Nuclear localization of γ-tubulin affects E2F transcriptional activity and S-phase progression

Greta Hög,1 Reihaneh Zarrizi,1 Kristoffer von Stedingk, Kristina Jonsson, and Maria Alvarado-Kristensson2

Center for Molecular Pathology, Department of Laboratory Medicine, Lund University, Skåne University Hospital, Malmö, Sweden

ABSTRACT We show that the centrosome- and microtubule-regulating protein γ-tubulin interacts with E2 promoter binding factors (E2Fs) to modulate E2F transcriptional activity and thereby control cell cycle progression. γ-Tubulin contains a C-terminal signal that results in its translocation to the nucleus during late G1 to early S phase. γ-Tubulin mutants showed that the C terminus interacts with the transcription factor E2F1 and that the E2F1–γ-tubulin complex is formed during the G1/S transition, when E2F1 is transcriptionally active. Furthermore, E2F transcriptional activity is altered by reduced expression of γ-tubulin or by complex formation between γ-tubulin and E2F1, E2F2, or E2F3, but not E2F6. In addition, the γ-tubulin C terminus encodes a DNA-binding domain that interacts with E2F-regulated promoters, resulting in γ-tubulin-mediated transient activation of E2Fs. Thus, we report a novel mechanism regulating the activity of E2Fs, which can help explain how these proteins affect cell cycle progression in mammalian cells.—Hög, G., Zarrizi, R., von Stedingk, K., Jonsson, K., Alvarado-Kristensson, M. Nuclear localization of γ-tubulin affects E2F transcriptional activity and S-phase progression. FASEB J. 25, 3815–3827 (2011). www.fasebj.org

Key Words: cell cycle progression · signal transduction · cell signaling

DURING CELL DIVISION, the point of no return is when a cell commits to divide at the G1/S transition. At the molecular level, during G1/S, the CDK2–cyclin E complex hyperphosphorylates retinoblastoma protein (RB), which leads to the dissociation of E2 promoter binding factor (E2F) from RB and initiation of E2F transcriptional activity. This event marks the onset of cell division, as E2F initiates the transcription of a number of genes necessary for cell cycle progression. Mutations in this RB–E2F-initiates the transcription of a number of genes necessary for cell cycle progression. Mutations in this RB–E2F

γ-Tubulin is a ubiquitously expressed protein that regulates interphase αβ-tubulin nucleation (2), centrosomal duplication (3), and spindle formation (4). γ-Tubulin occurs in the cytosol (5), centrosomes (3), and nucleus (6–8); in the latter location it associates with Rad51 during recombination repair (6). However, neither the mechanism underlying nuclear localization of γ-tubulin nor the nuclear functions of this protein have been elucidated. The present study detected a pool of γ-tubulin that is actively translocated to the nucleus of U2OS and NIH3T3 cells, where it modulates E2F transcriptional activity and cell cycle progression.

MATERIALS AND METHODS

Cell culture, transfection, and cell cycle analysis

Murine NIH3T3 embryonic fibroblasts and human U2OS osteosarcoma cells were cultured as reported elsewhere (3) and were transfected with various cDNAs using Lipofectamine Plus (Gibco BRL, Gaithersburg, MD, USA) and JetPei (Q-Biogene, Carlsbad, CA, USA). To obtain equal protein levels of the various ectopically expressed proteins, the following DNA amounts were used in transfection and cotransfection experiments: 960 ng of γ-tubulin-green fluorescent protein (γtubGFP), α-γ-tubulin, or C-γ-tubulin1–333, and/or 40 ng of hemagglutinin (HA)-E2F1, HA-E2F2, HA-E2F3, HA-E2F6, GFP, or HA-database of rice transcription factor polyepitope 1 (Dp1) in U2OS cells and 875 ng of γtubGFP in NIH3T3 cells was cotransfected with 188 ng of the various E2Fs or GFP. To achieve phase synchronization, cells were arrested in the G0 phase (3) and released for different periods of time. Phase distribution was examined by using propidium iodide to measure cell DNA content, as described previously (3).

Cell fractionation

To isolate chromatin, cells (0.5×10⁶) were treated as described previously (9). In brief, cells were first lysed in buffer

with Rad51 during recombination repair (6). However, neither the mechanism underlying nuclear localization of γ-tubulin nor the nuclear functions of this protein have been elucidated. The present study detected a pool of γ-tubulin that is actively translocated to the nucleus of U2OS and NIH3T3 cells, where it modulates E2F transcriptional activity and cell cycle progression.

MATERIALS AND METHODS

Cell culture, transfection, and cell cycle analysis

Murine NIH3T3 embryonic fibroblasts and human U2OS osteosarcoma cells were cultured as reported elsewhere (3) and were transfected with various cDNAs using Lipofectamine Plus (Gibco BRL, Gaithersburg, MD, USA) and JetPei (Q-Biogene, Carlsbad, CA, USA). To obtain equal protein levels of the various ectopically expressed proteins, the following DNA amounts were used in transfection and cotransfection experiments: 960 ng of γ-tubulin-green fluorescent protein (γtubGFP), α-γ-tubulin, or C-γ-tubulin1–333, and/or 40 ng of hemagglutinin (HA)-E2F1, HA-E2F2, HA-E2F3, HA-E2F6, GFP, or HA-database of rice transcription factor polyepitope 1 (Dp1) in U2OS cells and 875 ng of γtubGFP in NIH3T3 cells was cotransfected with 188 ng of the various E2Fs or GFP. To achieve phase synchronization, cells were arrested in the G0 phase (3) and released for different periods of time. Phase distribution was examined by using propidium iodide to measure cell DNA content, as described previously (3).

Cell fractionation

To isolate chromatin, cells (0.5×10⁶) were treated as described previously (9). In brief, cells were first lysed in buffer
A (9) containing 0.1% triton X-100. Nuclei in the first pellet were collected and lysed in buffer B (9). Insoluble chromatin was collected by centrifugation. The purified fractions were boiled in sample buffer (3) and analyzed by Western blotting using α-tubulin and histone as molecular markers for the cytosolic and nuclear fractions, respectively.

cDNA and reagents

Human (h)-α-tubulin pcDNA3-GFP was provided by Dr. Jiri Bartek (Institute of Cancer Biology, Danish Cancer Society, Copenhagen, Denmark; ref. 10); pcDNA3-hemagglutinin (HA)hE2F1 was furnished by Dr. Joseph R. Nevins (Duke University, Durham, NC, USA; ref. 11); pGL3-TATA-6xE2F-Luc, hE2F2, hE2F3 and hDp1 were from Dr. Kristian Helin (Biotech Research and Innovation Centre, University of Copenhagen, Copenhagen, Denmark; refs. 12, 13); hE2F6 was provided by Dr. David M. Livingston (Dana-Farber Cancer Institute, Boston, MA, USA; ref. 14), and cyclin E promoter (plasmid 8458; Addgene, Cambridge, MA, USA) was furnished by Dr. Robert A. Weinberg (Massachusetts Institute of Technology, Cambridge, MA, USA). Human α-tubulin N-α-tubulin 1–335 and C-α-tubulin 334–452 were amplified by PCR and subcloned in frame into pGEX2T (Amer sham, Picatony, NJ, USA) using the primer sets P1 (5′-GGCGCCGCCAGCATGCGGAGGAAAATCCTAG-3′) and P2 (5′-CGGCGGATCCGGAAGACTTGTGCCTCTG-3′), P1 and 5′-GGCGGATCCGGAAGACTTGTGCCTCTG-3′, and 5′-GGCCGCCAGCATGCGGAGGAAAATCCTAG-3′, respectively. Hu man E2F1, E2F2 (Δ306–426), and E2F3 (Δ194–246) were amplified by PCR and subcloned in frame into pET21d (Novagen, San Diego, CA, USA) using the primer sets P3 (5′-GGCGCCGAAATCCACGATGGGCAGCCTGC-3′) and P4 (5′-GGGAGGGATCCGGGATGGGCAGCCTGC-3′), P3 and 5′-GGCGCCGAAATCCACGATGGGCAGCCTGC-3′, and 5′-GGGAGGGATCCGGGATGGGCAGCCTGC-3′, respectively. Human HA-hE2F1 (Δ400–426), HA-hE2F2 (Δ12–126), and HA-hE2F3 (Δ195–426) were obtained by PCR and subcloned into HindIII/EcoRI sites of pcDNA3.1 using the primer sets P5 (5′-GGGAGGGATCCGGGATGGGCAGCCTGC-3′) and 5′-GGGAGGGATCCGGGATGGGCAGCCTGC-3′, and 5′-GGCGCCGAAATCCACGATGGGCAGCCTGC-3′, respectively. Human HA-hE2F1 (Δ400–426), HA-hE2F2 (Δ12–126), and HA-hE2F3 (Δ195–426) were obtained by PCR and subcloned into HintIII/EcoRII sites of pcDNA3.1 using the primer sets P5 (5′-GGGAGGGATCCGGGATGGGCAGCCTGC-3′) and 5′-GGCGCCGAAATCCACGATGGGCAGCCTGC-3′, and 5′-GGGAGGGATCCGGGATGGGCAGCCTGC-3′, respectively. The mutations were verified by sequencing.

The following antibodies and reagents were used: anti-Histone (Chemicon/Millipore, Billerica, MA, USA); anti-α-HA, anti-GFP, anti-RB, anti-pRB, anti-E2F1, anti-E2F2 anti-E2F3, and anti-GST (Amersham); Histone (Chemicon/Millipore, Billerica, MA, USA); anti-HA, anti-His (Abcam, Cambridge, MA, USA); protein G PLUS-Sagarose and anti-γ-Gal (Abcam); anti-α-tubulin (Calbiochem, San Diego, CA, USA); anti-His (Abcam, Cambridge, MA, USA); tubulin, N-tubulin and C-tubulin, anti-GFP, anti-RB, anti-pRB, anti-E2F1, anti-E2F2, anti-E2F3, and anti-HA (Novagen); anti-Dp1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit anti-γ-tubulin (Sigma, St. Louis, MO, USA) and anti-α-tubulin (Calbiochem, San Diego, CA, USA); anti-His (Abcam, Cambridge, MA, USA); pG PLUS-Sagarose and anti-γ-Gal (Abcam); because all the reagents were obtained from Sigma, unless otherwise indicated.

RNA interference

For human E2F1, E2F2, and E2F3 (15) shRNA, the annealed oligonucleotides 5′-GATCTGTGATACATCATATTTTTTGCAG-3′ and 5′-AGGTTTCAATTTTGTATGAGCAAGTTG-3′ were cloned into the pSilencer vector (Ambion, Austin, TX, USA). The human GST fusion proteins (N-terminal-GST E2F1, E2F1 (360–426), and E2F1 (193–359) were obtained by PCR and subcloned in frame into pGEX2T (Amer sham, Picatony, NJ, USA) using the oligo sets 5′-GGCGCTAGGCGAATCGGGAGGAAAATCCTAG-3′ and 5′-GGGCGGATCCGGAAGACTTGTGCCTCTG-3′, and 5′-GGCGCTAGGCGAATCGGGAGGAAAATCCTAG-3′, respectively. Human HA-hE2F1 (Δ400–426), HA-hE2F2 (Δ12–126), and HA-hE2F3 (Δ195–426) were obtained by PCR and subcloned into HintIII/EcoRIII sites of pcDNA3.1 using the primer sets P5 (5′-GGCGCTAGGCGAATCGGGAGGAAAATCCTAG-3′) and 5′-GGGCGGATCCGGAAGACTTGTGCCTCTG-3′, and 5′-GGCGCTAGGCGAATCGGGAGGAAAATCCTAG-3′, respectively.

Expression and purification of recombinant proteins

The human GST fusion proteins (N-terminal-γ-tubulin 1–335 and C-γ-tubulin 334–452) were expressed in Escherichia coli DH5a and C-terminal His-tagged E2F1, E2F2 (Δ306–426), and E2F3 (Δ194–246) were expressed in E. coli BL21 (DE3) (Strategen; ref. 3). Exponentially growing bacteria bearing these plasmids were induced (37°C, 2 h) with 0.2 and 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG), respectively. Recombinant proteins were purified under native conditions by nickel-affinity chromatography.
His-E2F1 was incubated for 45 min with or without His-E2F1 or Triton X-100, 1 mM MgCl₂, 0.25 mM GTP, 5 mM (total volume of 1 ml) containing 20 mM Tris (pH 7.5), 0.1% NCBI GEO accession number GSE1562). Genes are displayed in order of most positively enriched (genes 1–35) to the most negatively enriched (genes 37–77). Staining of endogenous NIH3T3 or U2OS cells were cultured and fixed as described previously (3).

**Microscopy**

NIH3T3 or U2OS cells were cultured and fixed as described previously (3). Staining of endogenous γ-tubulin was performed with the following antibodies: rabbit anti-γ-tubulin GTU-88. Fluorescence images were captured and processed using an Olympus BX 51 microscope (Olympus, Tokyo, Japan). A minimum of 100 cells were examined in each sample.

**Reconstitution of the E2F–γ-tubulin complex**

GST-γtub, GST-C-γtub334–452, or GST-N-γtub1–333 (500 ng) was incubated for 45 min with or without His-E2F1 or His-E2F1334–452 (500 ng); this was done on ice in a buffer (total volume of 1 ml) containing 20 mM Tris (pH 7.5), 0.1% Triton X-100, 1 mM MgCl₂, 0.25 mM GTP, 5 mM β-mercaptoethanol, 137 mM NaCl, and 5% glycerol. The GST-tag proteins were purified by adsorption on 40 ml of glutathione-Sepharose 4B (Amersham Pharmacia Biotech) or Ni²⁺ affinity resin (Qiagen, Valencia, CA, USA), according to the manufacturer’s instructions.

**Luciferase assays**

U2OS cells were transfected with 170 ng of the desired luciferase reporter construct, 1 ng of PRL-CMV-Renilla luciferase reporter construct (Promega, Madison, WI, USA), 20 ng of an E2F construct, and 200 ng of a γ-tubulin construct. If the total amount of plasmid DNA did not reach 400 ng, pCDNA 3.1 or pEGFP empty vectors were added, and an equal amount of plasmid DNA was used in the transfections. At 1 d after transfection, cells were harvested for determination of luciferase and Renilla activity. Double determination of luciferase activity was achieved using a dual-luciferase reporter assay system (Promega).

**Gene expression analysis**

Total RNA from transfected U2OS cells was extracted as previous described (3), followed by a cleaning step using the RNeasy Mini Kit (Qiagen). mRNA expression array analysis was performed using the human Illumina platform. The gene set enrichment analysis (GSEA) program (http://www.broadinstitute.org/gsea/) was used to generate a ranked gene list according to differential expression between control-shRNA- and γ-TUBULIN-shRNA-transfected samples. Genes were ranked in descending order with regard to level of expression in the γ-TUBULIN-shRNA-transfected cells. GSEA (19) was performed on the generated ranked gene list using an E2F1-upregulated gene set generated from the publically available data set (accession number GSE1562; http://www.ncbi.nlm.nih.gov/geo/) consisting of control (LacZ) and exogenous E2F1 expressing melanoma SK-MEL-2 cells (20). E2F1-upregulated genes were defined as all genes displaying ≥5-fold higher expression in the E2F1-expressing cells compared to control (Table 1).

To examine γ-tubulin-correlated gene expression in tumor material, two publically available datasets were examined including 315 breast tumors, 121 lung tumors [National Center for Biotechnology (NCBI) Gene Expression Omnibus (GEO) accession number GSE2109] and 290 colon tumors [GEO accession number GSE14333]. For each tumor type, all genes were ranked according to correlation to γ-tubulin expression. The top 100 correlated genes to γ-tubulin in each case were used to perform a Gene Ontology (GO) analysis, using the Functional Annotation tool in the online Database for Annotation, Visualization and Integrated Discovery (DAVID; refs.21, 22).

**Electrophoretic mobility shift assay (EMSA)**

The double-stranded oligonucleotides used for EMSA were derived from the dihydrofolate reductase promoter, an E2F

---

**Table 1. E2F1-upregulated target gene signature**

| No. | Gene     | No. | Gene     | No. | Gene     | No. | Gene     | No. | Gene     |
|-----|----------|-----|----------|-----|----------|-----|----------|-----|----------|
| 1.  | BMP2     | 17. | PKC1     | 33. | RORA     | 49. | HPGD     | 65. | INHBB    |
| 2.  | LRG1     | 18. | BRF1     | 34. | CDKN2C   | 50. | ZBTB1    | 66. | H2AFB1   |
| 3.  | EIF4A1   | 19. | MAP3K5   | 35. | KIAA0495 | 51. | CCNE2    | 67. | FOXF2    |
| 4.  | FOXD1    | 20. | NPTX2    | 36. | HOXD8    | 52. | ARR3     | 68. | MYB      |
| 5.  | RB1      | 21. | PEG10    | 37. | GATA2    | 53. | GZMB     | 69. | NR2F1    |
| 6.  | FST      | 22. | KLF10    | 38. | PTK7     | 54. | AMY2B    | 70. | CDKN1A   |
| 7.  | FOXA1    | 23. | GNRHR    | 39. | CYP26A1  | 55. | ATP8A1   | 71. | BAI2     |
| 8.  | VEGF     | 24. | FOXO1    | 40. | TCF7     | 56. | WIF1     | 72. | F3       |
| 9.  | PTHLH    | 25. | FGFR1    | 41. | PLCG1    | 57. | IRX5     | 73. | CDKN1B   |
| 10. | DUSP2    | 26. | AKAP5    | 42. | DLX2     | 58. | C8G      | 74. | SKP2     |
| 11. | NHLH2    | 27. | EFNB2    | 43. | GNAO1    | 59. | MPP6     | 75. | STX11    |
| 12. | ABCC4    | 28. | LOC652637| 44. | KITLG    | 60. | KIAA1107 | 76. | RBM38    |
| 13. | RPL35S   | 29. | TERF2    | 45. | CSPG5    | 61. | PTKL     | 77. | FG9      |
| 14. | ZIC3     | 30. | PLCL2    | 46. | LHX2     | 62. | CCNE1    | 78. |          |
| 15. | IRF1     | 31. | ESR2     | 47. | ABCA2    | 63. | SYNGR3   | 79. |          |
| 16. | FGFR3    | 32. | CHST1    | 48. | BCL2L11  | 64. | MAN1C1   | 80. |          |

All genes displaying a ≥5-fold higher expression in E2F1 overexpressing melanoma SK-MEL-2 cells compared to control SK-MEL-2 cells (data set NCBI GEO accession number GSE1562). Genes are displayed in order of most positively enriched (genes 1–35) to the most negatively enriched (genes 37–77) in the γ-Tubulin-shRNA-transfected cells. HOXD8 (gene 36) displayed no enrichment.
consensus site, and the sequences were as follows (23): nonmutated 5’AGTTTCTAGGCGTCAATTCGCGC5’ and 5’ATTCTCAGTTGGCGCAATTTTGC5’ (E2F site underscored); mutated 5’AGCTTTCATGGCAGTTCGCAACTTGG3’ (mutated bases underscored). The oligos were aligned and subcloned into HindIII/EcoRI sites of pcDNA3.1.

A 20 μl reaction containing 2 μg of each recombinant protein, 0.1 ng of the HindIII/EcoRI-fragmented 32P-labeled DNA probe, 0.25mg/ml poly(dI-dC), and DNA competitor (100-fold cold oligo; wild-type or mutated) or antibody (1.0 μl), was incubated in binding buffer (1.5 M Tris, pH 7.5; 1M KCl; 20% triton X-100; 0.5M EDTA; 5M NaCl; 30 mM NaN3VO4; 0.25 M PMSF; 3M KCl; and 500 mM NaF) at room temperature for 30 min and then analyzed on a 4% acrylamide gel in 0.5 M TBE at 4°C.

**Figure 1.** γ-Tubulin localizes to chromatin and interacts with E2F1. A) Localization of endogenous γ-tubulin (γTub) was examined by immunofluorescence staining with γTub (green), and nuclei were detected with DAPI (blue) in NIH3T3 and U2OS cells expressing human γ-TUBULIN-shRNA. Scale bars = 10 μm. B) Cells (0.5×10⁶) were biochemically divided into cytosolic (C), nuclear (N), and chromatin (CH) fractions and analyzed by Western blotting (WB) with an anti-γ-tubulin antibody (γTub), α-tubulin (αTub) and histone (Hist) (n=5 or 6). C) Extracts from synchronous NIH3T3 cells were prepared as in A and examined by WB using antibodies against E2F1, cyclin E (CyclE), phosphorelinoblastoma (pRB), total RB, γ- and α-tubulin, and histone (n=6). DNA content was determined by flow cytometry (percentage of S- or G₂/M-phase cells, indicated beneath blots). Graph illustrates densitometric analysis of γTub content in the chromatin fraction (means±sD; n=6). *P < 0.05. D) Using extracts from synchronous NIH3T3 cells, γ-tubulin was immunoprecipitated with an anti-γ-tubulin antibody, developed by WB with an anti-E2F1 antibody (top), and reprobed with GCP2, RB, Dp1, and γTub (bottom). Bottom panels illustrate DNA content of the cells detected by flow cytometry (percentage S-phase cells indicated, n=5). E) Immunoprecipitation with the indicated antibodies or with a control antibody (C) was performed using U2OS cells expressing human γ-TUBULIN- or E2F1-shRNA, or control plasmids. Some control cells were treated with aphidicolin (Aphid.). Total lysate was run as loading control (bottom panel; n=4), and WBs were analyzed with the indicated antibodies. F) NIH3T3 and U2OS cells were biochemically divided into cytosolic, nuclear membrane, and chromatin fractions. Each fraction was subjected to immunoprecipitation with an anti-γ-tubulin antibody and examined by WB.
**RESULTS**

During G1/S transition, γ-tubulin localizes to chromatin and interacts with E2F1

Immunofluorescence analysis using 3 different anti-γ-tubulin antibodies showed a constitutive localization of endogenous γ-tubulin to the nucleus of NIH3T3 and U2OS cells (6–8). In addition, the centrosomic, nuclear, and cytosolic immunofluorescence signal decreased with selective shRNA (Fig. 1A). Analysis of biochemical fractionations showed that part of the endogenous γ-tubulin was found in the chromatin fraction (Fig. 1B). A densitometric analysis of the different fractions showed that 29.3 ± 2.6 and 25.8 ± 2.6% (n=3) of the total amount of endogenous γ-tubulin was present in the chromatin of U2OS and NIH3T3, respectively. To determine variations in localization during the cell cycle, we investigated distribution of endogenous γ-tubulin in synchronized NIH3T3 cells (3). This revealed accumulation in the chromatin fraction of cells in late G1 and early S phase (6), in the latter reaching a maximum at 16 h (Fig. 1C and Supplemental Fig. S1A). Translocation of γ-tubulin to the chromatin coincided with a rise in E2F1 protein levels and an increase in hyperphosphorylated RB (Fig. 1C and Supplemental Fig. S1A), events that mark the onset of cell division (1). Analysis of endogenous γ-tubulin immunoprecipitates disclosed a cell cycle-dependent association of γ-tubulin with endogenous E2F1, but not with cyclin E, Dp1, or RB (Fig. 1D). By comparison to other γ-tubulin-interacting proteins, such as GCP2 (2), the E2F1–γ-tubulin complex appeared transiently at G1/S entry and in early S phase (Fig. 1D). Additional characterization showed that levels of E2F1–γ-tubulin complex decreased by shRNAi-induced reduction of either E2F1 or γ-tubulin (Fig. 1E) and increased when U2OS cells were aphidicolin arrested (3, 6) in early S phase (Fig. 1E). Next, we investigated the cellular localization of the E2F1–γ-tubulin by immunoprecipitating endogenous γ-tubulin from fractions of U2OS and NIH3T3 cells. In comparison with the microtubule associated GCP2–γ-tubulin complex, E2F1–γ-tubulin occurred mainly in the chromatin fraction, suggesting that γ-tubulin may modulate the nuclear transcriptional activity of E2F1 (Fig. 1F). Furthermore, E2F heterodimerization partner Dp1 was found associated with E2F1 immunoprecipitates but not with γ-tubulin immunoprecipitates (Fig. 1D, E). These findings support a specific nuclear association between γ-tubulin and E2F1 during early S phase.

**γ-Tubulin C terminus contains a nuclear localization signal that translocates γ-tubulin to the nucleus and regulates γ-tubulin association with E2F1**

Determinants of γ-tubulin nuclear targeting were elucidated using 2 fragments of human γ-tubulin fused to GFP: N-γtubGFP1–333 and C-γtubGFP334–452 (Fig. 2A). Both wild-type γtubGFP and the C-γtubGFP334–452 constructs exhibited stronger nuclear localization than N-γtubGFP1–333, although a pool of N-γtubGFP1–333 was still found in the chromatin fraction (Fig. 2B). The γ-tubulin sequence included a putative nuclear localization signal (NLS) located close to the C terminus that contained two short stretches of basic amino acids, one comprising residues K397, R399, K400, and R401, and the other encompassing R409 and K410. Sequence alignments showed that the residues in the NLS are highly conserved among species (Fig. 2A). Single or double mutations of R399A, K400A, R409A, and K410A partially blocked nuclear and chromatin accumulation of γtubGFP (Fig. 2B and Supplemental Fig. S1B). The amount of γtubGFP in the chromatin decreased significantly when R399A, K400A, and R409A were mutated (Fig. 2B and Supplemental Fig. S1B), confirming the implication of both stretches in the γ-tubulin NLS. Immunoprecipitation of E2F1 from cells transiently expressing γtubGFP, N-γtubGFP1–333, or C-γtubGFP334–452 showed that E2F1 associated with γtubGFP and C-γtubGFP334–452, but not with N-γtubGFP1–333 (Fig. 2C). Furthermore, bacterially produced GST-γtub and GST-C-γtub334–452, but not GST-N-γtub1–333, formed a complex with His-E2F1 (Fig. 2D), which further strengthens that the C terminus of γ-tubulin interacts directly with E2F1.

**γ-Tubulin moderates E2F1, E2F2, and E2F3 transcriptional activities in a RB-binding-independent manner**

Depending on their transcriptional effects, the human transcription factor E2F family can be divided into 2 groups: transcriptional activators, E2F1, E2F2, and E2F3; and transcriptional repressors, E2F4, E2F5, E2F6, E2F7, and E2F8 (26). To analyze the effect of γ-tubulin heterodimerization on E2F transcriptional activity, we performed an assay using luciferase reporter plasmids containing E2F binding sites (12) or the cyclin E promoter (27). The luciferase activity was measured in U2OS cells transfected with E2F activators E2F1, E2F2, and E2F3 or the transcriptional repressor E2F6 (1). Also, cells were transfected with γ-tubulin and/or the E2F heterodimerization partner, Dp1 (1). The lu-
transfected with GFP-NLS.

B. N-terminus of E2F1 was coexpressed with these mutants (Fig. 3D). The transcriptional activity was not blocked by deletion of the E2F1 repressor effect of C-terminal domain interaction with E2F moderates E2F transcriptional activities.

To identify the E2F1 domain important in the E2F1–γ-tubulin complex, we tested various E2F1 constructs (Fig. 3E). The effect of γ-tubulin on E2F1 transcriptional activity was not blocked by deletion of the E2F1 RB-binding domain (RBD; HA-E2F1Δ193–212), N-terminal domain (HA-E2F1Δ284–409), and transactivating domain (TAD; HA-E2F1Δ360–426). However, E2F1 transcriptional activity was reduced by deletion of the E2F1 region (HA-E2F1Δ193–426, Fig. 3E) that includes the predicted dimerization domain (DD; residues 195 to 284) (28–30). To determine whether E2F1Δ193–426 interacts with γ-tubulin in a cell-free system, we tested bacterially produced His-E2F1, His-E2F1Δ360–426, and His-E2F1Δ193–284 and GST-yub in vitro. E2F1–γ-tubulin complex formation occurred in the presence of His-E2F1Δ360–426 but not with His-E2F1Δ193–426 (Fig. 4A), showing that residues 193 to 360 are involved in the interaction. To ascertain the involvement of the DD (28–30); we deleted residues Ser193 to Ile284, Ile284 to Leu359, or Ser193 to Leu359 in E2F1 and tested each of the constructs in a luciferase reporter assay. The luciferase activity due to Flag-E2F1Δ193–284 or Flag-E2F1Δ284–359 expressions was decreased when γ-tubulin was coexpressed with these mutants (Fig. 3E). The repressor effect of γ-tubulin on E2F1 activity was abolished when residues 193–359 where deleted. However, it is worth noting that the luciferase activity of Flag-E2F1Δ193–359 was enhanced by increased levels of γ-tubulin, which is probably caused by the presence of additional sequence on E2F1 that could cause the observed effect of γ-tubulin on E2F2 activity, we studied variations in the protein levels of E2F. In the studied cells, the protein levels of endogenous E2F or ectopically expressed E2F varied, dependent on the γ-tubulin protein levels. Unexpectedly, increased protein levels of E2Fs were detected when γ-tubulin was coexpressed with E2F1, E2F1Δ193–284, E2F2, and E2F3, but not E2F6 (Fig. 4B) or E2F1Δ193–359 (Fig. 4C), suggesting that γ-tubulin protected E2F1, E2F1Δ193–284, E2F2, and E2F3 from degradation. Accordingly, γ-TUBULIN-shRNA-transfected cells showed
Figure 3. γ-Tubulin moderates E2F1, E2F2, and E2F3 transcriptional activity. Assay of the luciferase activity driven by 6 E2F promoter binding sites (A, C, E) or cyclin E promoter (B, D) on transient transfection of U2OS cells with a Renilla reporter construct and the following constructs: GFP, γ-TUBULIN-shRNA (shyTub), HA-E2F1, HA-E2F2, HA-E2F3, HA-E2F6, HA-Dp1, GFP-γ-tubulin (γTub), RNAi-resistant γ-TUBULIN gene (γTub-rest), E2F1-shRNA (shE2F1), E2F2-shRNA (shE2F2), E2F3-shRNA (shE2F3), Ala399-γtubGFP (R399A), Ala409-Ala410-γtubGFP (R409A-K410A), Ala399-Ala400-Ala409-γtubGFP (R399A-K400A-R409A), N-γTub, and C-γtubGFP1–333 (N-γTub), and C-γtubGFP334–452 (C-γTub), or various E2F1 mutants: HA-E2F1 Δ409–426, HA-E2F1 Δ2–126, HA-E2F1 Δ360–426, HA-E2F1 Δ193–426, HA-E2F1 Δ193–248, R399A, R409A, R409A-K410A, R409A-K400A-R409A, or various E2F1 mutants: HA-E2F1 Δ409–426, HA-E2F1 Δ2–126, HA-E2F1 Δ360–426, HA-E2F1 Δ193–426, Flag-E2F1 Δ193–248, Flag-E2F1 Δ193–359, or Flag-E2F1 Δ284–359, as indicated. Luciferase activity of cells transfected with control construct was set as 1, and relative activities were calculated (means ± s.d., n = 3–10). A–C Total lysates of transfected U2OS cells were analyzed by WB with the indicated antibodies. Arrowheads and arrows indicate GFP-γ-tubulin and endogenous γ-tubulin, respectively. E) Structure of wild-type E2F1 and various E2F1 constructs comprising the DNA-binding (DBD), dimerization (DD), transactivating (TAD), RB-binding (RBD), and γ-tubulin-interacting (γD) domains. *P < 0.05; **P < 0.01.
get decreased E2F1, E2F2, and E2F3 levels (Fig. 4D), but the protective effect of γ-tubulin on E2Fs was impaired in the presence of Dp1 (Fig. 3B and data not shown). Nonetheless, coexpression of Dp1 with E2Fs induced an increase in expression of E2Fs (Fig. 4E and data not shown). Interestingly, the E2F γD (residues 193–359) encloses the DD (residues 195–284), which suggests that Dp1 and γ-tubulin compete for the same E2F-interacting site and that the interaction of γ-tubulin with the DD of E2F protects E2F from degradation. These results indicate the existence of two different E2F complexes: an active complex composed of Dp1-E2F and a nonactive complex composed of γ-tubulin-E2F.

Association of E2Fs with RB regulates activities and turnover of E2Fs within cells (ref. 1 and Fig. 4F); consequently, we analyzed the effects of γ-tubulin on expression of an RBD-deficient E2F1 mutant (HA-E2F1Δ193–284). Indeed, the absence of RBD did not influence the γ-tubulin-induced increase in protein levels of HA-E2F1Δ193–284; instead, HA-E2F1Δ193–284 was rapidly degraded (Fig. 4G). To ascertain that the effect on E2F2 levels was dependent on the C terminus of γ-tubulin, we coexpressed HA-E2F1 or HA-E2F1Δ193–284 with γ-tubulin mutants and found that the γ-tubulin mutants did not affect HA-E2F1 or HA-E2F1Δ193–284 expression (Fig. 4H). Considering these findings, we concluded that γ-tubulin’s regulatory effect on E2Fs protein levels depends on the γ-tubulin C terminus in a RB-binding-independent manner.

γ-Tubulin binds to DNA on the same DNA-binding motif as E2F

To determine whether γ-tubulin, similar to Dp1 (1), binds to the E2F DNA binding site, we performed an electrophoretic mobility shift assay (EMSA; ref. 31). Surprisingly, GST-γtub and GST-C-γtub334–432, but not GST-N-γtub1–333, displayed binding to the radioactively labeled probe representing the E2F binding site, which was inhibited by an unlabeled competing wild-type E2F binding site (Fig. 5A). In addition, incubating His-E2F1 with GST-γtub increased the DNA-binding activity of GST-γtub relative to the amount of bound probe detected when the different components were incubated separately (Fig. 5B). To study γ-tubulin’s ability to bind to E2F-regulated promoters, a ChIP assay using γ-tubulin antibodies was performed (24). Endogenous γ-tubulin was present on the cyclin E promoter in aphidicolin-treated U2OS cells, and sequential ChIP assay (E2F ChIP followed by γ-tubulin re-ChIP or vice versa) showed that endogenous E2F1 and E2F2, but not Dp1, colocalized with endogenous γ-tubulin on cyclin E promoter (Fig. 5C). These results indicated that the C terminus of γ-tubulin may interact directly with the γD of E2Fs and thereby negatively modulate E2F transcriptional activity. All the above-mentioned experiments suggested that γ-tubulin and Dp1 compete for the same binding site on E2Fs. To confirm the hypothesis, we analyzed the Dp1 content on E2F1 immunoprecipitates from cells expressing variable protein...
levels of γ-tubulin (Fig. 5D). We detected that lower γ-tubulin levels were associated with higher levels of Dp1 in E2F1 immunoprecipitates (Fig. 5E). This result revealed that the mechanism by which γ-tubulin moderates E2F activity is by competing out Dp1 in the E2F–Dp1 complex.

**γ-Tubulin levels affect cell cycle progression and the expression of E2F-regulated genes**

To further confirm the effect of γ-tubulin on E2Fs, we altered γ-tubulin levels in cells expressing HA-E2F1 in a synchronized cell population. Increased γ-tubulin levels impaired the S-phase entry effect caused by HA-E2F1 by negatively regulating the expression of E2F transcriptional targets, such as cyclin E expression (Fig. 6A). Moreover, alterations of the γ-tubulin levels during cell cycle in U2OS cells expressing GFP, γtubGFP, γTUBULIN-shRNA, and NLS-γtubGFP mutants showed that a larger number of control cells expressing GFP or γtubGFP were in S phase compared to the cells with various γtubGFP mutants (Supplemental Fig. S2A). In a synchronized cell population, S-phase progression was delayed in NIH3T3 cells transfected with γTubulin-shRNA or any of the NLS-γtubGFP mutants, suggesting that γ-tubulin levels and localization determine optimal cell cycle progression (Fig. 6B and Supplemental Fig. S2B).

Intriguingly, low expression or mutations of γ-tubulin elevated E2F activity but delayed S-phase entry. To further elucidate the effect of reduced γ-tubulin levels on E2F activity, an mRNA expression array was performed on control shRNA- and γ-TUBULIN-shRNA-transfected cells, and the effect of γ-TUBULIN reduction on an E2F1 target gene signature was examined (Table 1). GSEA revealed that on reduced mRNA expression levels of γ-TUBULIN, a significant enrichment of E2F1 positively regulated target genes was found (enrichment score 0.34, nominal P=0.026; Fig. 6C). It can also be noted that RB lay among the most positively enriched E2F1 targets (Table 1). In accordance with this, we found increased protein levels of RB in γTUBULIN-shRNA- and γTubulin-shRNA-transfected cells (Fig. 6B, D), which provides a potential explanation for the observed delay in S-phase entry (Fig. 6B). These findings further support the moderating effect of γ-tubulin on E2F activities, as the increase in RB levels concurred with a decrease in levels of phosphorylated RB.

**High expression of γ-tubulin correlates with high expression of E2F1 and cell cycle-associated gene signatures in various tumor types**

To identify underlying biological processes associated with γ-tubulin expression in various tumor types, we made use of publicly available expression array datasets, including 351 breast cancers, 121 lung cancers (NCBI GEO accession number GSE2109), and 290 colon cancers (NCBI GEO GSE14333; ref. 32). For each tumor type, all genes were ranked according to correlation to γ-tubulin expression. As expected from our results, γ-tubulin expression was significantly positively correlated with E2F1 expression in all three-tumor types (Fig. 7). In addition, GO analysis was
performed using the top 100 positively correlated genes to γ-tubulin expression. In each tumor type, γ-tubulin-correlated gene expression displayed highly significant enrichment of cell cycle-related processes (Supplemental Tables S1 and S2). The fact that high levels of γ-tubulin coincide with high levels of cell cycle-regulated genes supports the hypothesis of γ-tubulin acting as a cell cycle regulator and also suggests that γ-tubulin may be involved in cell cycle regulation in multiple malignancies.

**NLS-γ-tubulin mutants do not affect centrosome or αβ-tubulin dynamics and E2F1 does not associate with γ-tubulin phosphorylated on Ser131**

To establish that the observed effect of γtubGFP mutants on E2F activities and cell cycle was not caused by defective centrosomes or altered αβ-tubulin dynamics, we examined the effect of γtubGFP-R399A-K400A-R409A on centrosomes and αβ-tubulin phosphorylation. Ectopic expression of γtubGFP-R399A-K400A-R409A in U2OS cells did not influence the number of centrosomes or mitotic spindles formed or altered interphase microtubules or astral microtubule regrowth (Fig. 8A, B). This finding suggests that the effects of γtubGFP mutants on cell cycle progression and E2F activity are not due to defective centrosomes or altered αβ-tubulin dynamics.

We have previously shown that γ-tubulin is phosphorylated on Ser131 (pSer131), and pSer131 enhances microtubule polymerization in the centriole (3). Thus, in search for the mechanism that regulates nuclear translocation of γ-tubulin, we examined the amount of...
E2F1 associated with immunoprecipitates of pSer131–γ-tubulin from synchronized NIH3T3 cells (Fig. 8C, D). E2F1 interacted with total γ9253-tubulin immunoprecipitates in a cell-cycle-dependent manner (Fig. 8C) but did not associate with endogenous pSer131–γ9253-tubulin immunoprecipitates (Fig. 8D), implying the existence of an alternative mechanism that translocates γ-tubulin to the nucleus.

**DISCUSSION**

We describe an NLS that mediates γ9253-tubulin translocation to the nucleus during S phase (6–8). In the nucleus, γ9253-tubulin governs E2F1, E2F2, and E2F3 transcriptional activities. We found that decreased γ9253-tubulin levels or expression of NLS–γ9253-tubulin mutants increase E2F transcriptional activity, while elevated γ9253-tubulin levels reduce...
E2F activity. Furthermore, the protein levels of recombinant E2F1, E2F2, and E2F3 vary depending on the γ-tubulin and Dp1 expression. Low E2F protein levels were found in cells with reduced γ-tubulin levels, despite the high measured E2F transcriptional activities. Increased expression of γ-tubulin caused high protein levels of E2F, but the transcriptional activity of E2F was low. Also, we found that high expression levels of Dp1 increased the total levels of E2Fs; considering that low protein levels of γ-tubulin decreased E2Fs expression, this finding suggest that the transient increase of E2Fs during G1 and S phase could be caused by the dynamic formation of the Dp1- and γ-tubulin–E2F complexes.

γTUBULINshRNA induced an increase in E2F transcriptional activity and caused an E2F-dependent increase in the protein levels of RB. The RB signal transduction pathways can trigger a G1 arrest (33, 34); consequently, an increase in RB may be responsible for causing the observed S-phase entry delay in cells with reduced γ-tubulin protein levels. In G1/S entry, γ-tubulin binds to cyclin E promoter to ensure a transient expression of cyclin E, and, in doing so, γ-tubulin coordinates the cell cycle. In addition, the chromatin-associated pool of γ-tubulin was not bound to microtubules or GCP2-containing fractions, suggesting that γ-tubulin has centrosome-independent functions. Altogether, these observations indicate that γ-tubulin plays a role in the regulation of E2F transcriptional activity during the cell cycle.

Our finding of an E2F-mediated increase of RB in response to reduced γ-tubulin levels implicates γ-tubulin as a regulator in the G1/S phase transition. In line with this view, the presence or absence of centrosomes promotes either proliferation or a G1 arrest (35). The mechanism by which centrosomes regulate the G1/S transition is unclear, but it remains possible that the triggering signal that translocates γ-tubulin to the nucleus may originate at the centrosomes. Once centrosome duplication is initiated and cyclin E activity is no longer needed, the translocation of γ-tubulin to the nucleus will inhibit the transcription of cyclin E, and in doing so, will prevent a cyclin E-dependent reduplication of the centrosomes (36). In this way, a timely regulation of cell cycle progression can be achieved.

Alteration in the RB-E2F signal transduction pathways are connected to cancer development in various tumor types, as uncontrolled E2F signaling may trigger cell division (1). Consequently, in these tumors, uncontrolled cell division coincides with expression of cell cycle-regulated genes, which should also include γ-tubulin. To test this possibility, we examined γ-tubulin-correlated gene expression in tumor material and found that γ-tubulin displayed significant positive correlation to E2F1 expression, as well as significant enrichment of cell cycle-associated processes on GO analysis. These trends did not seem to be tumor type specific, as similar patterns were observed in multiple tumor types, including breast, lung, and colon cancers. This may suggest a role for γ-tubulin in cell cycle regulation in not only normal cells but also in malignancies, however further investigations are required.

In summary, the mechanism that allows γ-tubulin to moderate E2F transcriptional activity involves direct binding to E2Fs. This, in turn, moderates E2F transcriptional activity during S-phase entry. In the absence of γ-tubulin, increased E2F activity elevates E2F-mediated expression of RB and delays S-phase entry (Fig. 9A). At the G1/S phase transition, RB releases E2Fs, which may induce E2F–γ-tubulin complex formation, ensuring a transient transcription of genes necessary for S-phase entry (Fig. 9B).

The authors thank Jiri Bartek (Institute of Cancer Biology, Danish Cancer Society, Yang Shi (Harvard Medical School, Boston, MA, USA), Kristian Helin (Biotech Research and Innovation Centre, University of Copenhagen, Copenhagen, Denmark), Marc van de Wetering (Hubrecht Laboratory, Centre for Biomedical Genetics, Utrecht, The Netherlands), Joseph R. Nevins (Duke University, Durham, NC, USA), Robert A. Weinberg (Massachusetts Institute of Technology, Cambridge, MA, USA), and David M. Livingston (Dana-Farber Cancer Institute, Boston, MA, USA) for reagents, and P. Ödman for editorial assistance. This work was supported by grants from the Royal Physiographic Society (Lund, Sweden); the Åke Wiberg, Thelma Zoegas, Per-Eric and Ulla Schyberg, O. E. and Edla Johansson, Gradflorska, H. and G. Jeassons, and O. and E. Ericsson Foundations; Gyllenstiernska Krapperupsstiftelsen; Universitetssjukhuset Malmö Allmänna Sjukhus (U-MAS) and the U-MAS Cancer Research Fund; and the Swedish Research Council. A fellowship from the Swedish Society for Medical Research provided additional support. The authors declare no conflicts of interest.
REFERENCES

1. Trimarchi, J. M., and Lees, J. A. (2002) Sibling rivalry in the E2F family. Nat. Rev. Mol. Cell. Biol. 3, 11–20
2. Schiebel, E. (2000) gamma-Tubulin complexes: binding to the centrosome, regulation and microtubule nucleation. Curr. Opin. Cell Biol. 12, 113–118
3. Alvarado-Kristensson, M., Rodriguez, M. J., Silio, V., Valpuesta, J. M., and Carrera, A. C. (2000) SADD phosphorylation of gamma-tubulin regulates centrosome duplication. Nat. Cell Biol. 11, 1081–1092
4. Muller, H., Fogeron, M. I., Lehmann, V., Leh Rach, H., and Lange, B. M. (2006) A centrosome-independent role for gamma-TuRC proteins in the spindle assembly checkpoint. Science 314, 654–657
5. Moudjou, M., Bordes, N., Paintrand, M., and Bornens, M. (1996) gamma-Tubulin in mammalian cells: the centrosomal and the cytosolic forms. J. Cell Sci. 109(Pt. 4), 875–887
6. Lesca, C., Germanier, M., Raynaud-Messina, B., Picheareaux, C., Etievant, C., Emond, S., Burlet-Schiltz, O., Monsarrat, B., Wright, M., and Defais, M. (2005) DNA damage induce gamma-tubulin-RAD51 nuclear complexes in mammalian cells. Oncogene 24, 5165–5172
7. Korver, W., Goevaara, C., Chen, Y., Neonitool, S., Bookstein, R., Liu, K., Luo, Y., Lin, F. T., and Lin, W. C. (2004) TopBP1 regulates centrosome duplication. Nat. Cell Biol. 11, 1081–1092
8. Kramer, A., Mailand, N., Lukas, C., Syljuasen, R. G., Wilkinson, C. J., Nigg, E. A., Bartek, J., and Lukas, J. (2004) Directed complexes in late mitosis. Nat. Cell Biol. 15, 113–118

19. Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., Paulovich, A., Pomeroy, S. L., Golub, T. R., Lander, E. S., and Mesirov, J. P. (2005) Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. U. S. A. 102, 15445–1550
20. Jamshidi-Parsian, A., Dong, Y., Zheng, X., Zhou, H. S., Zacharias, W., and McMasters, K. M. (2005) Gene expression profiling of E2F-1-induced apoptosis. Gene 344, 67–77
21. Dennis, G., Jr., Sherman, B. T., Hosack, D. A., Yang, J., Gao, W., Lane, H. C., and Lempicki, R. A. (2003) DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol. 4, P3
22. Huang da, W., Sherman, B. T., and Lempicki, R. A. (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 41–57
23. Qin, X. Q., Chittenden, T., Livingston, D. M., and Kaehn, W. G., Jr. (1992) Identification of a growth suppression domain within the retinoblastoma gene product. Genes Dev. 6, 953–964
24. Weinmann, A. S., and Farnham, P. J. (2002) Identification of unknown target genes of human transcription factors using chromatin immunoprecipitation. Methods 26, 37–47
25. Hosack, D. A., Dennis, G., Jr., Sherman, B. T., Lane, H. C., and Lempicki, R. A. (2003) Identifying biological themes within lists of genes with EASE[b]. Genome Biol. 4, R70
26. Wu, Z., Zhi, N., Song, S., Keyanfar, K., Liu, D., Raghavachari, N., Munson, P. J., Su, S., Malide, D., Kajigaya, S., and Young, N. S. (2010) Human parvovirus B19 causes cell cycle arrest of human erythroid progenitors via deregulation of the E2F family of transcription factors. J. Clin. Invest. 120, 3530–3544
27. Geng, Y., Eaton, E. N., Picon, M., Roberts, J. M., Lundberg, A. S., Gifford, A., Sardet, C., and Weinberg, R. A. (1996) Regulation of cyclin E transcription by E2Fs and retinoblastoma protein. Oncogene 12, 1173–1180
28. Q01094 (E2F1_HUMAN). Uniprot Protein Knowledgebase. Retrieved December 2, 2010, from http://www.uniprot.org/uniprot/Q01094
29. Helin, K., Wu, C. L., Fattore, A. R., Lees, J. A., D'Nalang, B. D., Ngw, C., and Harlow, E. (1993) Heterodimerization of the transcription factors E2F-1 and DP-1 leads to cooperative transactivation. Genes Dev. 7, 1850–1861
30. Vidal, M., Braun, P., Chen, E., Boeke, J. D., and Harlow, E. (1996) Genetic characterization of a mammalian protein-protein interaction domain by using a yeast reverse two-hybrid system. Proc. Natl. Acad. Sci. U. S. A. 93, 10321–10326
31. Mudryj, M., Hiebert, S. W., and Nevins, J. R. (1990) A role for the adenosin inducible E2F transcription factor in a proliferation dependent signal transduction pathway. EMBO J. 9, 2179–2184
32. Jorissen, R. N., Gibbs, P., Christie, M., Prakash, S., Lipton, L., Desai, J., Kerr, D., Alotenon, L. A., Arango, D., Kruehoffer, M., Ornot, T. F., Andersen, C. L., Gruell, M., Kamath, T. P., Eichholtz, S., Yeatman, T. J., and Sibber, O. M. (2000) Metastasis-associated gene expression changes predict poor outcomes in patients with Dukes stage B and C colorectal cancer. Clin. Cancer Res. 15, 7642–7651
33. Sancar, A., Lindsey-Boltz, L. A., Usmal-Kacrzak, K., and Linn, S. (2004) Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu. Rev. Biochem. 73, 39–85
34. Giacinti, C., and Giordano, A. (2006) RB and cell cycle progression. Oncogene 25, 5220–5227
35. Doyon, C., Mc Collum, D., and Theurkauf, W. (2005) Nm23somes in cellular regulation. Annu. Rev. Cell Dev. Biol. 21, 411–434
36. Carrera, A. C., and Alvarado-Kristensson, M. (2009) SADD kinases license centrosome replication. Cell. Cycle 8, 4005–4006

Received for publication April 20, 2011. Accepted for publication July 11, 2011.