the cells were cultured in normal growth medium for 9 h at 37°C. Thereafter, the cells were treated again with thymidine for 15 h, washed three times with PBS and cultured in normal growth medium. Dox (10 μg/ml) was added to the medium 24 h before the timing of the second release, and maintained until the time of harvest. The cell-cycle distribution was monitored by flow cytometry.

Immunoblotting
Total cell lysates, prepared in Nonidet P-40 (NP-40) lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 2 mM Na_3VO_4, 100 mM NaF, 10 mM sodium diphosphate decahydrate) with a protease-inhibitor cocktail, were analysed by immunoblotting as described elsewhere (Misawa *et al*., 2005), using anti-GFP antibody (MBL), anti-Plk1 antibody (Zymed Laboratories, San Francisco, CA, USA), anti-cyclin B1 antibody (Upstate, Lake Placid, NY, USA), anti-Aurora-B kinase antibody (Abcam, Cambridge, MA, USA), anti-p21 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p53 antibody (Ab-6) or anti-Aurora-A kinase antibody (kindly provided by Dr Hideyuki Saya; Marumoto *et al*., 2002).

In vitro *phosphatase reactions*
Total cell lysates, prepared from 40 ng/ml of nocodazole-treated mitotic phase GFP-RGC32-expressing HeLa Tet-On cells in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40) with a protease-inhibitor cocktail, were treated at 30°C for 2 h in phosphatase buffer (50 mM Tris-HCl, 0.1 mM Na_2EDTA, 5 mM dithiothreitol, 0.01% Brij 35, 2 mM $MnCl_2$, pH7.5) with 100 U of λ-PPase (New England Biolabs, Beverly, MA, USA) in the presence or absence of the PPase inhibitor vanadate (2 mM). The phosphorylation status of GFP-RGC32 was monitored by immunoblotting with anti-GFP antibody.

Immunoprecipitation
Full coding sequences of Aurora-A, Aurora-B, cyclin B1, Plk1 and RGC32, and deletion mutants of Plk1, Plk1(1-330) and Plk1(330-CT), were amplified by PCR and cloned into the pCMV-Tag2 and -Tag3 vectors (Stratagene) in-frame, along with the FLAG and Myc epitope, respectively. For immunoprecipitation, HEK 293-T cells co-transfected with expression constructs or HeLa Tet-On cells treated for 24 h with 10 μg/ml Dox were harvested, and solubilized using NP-40 lysis buffer with protease-inhibitor cocktail. Pre-cleared cell lysates were incubated with anti-FLAG or anti-GFP antibody for 2 h at 4°C, and then with Protein A Sepharose for 1 h at 4°C. The beads were washed five times with NP-40 lysis buffer. Immunoprecipitates and total input lysates were resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS—PAGE) gels, and subjected to immunoblotting.

Purification of His_6-tagged RGC32 protein and in vitro *kinase assay*
For synthesis of recombinant protein, the full coding sequence for RGC32 was cloned into pET-23d vector (Novagen, Madison, WI, USA). Recombinant His_6-tagged RGC32 protein was recovered from BL21-CodonPlus(DE3) cells (Stratagene) transformed with pET-23d-RGC32 using Ni–NTA superflow affinity resin (Qiagen, Hilden, Germany), and dialysed with PBS.

Human recombinant Plk1 (Invitrogen, Carlsbad, CA, USA) was incubated for 30 min at 30°C with His_6-RGC32 in Raf buffer (20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 0.1 mM EGTA, 25 mM KCl, 1 mM dithiothreitol) with [γ-^{32}P]ATP. After reactions were terminated by addition of 2 μl of 6 × sample buffer, phosphorylated substrates were detected by SDS–PAGE and autoradiography.

Acknowledgements

We thank Dr Bert Vogelstein (Howard Hughes Medical Institute, The Sidney Kimmel Comprehensive Cancer Center, and Program in Cellular and Molecular Medicine, The Johns Hopkins University Medical Institutions) for HCT116 ($p53^{+/+}$) and HCT116 ($p53^{-/-}$) cell lines and Dr Hideyuki Saya (Department of Tumor Genetics and Biology, Graduate School of Medical Sciences, Kumamoto University) for the anti-Aurora-A antibody. We also thank Ai Watanabe, Ayako Takahashi and Rumi Mori for technical assistance. This work was supported by Grants-in-Aid for Scientific Research on Priority Areas (C) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan; by a Grant-in-Aid from Core Research for Evolutional Science and Technology (CREST) of the Japan Science and Technology Corporation (JST); and by 21st Century Center of Excellence Program for Molecular Destruction and Reconstitution of Tooth and Bone.

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