Effects of human papillomavirus type 16 oncoproteins on survivin gene expression

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Survivin has recently been identified as a novel member of the inhibitor of apoptosis (IAP) gene family. The product of this gene not only suppresses apoptosis but also controls cell division. Survivin is undetectable in most terminally differentiated normal tissues but is expressed in embryonic and fetal organs and is present in most malignant tumours. Human papillomaviruses (HPV) are thought to play an important role in the development of cervical cancer. By interfering in the cell cycle, the viral oncoproteins (E6 and E7) can induce the immortalization of the host cell. The transcriptional effects of the HPV-16 E6 and E7 proteins on the survivin promoter in transiently transfected cell lines using luciferase tests were examined. HPV-16 E6, but not E7, was found to significantly transactivate the survivin promoter. Experiments performed in different cancer cell lines and with different E6 mutants indicated that the effect of E6 on the survivin promoter is largely dependent on p53 status. In accordance with this, the p53 tumour suppressor protein downregulated the expression of survivin. As E6 is able to interact with p53 and induces its ubiquitin-dependent degradation, it appears that the transactivation effect of E6 on survivin is mediated by the p53 degradation pathway. Transduction of HPV-16 E6 and E7 into human embryonic fibroblast cells showed that the HPV oncoproteins can upregulate endogenous survivin mRNA. Importantly, cell cycle synchronization experiments showed that the effect of HPV-16 E6 on survivin transcription is independent of the cell cycle.

INTRODUCTION

Survivin is a newly identified member of the inhibitor of apoptosis (IAP) protein family (Ambrosini et al., 1997; Deveraux & Reed, 1999). Besides the caspase-dependent inhibition of apoptosis, it has a role in the regulation of the cell cycle and cell division (Altieri, 2001). Survivin is upregulated in the G2/M phase of the cell cycle, when it is associated with the microtubules of the mitotic spindle (Li et al., 1998). It is not expressed in terminally differentiated adult tissues, but is abundant during embryonic development and is also re-expressed in most malignant tumours, including cervical cancer (Ambrosini et al., 1997; Saitoh et al., 1999; Kim et al., 2002).

Cancer of the uterine cervix is one of the most common female malignancies worldwide. Oncogenic or high-risk human papillomaviruses (such as HPV-16 and 18) are thought to play an important role in the development of cervical cancer. The E6 and E7 proteins of high-risk HPV's play a crucial role in the induction of malignant transformation of the host cells (Rapp & Chen, 1998; Münger et al., 2001). The HPV-16 E6 and E7 oncoproteins can bind and functionally inactivate the tumour suppressor proteins p53 and members of the retinoblastoma (Rb) tumour suppressor family (pRb, p107 and p130), respectively (Davies et al., 1993; Münger & Howley, 2002). E6 binds to E6AP, a ubiquitin protein ligase, and the resulted complex targets the p53 tumour suppressor protein for proteasome-mediated degradation (Münger & Howley, 2002). The interaction of hypophosphorylated Rb and E7 leads to the release of active E2F transcription factors, which in turn stimulate the expression of multiple genes encoding proteins essential for S phase progression (Münger & Howley, 2002). HPV-16 E6 was also shown to transactivate several viral (Desaintes et al., 1992) and cellular (Klingelhutz et al., 1996; Rapp & Chen, 1998; Lopez-Ocejo et al., 2000) promoters.

The expression of survivin was shown to be negatively regulated by the cellular tumour suppressor protein p53.
As the HPV-16 E6 oncoprotein is able to induce the degradation of p53, we assumed that it might have an effect on survivin transcription. Moreover, the expression of survivin was shown to be regulated by other viral (Punga & Akusjarvi, 2003) and cellular (Sommer et al., 2003) oncoproteins. These considerations led us to investigate the possible effects of the HPV-16 oncoproteins on the expression of the human survivin gene.

In this study, we found that HPV-16 E6 has a significant role in the regulation of survivin gene transcription, while HPV-16 E7 had no major effect on survivin transcription. E6 significantly upregulated survivin promoter activity. Consistently, HPV oncoproteins upregulated endogenous survivin mRNA levels in human embryonic fibroblast (HEF) cells. The effect of E6 on the survivin promoter was mediated largely by the p53 oncoprotein. In consideration of the potential role of survivin in cervical carcinogenesis, our findings suggest that the oncogenic property of HPV-16 may partially result from the upregulation of survivin expression by E6.

METHODS

Plasmid constructs. The luciferase reporter constructs pLuc-230c, pLuc-441c, pLuc-1430c and pLuc-2840c containing different fragments of the human survivin promoter were described previously (Li et al., 1998; Li & Altieri, 1999). The pcDNA-16E6 and pcDNA-16E7 expression vectors were described previously (Murvae et al., 2004).

The plasmid encoding wild-type HPV-16 E6 (pJ4E-16E6) and its deletion mutant derivatives (pJ4D-16E6A106-110, -166EΔ111-115 and -166EΔ128-132) were kindly provided by Dr Karen H. Vousden (Crook et al., 1991). The reporter construct p53CON-Luc containing a consensus p53-binding site cloned into the luciferase reporter vector pGUP.PA.8 was a gift from Dr Jerry W. Shay (Funk et al., 1992). The expression vector pcDNA3-p53Pro carrying the codon 72 proline variant of human p53 was provided by Dr Lawrence Banks (Thomas et al., 1999).

Cell culture and retroviral transduction. HeLa HPV-18-positive cervical adenocarcinoma cell line, MCF-7 human breast cancer cell line and Saos-2 human osteosarcoma cell line were obtained from the ATCC. HEF cells were prepared from an embryo removed during elective abortion. HEF cells have been routinely used in our laboratory to cultivate human cytomegalovirus strains. PA317-LXSN, -16E6, -16E7 and -16E6E7 cells are recombinant retrovirus producing cell lines obtained from the ATCC (Halbert et al., 1991). All these cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 2 mM l-glutamine and antibiotics (100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹).

Amphotropic retroviruses produced by the packaging cell lines were titrated on HeLa cells using G418 (500 μg ml⁻¹) as a selective agent and counting the number of drug-resistant colonies after methylene blue staining. HEF cells were transduced by the different retrovirus vectors and selected in media containing 200 μg G418 ml⁻¹.

Transient transfection and luciferase test. HeLa and MCF-7 cells were transfected with 2 μg of the reporter vectors and 1 μg of different expression vectors by using Lipofectamine 2000 (Invitrogen). Saos-2 cells were transfected with 5 μg of the reporter vectors together with 2 μg of the expression vectors by electroperoration (950 μF, 300 V) using GenePulser II (Bio-Rad). The cells were harvested 48 h after transfection by the addition of 250 μl Reporter lysis buffer (Promega) and one freeze-thaw cycle. The luciferase assay system of Promega was used to measure the luciferase activity of cell extracts. The Bradford protein assay was performed to standardize the protein concentration of the cell extracts. Each transfection experiment was performed independently at least three times.

Synchronization of the cell cycle. For G1 arrest, HEF-derived cell lines were treated with aphidicolin (10 μM) for 18 h and then for a further 24 h. For G2/M arrest, cells were treated with aphidicolin (10 μM) for 18 h and then released into nocodazole (0-2 μg ml⁻¹) for 24 h.

In the case of HeLa cells, G1, S and G2/M arrest were induced by treating the cells with 10 μM aphidicolin, 2 mM thymidine or 0-2 μg nocodazole ml⁻¹ for 24 h, respectively.

Semi-quantitative RT-PCR. Total cellular RNA was prepared from the cell lines by using TRI reagent (Sigma) according to the manufacturer’s protocol. The DuraScript RT-PCR kit (Sigma) was used to prepare cDNA. The reverse transcription reaction mixtures were incubated at 50 °C for 50 min. For reverse transcription, the anti-sense primers used for amplification of survivin, HPV-16 E6, E7 and GAPDH cDNA were used (see below). PCR amplification of target cDNA was performed in a final volume of 50 μl containing 5 μl first strand cDNA, 2-5 U RedTaq polymerase (Sigma), 1 × PCR buffer, 200 μM each dNTP and 20 pmol each forward- and reverse-specific oligonucleotide primers. The primer pair used for amplifying survivin cDNA was composed of a forward primer: 5'-GGCAGCCTTTCACAAGGACAGGC-3' and a reverse primer: 5'-GATGGCACCGCGCACTCTTCTGC-3' (Lühr et al., 2003). HPV-16 E6 was amplified using the primers 5'-TGGTTTCAGAGCCACAGGAG-3' and 5'-TTCTCAGAGCAGCATGGCT-3'. The primer pair 5'-GCAACCAGAGAACAATGCATCTCAG-3' and 5'-GTTCTTCAAAAGT-AGGAATGTCCTAGG-3' was used to amplify HPV-16 E7 cDNA. GAPDH was amplified using the primers 5'-AATCCATCCACCATCTTCCAG-3' and 5'-TCATGATCCCTCAGCTACCC-3'. Amplification of cDNAs was performed by an initial denaturation step at 95 °C for 2 min, followed by 23 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C (survivin) or 55 °C (E6, E7 and GAPDH) for 90 s, and extension at 72 °C for 90 s. These PCR conditions resulted in amplimer intensities proportional to the amount of cDNA templates.

Northern blot hybridization. RNA samples were run in formaldehyde/agarose gels, blotted onto Hybond-N+ nylon membranes (Amersham) and hybridized with radioactively labelled survivin and GAPDH probes. DNA probes were labelled by random priming (Prime-a-Genet labeling system; Promega) using 50 μCi (1-85 kBq) [α-32P]ATP (with a specific activity of 3000 Ci mmol⁻¹) and purified by gel filtration on DyeEx spin columns (Qiagen). Hybridization was performed in 5 × SSC, 50% formamide, 1% SDS, 5 × Denhardt’s solution and 100 μg denatured salmon sperm DNA ml⁻¹ at 42 °C overnight. The membranes were washed twice in 2 × SSC, 0-1% SDS for 5 min at 42 °C, and twice in 0-2 × SSC, 0-1% SDS for 15 min at 42 °C. Detection of hybridization signal was performed in a Bio-Rad phosphorimager (Personal Molecular Imager FX).

Flow cytometric analysis. For analysis of the cell cycle, cells were harvested by trypsinization, washed in PBS, fixed in 70% ethanol, stained (in PBS with 20 μg propidium iodide ml⁻¹, 200 μg RNAse A ml⁻¹ and 0-1% Triton-X 100) for 30 min and analysed for DNA content on a FACScan (Beckton Dickinson) cytomter. In order to assess the distribution of cells within the cell cycle, the ModFit LT software (Beckton Dickinson) was used.
RESULTS

HPV-16 E6 transactivates survivin promoter activity

HPV-16 is known to be involved in the aetiology of cervical tumorigenesis, and survivin appears to be involved in virus infection-induced tumorigenesis (Li, 2005). In this study, we investigated whether the E6/E7 oncogenes of HPV-16 have any effect on the expression of the human survivin gene. To test if these viral oncogenes have a direct effect on the survivin promoter, we performed transient transfection and luciferase assay experiments in different cancer cell lines using various survivin promoter-luciferase constructs (Fig. 1).

The results indicated that HPV-16 E6 but not E7 transactivates survivin promoter activity in HeLa cells, and that the minimal survivin promoter (represented by 230c) is necessary and sufficient for mediating this effect (Fig. 2a). Interestingly, in the transient transfection experiments performed in HeLa and MCF-7 cells (both carrying wild-type p53 protein), HPV-16 E6 consistently transactivated all the survivin reporter constructs tested (Fig. 2a and b). However, in Saos-2 cells, which express no p53 protein, 16E6 had no transactivation effect on the survivin reporter constructs (Fig. 2c). This may suggest that p53 has a role in mediating the transactivation effect of E6 on the survivin promoter.

Transactivation of survivin promoter by E6 is largely dependent on p53

To determine the underlying mechanism responsible for the E6-mediated upregulation of survivin promoter activity, and to clearly define the role of p53 in this process, we examined the function of endogenous p53 protein in HeLa, MCF-7 and Saos-2 cells using a p53-responsive luciferase reporter construct. Cells were transfected with either a

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**Fig. 1.** Schematic representation of transcriptional reporter constructs containing different fragments of the human survivin gene promoter cloned in front of the luciferase gene in the reporter vector pLuc. Nucleotide positions are given relative to the first nucleotide of the ATG start codon. Proven transcription factor recognition sites are indicated at the top. CDE, Cell cycle-dependent element; CHR, cell cycle homology region.

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**Fig. 2.** Effects of HPV-16 E6 and E7 on survivin reporter constructs. (a) HeLa, (b) MCF-7 or (c) Saos-2 cancer cell lines were transfected with the empty expression vector pcDNA (grey bars), expression plasmids encoding HPV-16 E6 (black bars) or E7 (white bars), along with the indicated survivin reporter constructs. The luciferase activity of cells transfected with the reporter construct p230c and the empty expression vector was set to 1, and the activities found in other transfections are shown relative to this. Data are from three independent experiments with standard errors shown as error bars.

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One of the most characteristic activities for high-risk HPV E6 proteins is degradation of the p53 protein. We found that, in Saos-2 cells, wild-type p53 downregulated all the survivin reporter constructs (Fig. 3b), which is consistent with previous findings by others (Hoffman et al., 2002; reportervector (p53CON-Luc) containing a consensus p53-binding site or empty control vector (pGUP.PA.8), and luciferase activity was measured. As shown in Fig. 3(a), luciferase activity of the p53 reporter construct in HeLa and MCF-7 cells but not in Saos-2 cells were strongly activated, suggesting that HeLa and MCF-7 cells but not Saos-2 cells possess a functionally active p53 protein.
Mirza et al., 2002). We obtained similar results using HeLa cells (data not shown). These results, in one way, suggest that the ability of 16E6 to induce the degradation of p53 has a role in the transactivation of the survivin promoter.

To further investigate the role of p53 in the regulation of the survivin promoter by E6, we tested some HPV-16 E6 deletion mutants that were previously characterized for p53 binding, in vitro p53 degradation and transactivation functions (Crook et al., 1991). HeLa cells were transfected with the survivin reporter construct pLuc-230c, along with either empty vector, expression vectors containing wild-type or different deletion mutants of 16E6 (Fig. 4). Two deletion mutants (Δ106–110 and Δ111–115), which lost p53 degradation activity but retained the ability to transactivate the adenovirus (Ad) E2 promoter, had no or very low transactivating effect on the survivin promoter. Conversely, a mutant (Δ128–132) that had showed almost wild-type p53 degradation, but very low AdE2 transactivation, retained near wild-type transactivation effect on the survivin promoter (Fig. 4). These results further suggest that the ability of E6 to bind p53 and induce its degradation has a major role in the transactivation of the human survivin promoter.

**HPV-16 oncogenes induce endogenous survivin transcription**

Next, we determined if expression of the HPV oncoproteins has an effect on endogenous survivin mRNA. We found very high survivin mRNA levels in different transformed cell lines (HeLa, C-33A and A549) (data not shown), so we chose to use non-transformed cells, which we expected to have lower basal survivin mRNA levels. HEF cells were transduced by control (LXSN) retrovirus or virus vectors containing HPV-16 E6, E7 or E6/E7 oncogenes. After drug selection, RNA was isolated from the different cell lines and subjected to Northern blot hybridization and semi-quantitative RT-PCR. Hybridization with a survivin-specific probe showed a very low level of survivin mRNA in control (LXSN) transduced HEF cells (Fig. 5a). Expression of HPV-16 E6, E7 or E6/E7 oncogenes led to substantial increases in the level of endogenous survivin mRNA in HEF cells. It was interesting to note that E7, which had no direct effect on the survivin promoter in transient expression assays, strongly induced endogenous survivin expression in HEF cells. Semi-quantitative RT-PCR analysis confirmed that HPV-16 E6 and/or E7 induced endogenous survivin transcription in HEF cells (Fig. 5b).
Effects of HPV oncogenes on survivin transcription is independent of the cell cycle

As the expression of survivin is strongly regulated by the cell cycle, it seemed possible that HPV E6 or E7 may increase survivin transcription through modulating the cell cycle. Therefore, we analysed the distribution of the phases of the cell cycle in control (LXSN transduced) HEF cells and in cells expressing HPV-16 E6/E7 (HEF-16E6E7). FACS analysis of cells stained with propidium iodide showed that in HEF cells expressing HPV-16 E6/E7, the distribution of cells in the different phases of the cell cycle was not significantly different from that found in control (LXSN) transduced cells (data not shown).

Next, we tested the effect of HPV-16 E6 on the activity of the survivin promoter in the different phases of the cell cycle. HeLa cells were co-transfected by the survivin reporter construct pLuc-230c along with vector control or HPV-16 E6 expression vector. After synchronization of the cell cycle, the activity of the survivin promoter was measured by luciferase activity assay. Cells were arrested in the G1, S and G2/M phases of the cell cycle by treatment with aphidicolin, thymidine or nocodazole, respectively (Fig. 6b). The results of luciferase tests performed in synchronized HeLa cells showed that HPV-16 E6 was able to transactivate the survivin promoter in all phases of the cell cycle (Fig. 6a). This indicates that the ability of E6 to transactivate the survivin promoter is independent of the cell cycle.

To further investigate whether the effect of the E6/E7 oncogenes on endogenous survivin transcription is modulated by the cell cycle, we measured survivin mRNA levels in HEF-LXSN and HEF-16E6E7 cells after arrest in the G1 and G2/M phases of the cell cycle. Semi-quantitative RT-PCR analysis showed that HPV-16 E6/E7 induced endogenous survivin transcription both in G1 and in G2/M arrested HEF cells (Fig. 7). This result strongly suggests that, at least in

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**Fig. 5.** Effects of HPV-16 E6/E7 on the transcription of the endogenous survivin gene in HEF cells. Cells were transduced by recombinant retroviruses carrying either the control vector (LXSN) or a vector encoding HPV-16 E6 and/or E7 as indicated. After G418 selection, total RNA was extracted from the cells and subjected to Northern blot hybridization using the indicated probes (a) or semi-quantitative RT-PCR specific for survivin, HPV-16 E6, E7 and GAPDH genes (b).

**Fig. 6.** (a) Effect of HPV-16 E6 on the survivin promoter is independent of the cell cycle. HeLa cells were co-transfected with 2 μg of the reporter construct pLuc-230c, and 1 μg of the empty expression vector pcDNA (grey bars) or 1 μg of the expression plasmid encoding HPV-16 E6 (black bars). Luciferase test was performed after cells were synchronized by treating with aphidicolin (10 μM), thymidine (2 mM) or nocodazole (0.2 μg ml⁻¹) for 24 h. The luciferase activity of cells transfected with the reporter construct pLuc-230c and the empty expression vector was set to 1, and activities found in other transfections are shown relative to this. Data are from three independent experiments with standard errors shown as error bars. (b) FACS analysis of cycling HeLa cells or cells arrested in the G1, S or G2/M phases of the cell cycle by 24 h treatment with aphidicolin, thymidine or nocodazole, respectively.
HEF cells, the effect of E6/E7 on the transcription of the endogenous survivin gene is independent of the cell cycle.

**DISCUSSION**

Our results demonstrate that the HPV-16 E6 oncoprotein is able to transactivate the human survivin promoter in human cancer cell lines. This transactivation appeared to be largely dependent on the presence of the cellular tumour suppressor protein p53. It is well established that high-risk HPV E6 proteins induce the proteosome-mediated degradation of p53, which seems to be important in the transformation of the host cells (Münger & Howley, 2002). On the other hand, we showed that expression of p53 results in downregulation of the survivin promoter constructs, which we showed to be transactivated by E6. Therefore, it seemed logical to assume that E6 transactivates the human survivin promoter through inducing the degradation of p53. Repression of the human survivin promoter by p53 was also demonstrated by others (Hoffman et al., 2002; Mirza et al., 2002; Löhr et al., 2003), although there were some discrepancies in the mechanisms proposed. A putative p53-binding site is found in the human survivin promoter, and one study found that this site binds p53 and is necessary for the p53-induced repression of the promoter (Hoffman et al., 2002). Other studies reported that repression of the survivin promoter by p53 is not dependent on direct binding of p53 to the promoter. It was suggested that p53 downregulates the survivin promoter by indirect mechanisms, either by altering the chromatin structure through recruiting histone deacetylases (Mirza et al., 2002) or by inducing the expression of the p21/waf1/cip1 gene (Löhr et al., 2003). Nevertheless, we have defined a role for p53 in the transactivation of the survivin promoter by E6.

In Saos-2 cells, which are p53 null, HPV-16 E6 had no transactivating effect on the survivin promoter. On the other hand, we found strong transactivation effect of E6 on the survivin promoter in HeLa and MCF-7 cells, which have functionally active p53 protein. This suggests that transactivation of the survivin promoter by E6 is a specific effect and is largely mediated by the degradation of p53 by E6. Our results with the E6 mutants also support this suggestion. The deletion mutants of E6 that are unable to bind and degrade p53 (Δ106–110 and Δ111–115) had no or very low transactivating effect on the survivin promoter. On the other hand, a mutant of E6 (Δ128–132) that is able to bind and degrade p53 retained the ability to transactivate the survivin promoter, in spite of the fact that it is unable to transactivate the AdE2 promoter (Crook et al., 1991). All these results indicate that HPV-16 E6 specifically transactivates the survivin promoter, largely through its ability to induce the degradation of p53.

We used HEF cells, which have native p53 and Rb, to study the effects of the HPV oncogenes on the transcription of the endogenous survivin gene. Control HEF cells had low survivin mRNA levels, while expression of HPV-16 E6 and/ or E7 by retroviral transduction was found to induce endogenous survivin transcription. This is in accordance with the results of the transient expression assays where we found E6 to transactivate the human survivin promoter. Although HPV-16 E7 did not transactivate the survivin promoter in transient expression assays, it induced endogenous survivin mRNA levels in HEF cells. Consistent with our findings, a previous study, using cDNA expression arrays also found that HPV-16 E6/E7 is able to induce survivin expression in human keratinocytes (Nees et al., 2000).

As the expression of survivin is strictly regulated by the cell cycle and the HPV oncoproteins have the ability to modify
the cell cycle, we studied whether the effect of the HPV oncoproteins on the expression of the survivin gene is dependent on the cell cycle. Experiments with HeLa cells where the cell cycle was synchronized showed that the transactivation effect of E6 on the human survivin promoter was independent of the cell cycle (Fig. 6). In accordance with this, we found that the induction of the endogenous survivin mRNA by HPV-16 E6/E7 in HEF cells was also independent of the cell cycle (Fig. 7). These results indicate that the HPV-16 E6 oncoprotein does not activate survivin transcription through modulation of the cell cycle. Interestingly, the human immunodeficiency virus-1 Vpr protein transactivates the survivin promoter through inducing arrest in G2/M of the cell cycle (Zhu et al., 2003).

Other cellular and viral oncoproteins have also been reported to activate survivin transcription. Most notably, the Ad2 E1B-55K protein was shown to relieve p53-mediated transcriptional repression of the survivin promoter (Punga & Akusjarvi, 2003). This finding is in accordance with our results, since the high-risk HPV E6 oncoproteins have several homologous functions with the Ad E1B proteins, including binding to p53, transactivation or repression of different gene promoters and inhibition of programmed cell death (apoptosis) (Rapp & Chen, 1998; Finzer et al., 2002; Cuconati & White, 2002). These conserved functions are thought to have important roles in the oncogenic activity of these oncoproteins.

High-risk HPV oncoproteins have effects on apoptosis, but these effects are largely modulated by the cell type, apoptosis inducing agents and experimental conditions. HPV-16 E6 tends to inhibit apoptosis in the natural host cells of the virus (primary keratinocytes), when apoptosis is induced by relatively physiological stimuli such as differentiation (by virus (primary keratinocytes), when apoptosis is induced by relatively physiological stimuli such as differentiation (Fig. 6). In accordance with this, we found that the induction of the endogenous survivin mRNA by HPV-16 E6/E7 in HEF cells was also independent of the cell cycle (Fig. 7). These results indicate that the HPV-16 E6 oncoprotein does not activate survivin transcription through modulation of the cell cycle. Interestingly, the human immunodeficiency virus-1 Vpr protein transactivates the survivin promoter through inducing arrest in G2/M of the cell cycle (Zhu et al., 2003).

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