MOL
PHARM

MOLECULAR PHARMACOLOGY

Reversal of Stathmin-Mediated Resistance to Paclitaxel and Vinblastine in Human Breast Carcinoma Cells

Elizabeth Alli, Jin-Ming Yang, James M. Ford, and William N. Hait

The Cancer Institute of New Jersey, University of Medicine and Dentistry of New Jersey–Robert Wood Johnson Medical School, Departments of Medicine (J.-M.Y, W.N.H.) and Pharmacology (E.A., J.-M.Y, W.N.H.), New Brunswick, New Jersey; and Division of Oncology, Stanford University School of Medicine, Stanford, California (J.F.)

ABSTRACT

Antimicrotubule agents are commonly used chemotherapy drugs for the treatment of breast and other cancers. However, these agents have variable activity partly because of microtubule regulatory proteins. Stathmin, an 18-kDa phosphoprotein that promotes microtubule depolymerization, was found to be frequently overexpressed in breast cancer. We previously identified stathmin-mediated mechanisms of resistance to antimicrotubule agents, including altered drug binding and delayed transit from G_2 into M phase, where these agents are effective in disrupting microtubule dynamics. We hypothesized that by reversing stathmin-mediated depolymerization of microtubules or by promoting entry into mitosis, this could increase sensitivity to antimicrotubule agents in human breast cancer cells overexpressing stathmin. We found that targeting stathmin or wee-1 expression with RNA interference can induce microtubule polymerization and promote G_2/M progression, respectively, and sensitize stathmin-overexpressing breast cancer cells to paclitaxel and vinblastine. Furthermore, targeting wee-1 led to the phosphorylation of stathmin, which is known to attenuate its activity. Therefore, these data suggest a novel approach to improving the efficacy of certain antimicrotubule agents against breast cancer by regulating the function of stathmin.

Antimicrotubule agents are among the most active drugs in the treatment of breast cancer. These drugs can be classified broadly into two groups based on their mechanism of action: the microtubule stabilizers (e.g., taxanes, such as paclitaxel and docetaxel) and the microtubule destabilizers (e.g., the *vinca* alkaloids, such as vinblastine, vincristine, and vinorelbine). Microtubules are dynamic structures that fluctuate between microtubule polymer and tubulin heterodimers. The stabilizers bind microtubule polymer, whereas the destabilizers bind tubulin. Regardless of drug target, both groups of antimicrotubule agents promote cell death by suppressing microtubule dynamics. They are referred to as antimitotic agents because they are active during mitosis or M phase, where the microtubules that comprise the mitotic spindle are up to 100 times more dynamic than interphase microtubules (Zhai et al., 1996; Rusan et al., 2001).

Resistance to antimicrotubule agents is complex and includes nonspecific mechanisms of drug resistance and those specific to these agents. General, nonspecific mechanisms include those mediated by the overexpression of drug efflux pumps (e.g., P-glycoprotein, multidrug resistance protein), whereas mechanisms specific to antimicrotubule agents include alterations in tubulin through mutation, differential expression of tubulin isotypes, post-translational tubulin modifications (Dumontet and Sikic, 1999; Jordan and Wilson, 2004), and altered expression of microtubule regulatory proteins (Zhang et al., 1998; Alli et al., 2002; Balachandran et al., 2003; Martello et al., 2003).

One such microtubule regulator is stathmin, an 18-kDa protein that destabilizes microtubules through catastrophe promotion (Belmont and Mitchison, 1996) and/or tubulin sequestration (Curmi et al., 1997). Stathmin was originally identified as a phosphoprotein up-regulated in leukemia (Sobel and Tashjian, 1983; Hanash et al., 1988). Since then, it has also been reported to be overexpressed in solid tumors, including breast cancer (Bieche et al., 1998; Curmi et al., 2000).

Stathmin is regulated through transcriptional and post-

ABBREVIATIONS: cdk1, cyclin dependent kinase 1; RT, reverse transcriptase; qPCR, quantitative polymerase chain reaction; siRNA, small interfering RNA; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; P:S, ratio of microtubule polymer to soluble tubulin; $\Delta\Delta C_T$, comparative threshold.

translational mechanisms. Expression of stathmin can be repressed by wild-type p53 after DNA damage (Ahn et al., 1999; Murphy et al., 1999; Alli et al., 2007). Phosphorylation of stathmin on up to four serine residues (Ser16, Ser25, Ser38, and Ser63) by various kinases [mitogen-activated protein kinase, cyclin dependent kinase 1 (cdk1), cyclin-dependent kinase 2, calcium calmodulin-dependent protein kinase II, calcium calmodulin-dependent protein kinase IV, and protein kinase A] attenuates the microtubule destabilizing effects of the protein (Cassimeris, 2002). At the G_2/M transition, cdk1 phosphorylates Ser25 and Ser38 of stathmin to generate a transient diphospho form that is rapidly phosphorylated on Ser16 followed by Ser63 to produce a completely inactive tetraphospho form of stathmin required for entry into M phase (Larsson et al., 1995; Marklund et al., 1996; Di Paolo et al., 1997). Dephosphorylation of stathmin by phosphatases PP1, PP2a, and/or PP2b occurs as cells exit mitosis (Guy et al., 1992; Tournebize et al., 1997; Mistry et al., 1998; Mistry and Atweh, 2001). These events, summarized in Fig. 1, are believed to contribute to the formation and function of the mitotic spindle.

We previously found that overexpression of stathmin markedly affects the response to antimicrotubule agents through at least two possible mechanisms (Alli et al., 2002). First, by promoting microtubule destabilization, overexpression of stathmin decreased the cellular binding of paclitaxel and increased the binding of vinblastine. Second, stathmin overexpression delayed entry into M phase. Together, these mechanisms led to stathmin-mediated resistance to paclitaxel and, to a lesser extent, to vinblastine. Therefore, we hypothesized that reversing stathmin-mediated destabilization of microtubules and/or promoting entry into mitosis should increase sensitivity to antimicrotubule agents in human breast cancer cells overexpressing stathmin.
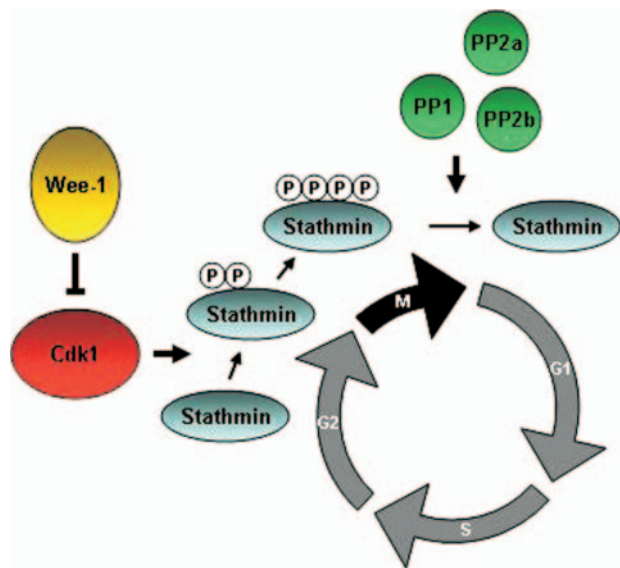

Fig. 1. Regulation of stathmin phosphorylation through the cell-cycle. Stathmin harbors four phosphorylation sites (Ser16, Ser25, Ser38, and Ser63). During interphase (G_1/S/G_2), stathmin remains unphosphorylated. At the G_2/M transition, cdk1 initiates the phosphorylation of stathmin at Ser25 and Ser38 to generate the diphospho form of the protein, which is rapidly phosphorylated at Ser16 and Ser63. The fully phosphorylated form of stathmin is inactive, and allows transition from G_2 into M phase. Phosphorylation of stathmin presumably facilitates formation of the mitotic spindle. Upon completion of M phase, stathmin is dephosphorylated by protein phosphatases PP1, PP2a, and P2b.

Materials and Methods

Cell Lines and Culture Conditions. The BT549 human breast epithelial cancer cell line was cultured in RPMI 1640 medium with 0.023 units/ml insulin. V1 and S1 human breast cancer cell lines were derived from the BT20 human breast carcinoma cell line as described previously (Alli et al., 2002). In brief, BT20 cells were transfected with pcDNA3.1 vector or pcDNA3.1 vector containing stathmin and isolated as single colony expansions to generate control (V1, formerly BT20V1) and stathmin-transfected (S1, formerly BT20ST1) stable cell lines, respectively. V1 and S1 cell lines were cultured in minimum essential medium Eagle's containing nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, and 200 μg/ml G418 (Geneticin). All culture media were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cell lines were maintained at 37° with 5% CO_2.

Reverse Transcriptase-Quantitative Polymerase Chain Reaction. Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) and purified by RNeasy MinElute Cleanup kit (QIAGEN, Valencia, CA) according to the manufacturers' instructions. RT-qPCR was carried out using one-step RT-qPCR kit for SYBR green I (Eurogentec, San Diego, CA) with 100 ng of RNA template per reaction. Controls included reactions free of reverse transcriptase and RNA template. Each reaction was carried out in quadruplicate. Human acidic ribosomal protein P0 was used as the normalization control. The primers and cycling protocol were described previously (Bieche et al., 2003). The comparative threshold ($\Delta\Delta C_T$) method (Livak and Schmittgen, 2001) was used to determine relative RNA expression.

Western Blotting. Whole-cell lysates were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, blocked, and probed with the appropriate antibody. Protein was detected by horseradish peroxidase-conjugated secondary antibody and ECL detection system (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Antibodies included anti-stathmin (Calbiochem, La Jolla, CA), anti-β-actin (Sigma-Aldrich, St. Louis, MO), anti-wee-1 (Cell Signaling Technology, Danvers, MA), anti-α-tubulin (clone B5-1-2; Sigma-Aldrich), anti-cdk1 (Calbiochem, La Jolla, CA), anti-phospho-cdk1-Tyr15 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-MPM2 (Upstate, Lake Placid, NY), anti-phospho-stathmin-S16 (Santa Cruz Biotechnology, Inc.), and anti-phospho-stathmin-Ser63 (Santa Cruz Biotechnology, Inc.). Where indicated, densitometry was carried out using NIH Image J software (http://rsb.info.nih.gov/ij/).

Immunofluorescent Staining of Microtubules. Cells grown in tissue culture chamber slides were transfected with 100 nM small interfering RNA (siRNA), fixed with methanol, blocked with 3% bovine serum albumin, and stained with α-tubulin (clone DM1A; Sigma-Aldrich) using a FITC-conjugated secondary antibody (Sigma-Aldrich). Slides were mounted with ProLong Antifade Mounting Media containing 4′,6-diamidino-2-phenylindole (Invitrogen) according to the manufacturer's instruction. Slides were then analyzed under 40X oil immersion using a Zeiss HBO 100 W/2 Axioskop fluorescent microscope (Thornwood, NY) and ScionImage software (Scion Corporation, Frederick, MD).

Determination of Microtubule Polymer: Soluble Tubulin Ratio. Separation of microtubule polymer from soluble tubulin was done as described previously (Galmarini et al., 2003). In brief, cells were lysed in buffer (20 mM Tris-HCl, pH 6.8, 1 mM $MgCl_2$, and 2 mM EGTA) for 5 min in the dark and centrifuged at 14,000 rpm. The supernatant (soluble tubulin) was stored on ice, and the pellet (polymerized microtubules) was resuspended in Ling's buffer (10 mM Tris-HCl, pH 7.5, 1.5 mM $MgCl_2$, and 10 mM KCl) in a volume equivalent to that of the supernatant fraction. Equal amounts of each fraction were subjected to Western blot analysis for tubulin using a pan-specific antibody (Clone T2.1; Cytoskeleton, Denver, CO). Polymerized and soluble tubulin for each sample was quantified

by Image J software and expressed as a ratio of polymerized microtubules to soluble tubulin (Galmarini et al., 2003).

Cell-Cycle Analysis. Cells were collected by centrifugation, washed with phosphate-buffered saline (PBS), and fixed with ice-cold 70% ethanol. Cells were then washed with PBS and incubated with RNase A/propidium iodide in PBS for 30 min at room temperature in the dark. Flow cytometric analysis was carried out on a Cytomics FC flow cytometer (Beckman Coulter, Miami, FL).

siRNA and Transfection. siRNA sequences, empirically designed based on the recommendations of Dharmacon RNA Technologies (Lafayette, CO) and T. Tuschl (Rockefeller University, New York, NY) (http://www.rockefeller.edu/labheads/tuschl/sirna.html), included stathmin sequence 5′-CUCCAGGGAAAGAUCCUUCdTdT and scrambled control sequence 5′-CUCAAGCGACGAUAGCUUC-dTdT. The sequence of wee-1 siRNA was described previously (Yuan et al., 2004), and its control siRNA refers to NonTargeting Control Sequence XI available through Dharmacon. All siRNAs were chemically synthesized as double stranded RNA (Dharmacon) and introduced into cell lines with liposome based-transfection reagent. Transfection was carried out with Oligofectamine reagent (Invitrogen) according to the manufacturer's instructions.

Combination Treatment with Stathmin siRNA. Scrambled control or stathmin siRNA was transfected into cells as described above. After 48 h, cells were treated with vehicle or increasing concentrations of the appropriate drug for 72 h. Drugs included paclitaxel, vinblastine, doxorubicin, cisplatin, or etoposide (all from Sigma-Aldrich).

Combination Treatment with Wee-1 siRNA. Nontargeting control or wee-1 siRNA was transfected into cells as described above.

After 12 h, cells were treated with vehicle or increasing concentrations of paclitaxel, vinblastine, cisplatin, or doxorubicin for 6 days (~two doubling times).

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium Bromide Assay. MTT (Sigma-Aldrich) was added to each well and incubated for 3 to 4 h at 37° and 5% CO_2; formazan crystals were made soluble with dimethyl sulfoxide. Optical density was determined at 570 nm using a Dynatech MR5000 plate reader (Dynex Technologies, Vienna, VA). Viability was expressed as a percentage of control treated cells by dividing the optical density of each treated well by the mean of the control wells. Viability was determined in quadruplicate.

Results

Effect of Silencing Stathmin on Microtubule Polymerization. Stathmin destabilizes microtubules and promotes depolymerization. Therefore, to reverse the destabilizing effect of stathmin on microtubules, we tested the ability of RNA interference to knock down stathmin expression and induce microtubule polymerization. The BT549 human breast cancer cell line expresses 11-fold more stathmin protein than normal cells (Alli et al., 2002) and has high transfection efficiency. These cells were transfected with 100 nM stathmin siRNA (nontoxic concentration), scrambled-control siRNA, and/or transfection reagent alone, and assayed for stathmin expression and microtubule polymerization. Knock-
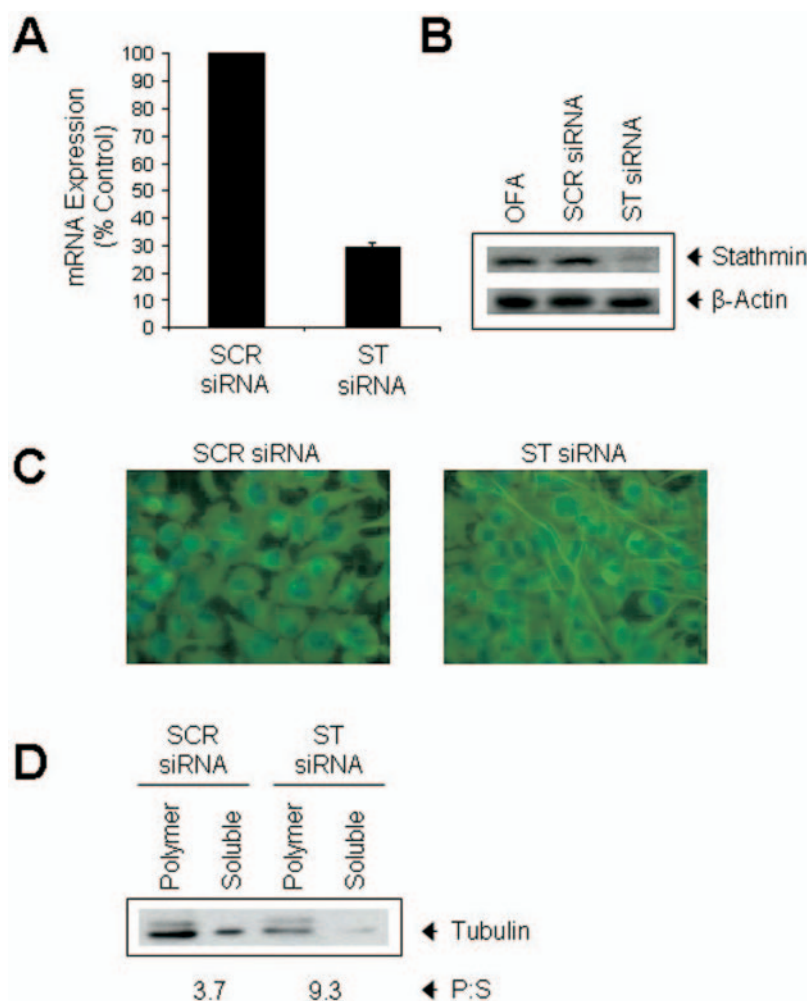

Fig. 2. Silencing of stathmin expression induces microtubule polymerization. BT549 cells were treated with transfection reagent alone (OFA), 100 nM scrambled-control siRNA (SCR), or 100 nM stathmin siRNA (ST). Knockdown of stathmin mRNA expression was determined by RT-qPCR (A). Each value represents a normalized average of quadruplicate readings determined by the comparative threshold ($\Delta\Delta C_T$) method. These data are representative of three independent experiments. Knock-down of stathmin protein expression was determined by Western blot for stathmin (B). β-Actin was used as a loading control. Images are representative of three independent experiments. Microtubule polymer was visualized by fluorescent microscopy (C). Cells were fixed and permeabilized with methanol, stained for α-tubulin using FITC-conjugated secondary antibody (green), and visualized by fluorescent microscopy with a 40× oil immersion lens. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (blue). Images are representative of two independent experiments. Microtubule polymerization was determined from the ratio of microtubule polymer to soluble tubulin (D). Transfected cells were lysed and collected into an insoluble and soluble protein fraction. Equal amounts of each fraction were subjected to Western blot with a pan-specific tubulin antibody. P:S was measured by densitometry. Data are representative of two independent experiments.

down of stathmin expression was observed by RT-qPCR (Fig. 2A) and by Western blot (Fig. 2B). After transfection with stathmin siRNA, stathmin mRNA expression decreased to $29 \pm 2\%$ of control (Fig. 2A), and stathmin protein expression markedly decreased compared with controls (Fig. 2B).

We examined the effect of stathmin siRNA on microtubules by immunofluorescent staining of the microtubule network and by quantifying the ratio of polymerized to soluble tubulin (Fig. 2, C and D). To analyze the microtubule network, transfected cells were fixed and permeabilized to allow free tubulin to diffuse from the cell, and stained with anti-α-tubulin and FITC-conjugated secondary antibody to visualize tubulin polymer. Cells transfected with stathmin siRNA showed long and bundled microtubule polymers, whereas cells transfected with scrambled-control siRNA showed short and diffuse microtubule polymers (Fig. 2C). To quantify the ratio of polymerized to soluble tubulin, transfected-cells were lysed and separated into insoluble and soluble fractions. Equal amounts of each fraction were probed for tubulin by Western blot, and tubulin content was quantified by densitometry. The ratio of microtubule polymer to soluble tubulin (P:S) was approximately three times greater for cells transfected with stathmin siRNA (P:S = 9.3) compared with control siRNA (P:S = 3.7) (Fig. 2D).

Effect of Silencing Stathmin on Drug Sensitivity. We found previously that decreased microtubule polymerization by stathmin overexpression led to paclitaxel resistance, and that decreased mitotic accumulation by stathmin overexpression led to paclitaxel and vinblastine resistance (Alli et al., 2002). Because of the effects of silencing stathmin on microtubule polymerization described above and our previous finding that silencing stathmin induces mitotic accumulation (Alli et al., 2007), we sought to determine whether or not silencing stathmin could increase sensitivity to antimicrotu-

bule agents. BT549 cells were transfected with 100 nM stathmin siRNA or scrambled-control siRNA, and analyzed for sensitivity to paclitaxel and vinblastine by MTT assay. BT549 cells transfected with stathmin siRNA were ~10-fold more sensitive to paclitaxel (IC$_{50}$, ~0.1 versus ~1 nM) than cells transfected with scrambled-control siRNA (Fig. 3A), and ~2-fold more sensitive to vinblastine (IC$_{50}$, ~0.5 versus ~0.9 nM) (Fig. 3B).

We also tested the effect of stathmin siRNA on sensitivity to drugs that do not affect microtubule dynamics. As shown in Fig. 3C, silencing stathmin had no effect on the sensitivity to doxorubicin, cisplatin, and etoposide.

Effect of Silencing Wee-1 on M Phase Accumulation. Overexpression of stathmin delays transition of breast cancer cells from G$_2$ into M phase (Alli et al., 2002). Cdk1 phosphorylates stathmin at the G$_2$/M transition and promotes entry into mitosis (Larsson et al., 1995; Marklund et al., 1996; Di Paolo et al., 1997). Wee-1 protein kinase phosphorylates cdk1 at Tyr15, which renders cdk1 inactive (Berry and Gould, 1996; Porter and Donoghue, 2003) and sustains G$_2$/M arrest. Therefore, we tested whether or not silencing wee-1 expression could promote entry into mitosis. S1 stathmin-overexpressing cells [described previously as BT20ST1 (Alli et al., 2002)] were transfected with 50 or 100 nM wee-1 siRNA or nontargeting control siRNA and examined by Western blotting for expression and activity of wee-1 and for cellular accumulation in mitosis (Fig. 4). Wee-1 siRNA silenced wee-1 expression in a dose-dependent manner compared with nontargeting control siRNA (Fig. 4A). To measure the activity of silencing wee-1 expression, we used a phosphorylation-specific antibody to Tyr15 of cdk1 to detect the presence of the inactive form and found that wee-1 siRNA decreased phosphorylation of cdk1 on Tyr15 (Fig. 4B). To determine the effect of silencing wee-1 on mitotic accumula-
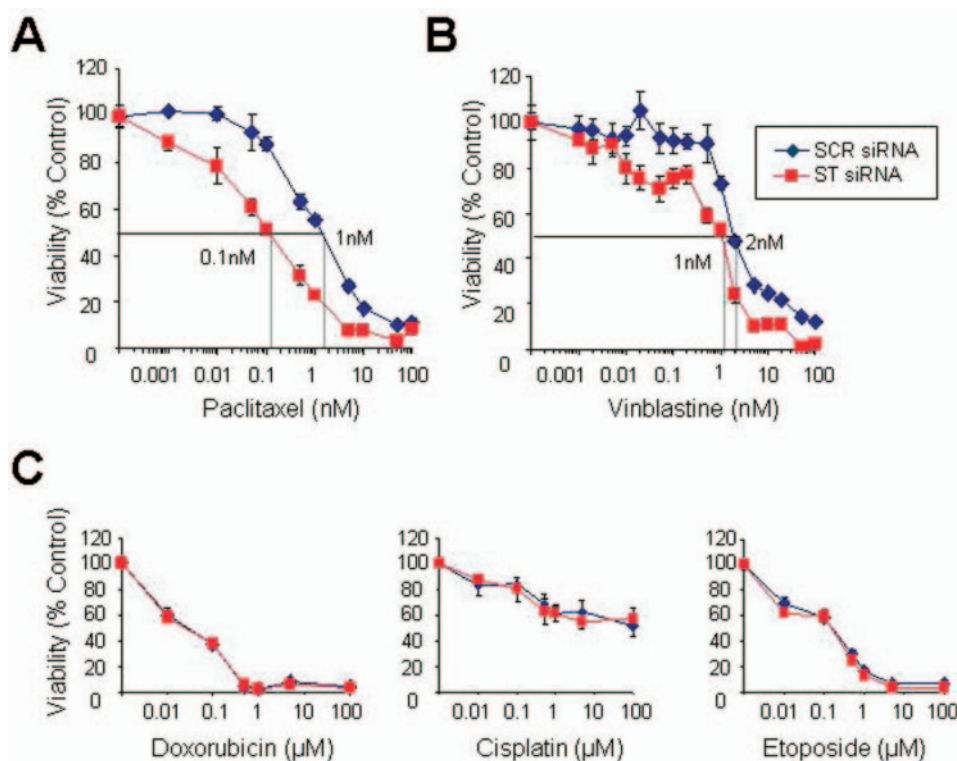
Fig. 3. Silencing of stathmin expression sensitizes stathmin-overexpressing cells to antimicrotubule agents but not to non-antimicrotubule agents. BT549 cells transfected with 100 nM scrambled-control (SCR) or stathmin (ST) siRNA were treated with paclitaxel (A), vinblastine (B), doxorubicin (C, left), cisplatin (C, middle), or etoposide (C, right) for 72 h. Drug sensitivity was determined by MTT assay. Each point represents the mean $\pm$ S.D. from quadruplicate determinations obtained from at least two independent experiments.

tion, we first analyzed the cell cycle by flow cytometry, which revealed a small but statistically insignificant decrease in the G_2/M population after transfection with wee-1 siRNA compared with control (data not shown). Given that flow cytometry does not distinguish between G_2 and M phases of the cell cycle, we focused on the accumulation of cells in mitosis by analyzing MPM2, a marker phosphoprotein specific to mitosis. Phosphoproteins recognized by MPM2 antibody (molecular mass = ~220, ~130, and ~85 kDa) increased in a dose-dependent manner after transfection with wee-1 siRNA compared with control (Fig. 4C). We next analyzed the effects of wee-1 siRNA on stathmin. S1 cells were transfected with 50 or 100 nM wee-1 siRNA or nontargeting control siRNA and analyzed for stathmin expression and phosphorylation by Western blot. Phosphorylation of stathmin was detected using phosphorylation-specific antibodies to Ser16 and Ser63 of stathmin. Wee-1 siRNA did not alter protein expression of stathmin, but increased phosphorylation of stathmin on Ser16 and Ser63 in a dose-response manner (Fig. 4D). Thus, knockdown of wee-1 increased phosphorylation of stathmin and promoted entry and cell accumulation in mitosis.

Effect of Silencing Wee-1 on Drug Sensitivity. We next studied whether the increased mitotic population of cells produced by silencing of wee-1 expression altered the sensitivity to antimicrotubule agents. In these experiments, S1 cells transfected with 100 nM wee-1 or nontargeting control siRNA were treated with increasing concentrations of paclitaxel or vinblastine and analyzed for cell viability by MTT. S1 cells transfected with wee-1 siRNA were significantly more sensitive to paclitaxel and vinblastine than cells treated with nontargeting control siRNA (Fig. 5, B and C).

To compare the effect of wee-1 siRNA on antimicrotubule drug sensitivity in stathmin-overexpressing cells to that of nonoverexpressing cells, we treated the V1 control cell line in an identical manner as described above for the S1 cell line. The S1 cell line expresses ~8-fold more stathmin than the isogenic V1 cell line [vector control described previously as BT20V1 (Alli et al., 2002)] (Fig. 5A). Wee-1 siRNA had little effect on the sensitivity to paclitaxel and vinblastine in V1 cells (Fig. 5, B and C).

We also tested the effect of wee-1 siRNA on drug sensitivity to nonantimicrotubule agents in both stathmin-overexpressing cells and nonoverexpressing cells. S1 and V1 cells transfected with wee-1 siRNA showed similar sensitivity to cisplatin and doxorubicin compared with cells treated with nontargeting control siRNA (Fig. 5D).

Discussion

Stathmin, a microtubule-destabilizing protein often overexpressed in breast cancer, was previously reported by our laboratory to decrease sensitivity to paclitaxel and vinblastine through altered drug-target interaction and cell-cycle regulation (Alli et al., 2002). Overexpression of stathmin
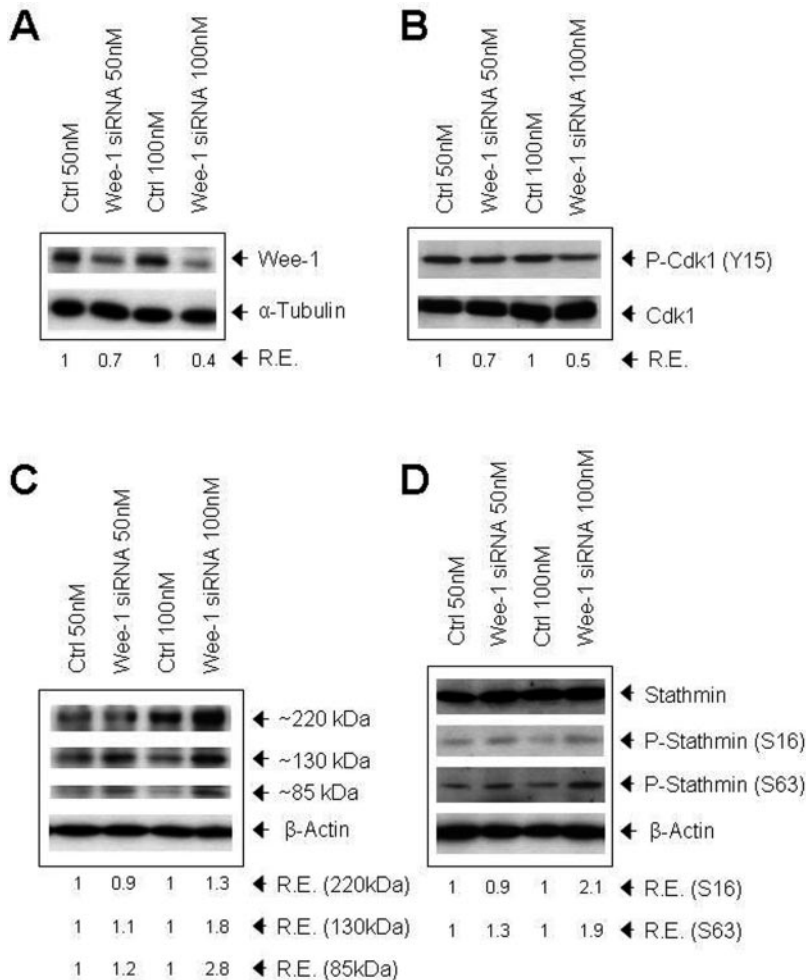

Fig. 4. Silencing of wee-1 expression induces M phase accumulation and stathmin phosphorylation. S1 cells were treated with 50 or 100 nM control (ctrl) or wee-1 siRNA for 96 h. Whole-cell lysates were collected and subjected to Western blot. Expression of wee-1 protein was determined (A). α-Tubulin was used as a loading control. Activity of wee-1 was determined by cdk1 phosphorylation using a phosphorylation-specific antibody for cdk1 at Tyr15 (B). β-Actin was used as a loading control. Expression and phosphorylation of stathmin were determined (C). Total cdk1 expression was used as a control. M phase accumulation was determined by Western blot for mitotic phosphoproteins using MPM2 antibody (D). Phosphorylation was determined using phosphorylation-specific antibodies for stathmin at Ser16 and Ser63. Images are representative of at least two independent experiments. Expression was normalized to the appropriate loading control by densitometry. Relative expression (R.E.) was calculated as the ratio of normalized expression relative to that of the control siRNA.

favored microtubule destabilization, decreased cellular binding of paclitaxel, and delayed cellular transit from G_2 into M phase. In the current studies, we attempted to reverse stathmin-mediated mechanisms of resistance to antimicrotubule agents and found that targeting stathmin or wee-1 expression can induce microtubule polymerization or $G_2 \rightarrow M$ progression, respectively, and sensitize stathmin-overexpressing breast cancer cells to paclitaxel and vinblastine.

Silencing stathmin induces microtubule polymerization and sensitizes stathmin-overexpressing breast cancer cells to antimicrotubule agents. Decreased expression of stathmin by siRNA (Fig. 2, A and B) favored elongated, bundled microtubules (Fig. 2C) and an increased ratio of polymerized to soluble tubulin (Fig. 2D). These data are consistent with the known functions of stathmin as well as previous attempts to target stathmin using antibodies or antisense (Howell et al., 1999). The shift toward polymerized microtubule dynamics produced by stathmin siRNA predicts that silencing stathmin would increase sensitivity to paclitaxel (a microtubule-stabilizing agent) and decrease sensitivity to vinblastine (a microtubule-destabilizing agent). However, we recently reported that silencing stathmin induces mitotic accumulation (Alli et al., 2007), which led to the hypothesis that silencing stathmin can increase susceptibility to both classes of antimicrotubule agents. We consistently observed that silencing stathmin increases sensitivity to both paclitaxel and vinblas-

tine, although the magnitude of change was greater for paclitaxel (Fig. 3, A and B). The greater effect of stathmin siRNA on paclitaxel sensitivity is consistent with our previous data showing that overexpression of stathmin created far greater paclitaxel resistance than vinblastine resistance (Alli et al., 2002). This is consistent with the interpretation that the effect of silencing stathmin on taxane sensitivity was due to both increased target for the drug as well as favorable cell-cycle kinetics. In contrast, the effect of silencing stathmin on vinblastine sensitivity was probably due only to favorable cell-cycle kinetics. These data are also supported by those of Iancu et al. (2000), who showed that stathmin antisense RNA sensitized K562 leukemia cells to paclitaxel. Furthermore, Balachandran et al. (2003) and Martello et al. (2003) found that stathmin is overexpressed in paclitaxel-resistant ovarian cancer and human non–small-cell lung carcinoma cell lines, respectively. Although this study is limited to paclitaxel and vinblastine, there is reason to suspect that stathmin may also influence the effect of other microtubule stabilizers, such as the epothilones, and destabilizers, such as halichondrin B. Work on these drugs is in progress. Knockdown of stathmin had no effect on doxorubicin, cisplatin, or etoposide (i.e., drugs that target DNA and topoisomerase) (Fig. 3C), suggesting that the effect of silencing stathmin on drug sensitivity is specific to antimicrotubule agents.

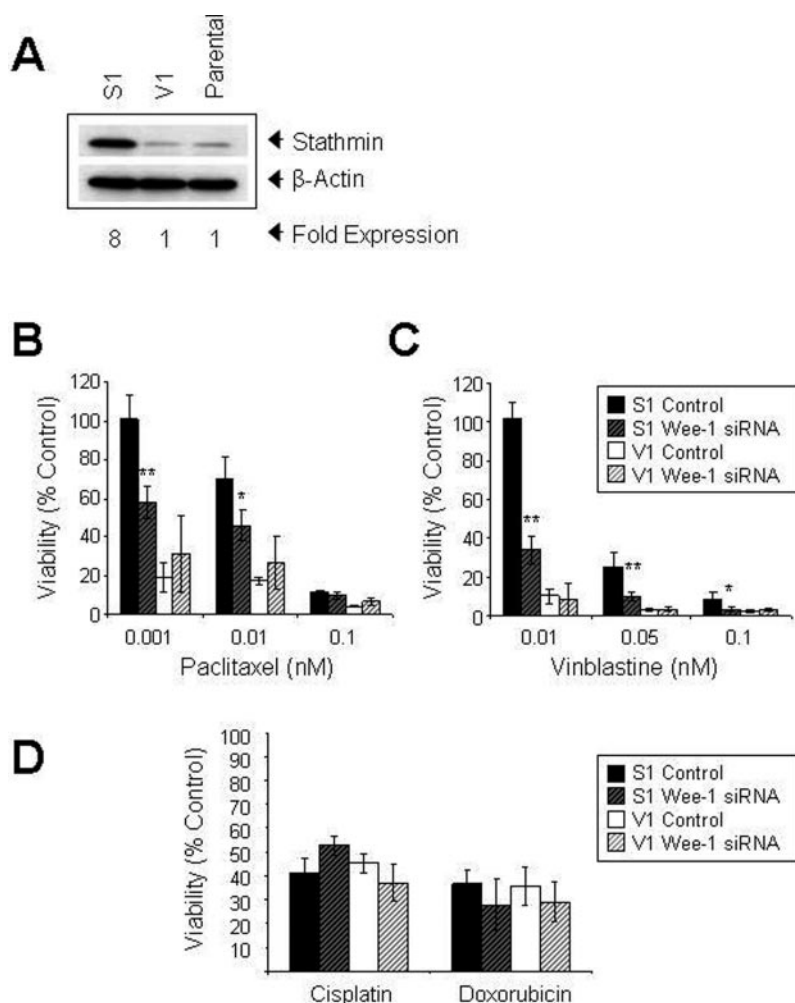Wee-1 inactivates cdk1 by phosphorylating cdk1 at Tyr15

Fig. 5. Silencing of wee-1 expression selectively sensitizes stathmin-overexpressing cells to antimicrotubule agents. Stathmin expression for S1, V1, and parental (BT20) cells growing in log phase was determined by Western blot (A). β-Actin was used as a loading control. Stathmin expression was normalized to β-actin by densitometry. -Fold expression was calculated as the ratio of normalized stathmin relative to the parental line. Sensitivity to paclitaxel (B) vinblastine (C), cisplatin (D, left), or doxorubicin (D, right) was determined for S1 and V1 cells treated with wee-1 siRNA. Cells were transfected with 100 nM control or wee-1 siRNA, exposed to antimicrotubule agent, 100 μM cisplatin, or 10 nM doxorubicin for 6 days, and analyzed for drug sensitivity by MTT assay. Each point represents the mean ± S.D. from quadruplicate determinations obtained from at least two independent experiments. $*$, $p < 0.05$; $**$, $p < 0.01$.

and prevents the G$_2$/M transition. Silencing wee-1 activates cdk1, induces stathmin phosphorylation, and promotes cellular accumulation in mitosis. We found that silencing wee-1 by siRNA (Fig. 4A) decreases phospho-cdk1 at Tyr15 (Fig. 4B), increases phospho-stathmin on Ser16 and Ser63 (Fig. 4D), and increases mitotic progression as measured by increased MPM2 expression (Fig. 4C) in stathmin-overexpressing cells, which were previously found to arrest at G$_2$ (Alli et al., 2002). These results are consistent with the findings of Yuan et al. (2004), who showed that silencing wee-1 overcame the G$_2$ arrest induced by HIV protein Vpr, and Tominaga et al. (2006), who showed that wee-1 knockout MEFs exhibit increased cdk1 activity, a defective G$_2$/M checkpoint, and premature mitotic entry upon DNA damage. Likewise, Larrson et al. (1995) showed that phosphorylation of stathmin by cdk1 on Ser25 and Ser38 precedes phosphorylation of stathmin by unknown kinase(s) on Ser16 and Ser63, which leads to the inactive phospho form of stathmin and subsequent entry into mitosis (Marklund et al., 1996; Di Paolo et al., 1997). At the G$_2$/M transition, interphase microtubules are depolymerized and then repolymerized into the mitotic spindle, suggesting that phosphorylation of stathmin functions in the assembly of the mitotic spindle (Fig. 1). Thus, we propose that decreasing wee-1 expression activates cdk1, which phosphorylates and inhibits stathmin, thereby promoting formation of the mitotic spindle.

Silencing wee-1 restores sensitivity to antimicrotubule agents in stathmin-overexpressing cells (Fig. 5). As previously reported, stathmin-overexpressing S1 cells are relatively resistant to paclitaxel and vinblastine compared with empty-vector control cells (V1). Silencing wee-1 by siRNA sensitizes S1 cells to paclitaxel and vinblastine, and the sensitivity of the combined treatment approaches that of V1 cells treated with either of these agents alone (Fig. 5, B and C). Furthermore, unlike results obtained with silencing stathmin, silencing wee-1 in S1 cells increases the sensitivity to vinblastine to a greater extent than that to paclitaxel (Fig. 5, B and C). This result is consistent with our previous finding that stathmin-induced resistance to vinca alkaloids was due primarily to a delay in G$_2$ to M transition, whereas resistance to paclitaxel as a result of stathmin overexpression was associated with a dramatic decrease in drug binding (Alli et al., 2002). Therefore, by silencing wee-1, we anticipated a greater effect on vinblastine sensitivity than on paclitaxel sensitivity. The microtubule composition of the mitotic spindle, the dynamics of mitotic microtubules, and the metaphase-anaphase block induced by antimicrotubule drugs supports the importance of cellular progression into mitosis in determining sensitivity to these drugs (Jordan and Wilson, 2004; Zhou and Giannakakou, 2005). Furthermore, Skladanowski et al. (2005) recently showed that prolonged G$_2$ and early M phases increased resistance to vincristine in leukemic cells. We found that okadaic acid, a PP1 and PP2a inhibitor known to be associated with G$_2$/M abrogation (Tournebize et al., 1997) and phosphorylation of stathmin (Guy et al., 1992; Tournebize et al., 1997; Mistry et al., 1998; Mistry and Atweh, 2001), sensitized S1 but not V1 cells to paclitaxel and vinblastine (data not shown). These data support the importance of stathmin inactivation in producing sensitivity to antimicrotubule agents. Finally, silencing wee-1 had no effect on sensitivity to cisplatin or doxorubicin in stathmin-overexpressing or nonoverexpressing cells (Fig. 5D), suggest-

ing that the enhanced sensitivity by silencing of wee-1 is specific to antimicrotubule agents.

In summary, we found that decreasing stathmin expression by siRNA can diminish stathmin-mediated mechanisms of resistance to paclitaxel and vinblastine and sensitize stathmin-overexpressing breast cancer cells to these drugs. In addition, we demonstrated for the first time that it is possible, by targeting wee-1, to increase sensitivity to antimicrotubule agents at least partly because of inactivation of stathmin. These results suggest that developing a means to target stathmin directly or indirectly via wee-1 might lead to increased sensitivity to microtubule-stabilizing agents or increased sensitivity to microtubule-stabilizing and -destabilizing agents, respectively. With the recent detailed analysis of the interaction of three stathmin-like domains with tubulin (Jourdain et al., 2004), small molecules might also be designed to prevent the stathmin-tubulin interaction. Our laboratory previously found that stathmin is overexpressed in human breast cancer cell lines harboring mutant p53 (Alli et al., 2002). Furthermore, stathmin tends to be overexpressed in leukemia (Sobel and Tashjian, 1983; Hanash et al., 1988), ovarian cancer (Alaiya et al., 1997; Price et al., 2000), and prostate cancer (Friedrich et al., 1995), suggesting applicability to cancers other than those of breast origin. Overall, our studies contribute to an understanding of the complexities of drug resistant mechanisms present in human cancers and suggest new approaches for a more effective use of certain antimicrotubule agents.

References

Ahn J, Murphy M, Kratowicz S, Wang A, Levine AJ, and George DL (1999) Down-regulation of the stathmin/Op18 and FKBP25 genes following p53 induction. *Oncogene* **18:**5954–5958.

Alaiya AA, Franzen B, Fujioka K, Moberger B, Schedvins K, Silfversvard C, Linder S, and Auer G (1997) Phenotypic analysis of ovarian carcinoma: polypeptide expression in benign, borderline and malignant tumors. *Int J Cancer* **73:**678–683.

Alli E, Bash-Babula J, Yang JM, and Hait WN (2002) Effect of stathmin on the sensitivity to antimicrotubule drugs in human breast cancer. *Cancer Res* **62:**6864–6869.

Alli E, Yang JM, and Hait WN (2007) Silencing of stathmin induces tumor-suppressor function in breast cancer cell lines harboring mutant p53. *Oncogene* **26:**1003–1012.

Balachandran R, Welsh MJ, and Day BW (2003) Altered levels and regulation of stathmin in paclitaxel-resistant ovarian cancer cells. *Oncogene* **22:**8924–8930.

Belmont LD and Mitchison TJ (1996) Identification of a protein that interacts with tubulin dimers and increases the catastrophe rate of microtubules. *Cell* **84:**623–631.

Berry LD and Gould KL (1996) Regulation of Cdc2 activity by phosphorylation at T14/Y15. *Prog Cell Cycle Res* **2:**99–105.

Bieche I, Lachkar S, Becette V, Cifuentes-Diaz C, Sobel A, Lidereau R, and Curmi PA (1998) Overexpression of the stathmin gene in a subset of human breast cancer. *Br J Cancer* **78:**701–709.

Bieche I, Manceau V, Curmi PA, Laurendeau I, Lachkar S, Leroy K, Vidaud D, Sobel A, and Maucuer A (2003) Quantitative RT-PCR reveals a ubiquitous but preferentially neural expression of the KIS gene in rat and human. *Brain Res Mol Brain Res* **114:**55–64.

Cassimeris L (2002) The oncoprotein 18/stathmin family of microtubule destabilizers. *Curr Opin Cell Biol* **14:**18–24.

Curmi PA, Andersen SS, Lachkar S, Gavet O, Karsenti E, Knossow M, and Sobel A (1997) The stathmin/tubulin interaction in vitro. *J Biol Chem* **272:**25029–25036.

Curmi PA, Nogues C, Lachkar S, Carelle N, Gonthier MP, Sobel A, Lidereau R, and Bieche I (2000) Overexpression of stathmin in breast carcinomas points out to highly proliferative tumours. *Br J Cancer* **82:**142–150.

Di Paolo G, Antonsson B, Kassel D, Riederer BM, and Grenningloh G (1997) Phosphorylation regulates the microtubule-destabilizing activity of stathmin and its interaction with tubulin. *FEBS Lett* **416:**149–152.

Dumontet C and Sikic BI (1999) Mechanisms of action of and resistance to antitubulin agents: microtubule dynamics, drug transport, and cell death. *J Clin Oncol* **17:**1061–1070.

Friedrich B, Gronberg H, Landstrom M, Gullberg M, and Bergh A (1995) Differentiation-stage specific expression of oncoprotein 18 in human and rat prostatic adenocarcinoma. *Prostate* **27:**102–109.

Galmarini CM, Kamath K, Vanier-Viornery A, Hervieu V, Peiller E, Falette N, Puisieux A, Ann Jordan M, and Dumontet C (2003) Drug resistance associated with loss of p53 involves extensive alterations in microtubule composition and dynamics. *Br J Cancer* **88:**1793–1799.

Guy GR, Cao X, Chua SP, and Tan YH (1992) Okadaic acid mimics multiple changes in early protein phosphorylation and gene expression induced by tumor necrosis factor or interleukin-1. *J Biol Chem* **267:**1846–1852.

Hanash SM, Strahler JR, Kuick R, Chu EH, and Nichols D (1988) Identification of a polypeptide associated with the malignant phenotype in acute leukemia. *J Biol Chem* **263:**12813–12815.

Howell B, Deacon H, and Cassimeris L (1999) Decreasing oncoprotein 18/stathmin levels reduces microtubule catastrophes and increases microtubule polymer in vivo. *J Cell Sci* **112:**3713–3722.

Iancu C, Mistry SJ, Arkin S, and Atweh GF (2000) Taxol and anti-stathmin therapy: a synergistic combination that targets the mitotic spindle. *Cancer Res* **60:**3537–3541.

Jordan MA and Wilson L (2004) Microtubules as a target for anticancer drugs. *Nat Rev Cancer* **4:**253–265.

Jourdain I, Lachkar S, Charbaut E, Gigant B, Knossow M, Sobel A, and Curmi PA (2004) A synergistic relationship between three regions of stathmin family proteins is required for the formation of a stable complex with tubulin. *Biochem J* **378:**877–888.

Larsson N, Melander H, Marklund U, Osterman O, and Gullberg M (1995) G2/M transition requires multisite phosphorylation of oncoprotein 18 by two distinct protein kinase systems. *J Biol Chem* **270:**14175–14183.

Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* **25:**402–408.

Marklund U, Larsson N, Gradin HM, Brattsand G, and Gullberg M (1996) Oncoprotein 18 is a phosphorylation-responsive regulator of microtubule dynamics. *EMBO (Eur Mol Biol Organ) J* **15:**5290–5298.

Martello LA, Verdier-Pinard P, Shen HJ, He L, Torres K, Orr GA, and Horwitz SB (2003) Elevated levels of microtubule destabilizing factors in a Taxol-resistant/dependent A549 cell line with an alpha-tubulin mutation. *Cancer Res* **63:**1207–1213.

Mistry SJ and Atweh GF (2001) Stathmin inhibition enhances okadaic acid-induced mitotic arrest: a potential role for stathmin in mitotic exit. *J Biol Chem* **276:**31209–31215.

Mistry SJ, Li HC, and Atweh GF (1998) Role for protein phosphatases in the cell-cycle-regulated phosphorylation of stathmin. *Biochem J* **334:**23–29.

Murphy M, Ahn J, Walker KK, Hoffman WH, Evans RM, Levine AJ, and George DL (1999) Transcriptional repression by wild-type p53 utilizes histone deacetylases, mediated by interaction with mSin3a. *Genes Dev* **13:**2490–2501.

Porter LA and Donoghue DJ (2003) Cyclin B1 and CDK1: nuclear localization and upstream regulators. *Prog Cell Cycle Res* **5:**335–347.

Price DK, Ball JR, Bahrani-Mostafavi Z, Vachris JC, Kaufman JS, Naumann RW, Higgins RV, and Hall JB (2000) The phosphoprotein Op18/stathmin is differentially expressed in ovarian cancer. *Cancer Investig* **18:**722–730.

Rusan NM, Fagerstrom CJ, Yvon AM, and Wadsworth P (2001) Cell cycle-dependent changes in microtubule dynamics in living cells expressing green fluorescent protein-alpha tubulin. *Mol Biol Cell* **12:**971–980.

Skladanowski A, Come MG, Sabisz M, Escargueil AE, and Larsen AK (2005) Down-regulation of DNA topoisomerase IIα leads to prolonged cell cycle transit in G_2 and early M phases and increased survival to microtubule-interacting agents. *Mol Pharmacol* **68:**625–634.

Sobel A and Tashjian AH Jr (1983) Distinct patterns of cytoplasmic protein phosphorylation related to regulation of synthesis and release of prolactin by GH cells. *J Biol Chem* **258:**10312–10324.

Tominaga Y, Li C, Wang RH, and Deng CX (2006) Murine wee1 plays a critical role in cell cycle regulation and pre-implantation stages of embryonic development. *Int J Biol Sci* **2:**161–170.

Tournebize R, Andersen SS, Verde F, Doree M, Karsenti E, and Hyman AA (1997) Distinct roles of PP1 and PP2A-like phosphatases in control of microtubule dynamics during mitosis. *EMBO (Eur Mol Biol Organ) J* **16:**5537–5549.

Yuan H, Kamata M, Xie YM, and Chen IS (2004) Increased levels of Wee-1 kinase in G(2) are necessary for Vpr- and gamma irradiation-induced G(2) arrest. *J Virol* **78:**8183–8190.

Zhai Y, Kronebusch PJ, Simon PM, and Borisy GG (1996) Microtubule dynamics at the G2/M transition: abrupt breakdown of cytoplasmic microtubules at nuclear envelope breakdown and implications for spindle morphogenesis. *J Cell Biol* **135:**201–214.

Zhang CC, Yang JM, White E, Murphy M, Levine A, and Hait WN (1998) The role of MAP4 expression in the sensitivity to paclitaxel and resistance to vinca alkaloids in p53 mutant cells. *Oncogene* **16:**1617–1624.

Zhou J and Giannakakou P (2005) Targeting microtubules for cancer chemotherapy. *Curr Med Chem Anti-Canc Agents* **5:**65–71.

Address correspondence to: William N. Hait, The Cancer Institute of New Jersey, University of Medicine and Dentistry of New Jersey/Robert Wood Johnson Medical School, 195 Little Albany Street, New Brunswick, NJ 08901. E-mail: haitwn@umdnj.edu